Elżbieta Maria Gniazdowska

Opracowanie nowych metod bioanalitycznych oraz optymalizacja sposobu ich walidacji

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu w dyscyplinie nauki farmaceutyczne

Promotor: **dr hab. n. farm. Joanna Giebułtowicz**
Promotor pomocniczy: **dr Katarzyna Buś-Kwaśnik**

Zakład Chemii Leków

WYDZIAŁ FARMACEUTYCZNY WUM

Laboratorium Analityki Farmaceutycznej Sekcja Farmakokinetyki

Łukasiewicz Instytut Chemii Przemysłowej

Projekt badawczy realizowany w ramach programu „Doktorat wdrożeniowy” DW/03/2019

Obrona rozprawy doktorskiej przed Radą Dyscypliny Nauk Farmaceutycznych Warszawskiego Uniwersytetu Medycznego

Warszawa 2023 rok

Słowa kluczowe: walidacja metody bioanalitycznej, wpływ matrycy, stabilność, farmakokinetyka

Keywords: *bioanalytical method validation, matrix effect, stability, pharmacokinetics*

Składam serdeczne podziękowania mojej Promotor
dr hab. n. farm. Joannie Giebułtowicz za możliwość konsultacji, poświęcony czas, wszelkie okazane wsparcie, dzielenie się wiedzą w trakcie wykonywania badań, przygotowania publikacji oraz uwagi merytoryczne podczas pisania pracy.

Bardzo dziękuję Promotorowi pomocniczemu dr Katarzynie Buś-Kwaśnik za możliwość konsultacji, cenne uwagi, za wszechstronną pomoc, która była dużym wsparciem podczas planowania badań i pisania publikacji i pracy.

Bardzo dziękuję poprzedniemu Kierownikowi Zakładu Farmakokinetyki, Sieć Badawcza Łukasiewicz – Instytut Farmaceutyczny, dr hab. Piotrowi J. Rudzkiemu za współpracę i udzieloną pomoc podczas narodzin tematyki mojej pracy doktorskiej

Dziękuję Dyrektorowi Sieć Badawcza Łukasiewicz – Instytut Chemii Przemysłowej imienia Profesora Ignacego Mościckiego, dr Pawłowi Bielskiemu,
Dyrektorowi do spraw naukowych, dr Ewie Śmigierze,
Kierownikowi Laboratorium Analiz Farmaceutycznych dr Anecie Łukomskiej,
Kierownikowi Sekcji Farmakokinetyki mgr Edycie Peście,
poprzedniemu Kierownikowi Zakładu Bioanalizy i Analizy Leków, Warszawskiego Uniwersytetu Medycznego prof. dr hab. n. farm. Piotrowi Wroczyńskiemu
oraz Kierownikowi Zakładu Chemii Leków, Warszawskiego Uniwersytetu Medycznego prof. dr hab. Tomaszowi Pawińskiemu, za możliwość realizowania pracy doktorskiej.

Podziękowania kieruję do współautorów publikacji wchodzących w skład rozprawy, współpracowników Sekcji Farmakokinetyki, Zakładu Bioanalizy i Analizy Leków za współpracę i dzielenie się wiedzą.

Szczególne podziękowania składam mojej Rodzinie i Przyjaciołom za życzliwość i duchowe wsparcie. Wyrażam również wdzięczność Wszystkim Tym, którzy okazali mi wsparcie i życzliwość przy realizacji niniejszej pracy.

Wykaz publikacji stanowiących pracę doktorską

1. Gniazdowska E., Korytowska N., Kłudka G., Giebułtowicz J. Determination of antidepressants in human plasma by modified cloud-point extraction coupled with mass spectrometry. Pharmaceuticals 2020; 13:1-25. IF = 5,863; MEiN = 100.

2. Gniazdowska E., Kaza M., Buś-Kwaśnik K., Giebułtowicz J. LC-MS/MS determination of dutasteride and its major metabolites in human plasma. Journal of Pharmaceutical and Biomedical Analysis 2021; 206:114362. IF= 3,571; MEiN = 100.

3. Gniazdowska E., Goch W., Giebułtowicz J., Rudzki P.J. Replicates number for drug stability testing during bioanalytical method validation – experimental and retrospective approach. Molecules 2022; 27: 457. IF= 4,600; MEiN = 140.

4. Gniazdowska E.M., Giebułtowicz J. and Rudzki P.J. How does the order of sample analysis influence the matrix effect during LC-MS bioanalysis? Journal of Chromatography B 2023; 1227:123800. IF= 3,000; MEiN = 100.

5. Gniazdowska E., Gilant E., Buś-Kwaśnik K. Wytyczna ICH M10 – Jednolite podejście do bioanalizy na całym świecie. Prospects in Pharmaceutical Sciences 2023; 21(3):57-63. IF= 0,100; MEiN: 20.

Spis treści

Wykaz skrótów i symboli

AUC	*(ang. Area Under the Curve)* pole powierzchni pod krzywą
CE	(*ang. Collision Energy*) energia zderzeń
CV	(*ang. Coefficient of Variation*) współczynnik zmienności
CPE	(*ang. Cloud Point Extraction*) ekstrakcja w punkcie zmętnienia
CXP	(*ang. Collision Exit Potential*) potencjał wyjścia z komory zderzeń
CTD	(ang. *Common Technical Document*) Wspólny Dokument Techniczny
DP	(*ang. Declustering Potential*) potencjał, pod wpływem którego następuje rozgrupowanie klastrów
EMA	(*ang. European Medicines Agency*) Europejska Agencja Leków
EP	(*ang. Entrance Potential*) potencjał wejściowy
ESI	(*ang. Electrospray ionization*) jonizacja przez rozpylanie w polu elektrycznym
FDA	(*ang. Food and Drug Administration*) Agencja ds. Leków i Żywności
GCP	(*ang. Good Clinical Practice)* Dobra Praktyka Kliniczna
GLP	(*ang. Good Laboratory Practice)* Dobra Praktyka Laboratoryjna
ICH	(*ang. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use)* Międzynarodowa Rada Harmonizacji Wymagań Technicznych dla Rejestracji Produktów Leczniczych Stosowanych u Ludzi
LC-MS/MS	(*ang. Liquid Chromatography - Mass Spectrometry*) chromatografia cieczowa sprzężona ze spektrometrią mas
LLOQ	(*ang. Lower Limit of Quantification*) dolna granica oznaczalności
OECD	*(ang. Organisation for Economic Cooperation and Development – OECD)* Organizacja Współpracy Gospodarczej i Rozwoju
QC	(*ang. Quality Control sample*) próbka kontrolna
WHO	(*ang. World Health Organization*) Światowa Organizacja Zdrowia

Streszczenie

Ważnym elementem prac badawczo-rozwojowych nad nowymi produktami leczniczymi są badania farmakokinetyczne i toksykologiczne. Badania te wymagają oznaczenia stężeń substancji czynnych badanych produktów leczniczych w materiale biologicznym, najczęściej w osoczu i wykonywane są za pomocą zwalidowanych metod bioanalitycznych. Istnieje zatem ciągłe zapotrzebowanie na nowe metody bioanalityczne. Walidacja metod bioanalitycznych była do 2023 roku wykonywana zgodnie z wytycznymi Europejskiej Agencji Leków (*ang. European Medicines Agency* - EMA) lub Amerykańskiej Agencji ds. Leków i Żywności (*ang. Food and Drug Administration* - FDA) i dokumentowana zgodnie z zasadami Dobrej Praktyki Laboratoryjnej (*ang. Good Laboratory Practice* - GLP*)*. W odpowiedzi na postulowane od wielu lat uwagi o harmonizacji na poziomie międzynarodowym zaleceń dotyczących walidacji metod bioanalitycznych, w 2019 roku powstał projekt wytycznej ICH M10, opracowany przez Międzynarodową Radę Harmonizacji Wymagań Technicznych dla Rejestracji Produktów Leczniczych Stosowanych u Ludzi (ang. *The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use – ICH*). W świetle trwających prac nad ostateczną wersją wytycznej ICH M10, widoczna była potrzeba optymalizacji testów walidacyjnych z zakresu zalecanej liczby próbek kontrolnych
(ang. Quality Control – QC) w teście stabilności, kolejności próbek w sekwencji analitycznej w teście oceniającym wpływ matrycy oraz liczby użytych źródeł matrycy. Innym zagadnieniem, które jest niedostateczne poznane jest stabilność długoterminowa analitów w próbkach biologicznych.

Celem niniejszej pracy było: 1) opracowanie nowych metod bioanalitycznych, 2) optymalizacja sposobu ich walidacji w zakresie badania wpływu matrycy (kolejność próbek w sekwencji analitycznej) oraz testu stabilności (liczba próbek kontrolnych), 3) ocena stabilności długoterminowej wybranych analitów (dutasterydu i arypiprazolu) w próbkach osocza ludzkiego.

Badania pozwoliły na opracowanie dwóch precyzyjnych i selektywnych metod oznaczania 21 substancji czynnych leków przeciwdepresyjnych oraz dutasterydu (syntetycznego analogu testosteronu) wraz z metabolitami w osoczu ludzkim. Metody te mogą być stosowane w praktyce np. w badaniu równoważności biologicznej. W toku prac wykazano także stabilność długoterminową dutasterydu (3 lata) i arypiprazolu (7 lat) w osoczu ludzkim. Wyniki badania z zakresu optymalizacji testów walidacyjnych pokazały, że ze względu na duże różnice w profilach fosfolipidów, uważanych za istotny czynnik powodujący efekt matrycowy, w osoczach pochodzących z różnych źródeł, użycie jednego osocza lipemicznego zalecane przez ICH M10 jest niewystarczające, żeby ocenić wiarygodność metody bioanalitycznej. Z zakresu optymalizacji przeprowadzania testu stabilności, za optymalną liczbę powtórzeń próbek QC wykazano liczbę pięć, uznając liczbę trzy, sugerowaną przez wytyczną ICH M10, jako niewystarczającą do wykazania stabilności analitu w materiale biologicznym. Wyniki przeprowadzonych badań znajdują odzwierciedlenie w nowym wydaniu Standardowej Procedury Operacyjnej Sekcji Farmakokinetyki dotyczącej walidacji metody bioanalitycznej pt. „Walidacja metod bioanalitycznych”.

Title: Development of new bioanalytical method and optimization of validation procedures

Abstract

A crucial component of the research and development of new medicinal products involves pharmacokinetic studies. These studies aim to determine the concentrations of the active substance and its metabolites in the biological matrix, typically in human plasma, utilizing validated bioanalytical methods. Nevertheless, there remains an unmet need for the development of new bioanalytical methods.The validation of bioanalytical methods adheres to the guidelines set forth by regulatory agencies such as the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA), while documentation is in accordance with the principles of Good Laboratory Practice (GLP). In 2019, the International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) introduced the draft of the ICH M10 guideline, representing an effort to globally standardize the validation of bioanalytical methods—an aspiration held for several years. In light of ongoing work to finalize the ICH M10 guideline, it became evident that optimization of certain aspects of validation tests was necessary. These aspects included the recommended number of quality control (QC) samples in stability tests, the order of sample order within the analytical sequence during matrix effect tests, and the utilization of multiple sources for matrix effect tests. Additionally, a less understood area pertained to the long-term stability of analytes within biological samples.

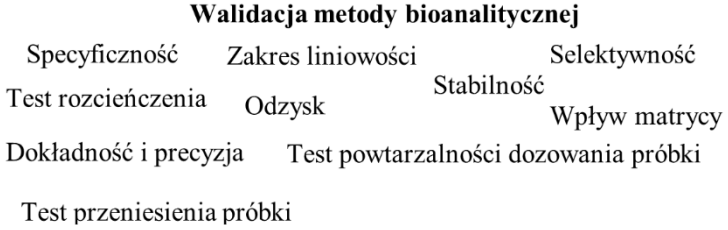
This study aimed to achieve several objectives: 1) Develop new bioanalytical methods. 2) Optimize validation tests, specifically within the matrix effect test (considering the order in the analytical batch) and the stability test (determining the optimal number of QC samples). 3) Evaluate the long-term stability of analytes, specifically dutasteride and aripiprazole, in human plasma.

Two new methods were successfully developed validated for the determination of 21 antidepressants, as well as for dutasteride (a synthetic analog of testosterone) and its metabolites in human plasma. These methods can be applied in bioequivalence studies. Furthermore, the long-term stability of dutasteride (over a period of 3 years) and aripiprazole (over a period of 7 years) in human plasma was proved.

The results of the optimization study for matrix effect tests highlighted the importance of considering the diverse phospholipid profiles present in lipemic plasma samples, which significantly contribute to the observed matrix effect. A single source of lipemic plasma was deemed insufficient for assessing the reliability of bioanalytical methods. Regarding the stability test, it was examined whether the draft ICH guideline M10's recommendation of a minimum of three QC samples adequately demonstrated analyte stability. The study results indicated that five QC samples represent the optimal number for this test. The findings generated from these comprehensive studies were incorporated into the updated edition of the Standard Operating Procedure for bioanalytical method validation within the Pharmacokinetics Section.

1. Wprowadzenie

Ważnym elementem prac badawczo–rozwojowych nad nowymi produktami leczniczymi są badania toksykologiczne i farmakokinetyczne. Badania te wymagają oznaczenia stężeń substancji czynnych badanych produktów leczniczych w materiale biologicznym, najczęściej w osoczu, za pomocą zwalidowanych metod bioanalitycznych. Walidacja metod bioanalitycznych prowadzona była do 2023 roku zgodnie z wytycznymi Europejskiej Agencji Leków (*ang. European Medicines Agency* - EMA) [1] lub Amerykańskiej Agencji ds. Leków i Żywności (*ang. Food and Drug Administration* - FDA) [2] i dokumentowana zgodnie z zasadami Dobrej Praktyki Laboratoryjnej (*ang. Good Laboratory Practice - GLP)* [3]. Wyniki walidacji sprawozdawane są zgodnie z formatem Wspólnego Dokumentu Technicznego (*ang. Common Technical Document* - CTD) [4] – dla badań prowadzonych w materiale biologicznym ludzkim (CTD 5.3.1.2) oraz dla badań prowadzonych w materiale biologicznym zwierzęcym (CTD 4.2.2.1). Dokument ten jest przedstawiany organom rejestracyjnym w procesie dopuszczenia produktu leczniczego do obrotu. W 2019 roku, Międzynarodowa Rada Harmonizacji Wymagań Technicznych dla Rejestracji Produktów Leczniczych Stosowanych u Ludzi (*ang. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use – ICH)* wydała projekt wytycznej ICH M10 [5] w celu uzyskania opinii środowiska naukowego i przedsiębiorców z zakresu zaproponowanych jednolitych rekomendacji ogólnoświatowych do prowadzenia walidacji metod bioanalitycznych. Walidacja metody bioanalitycznej polega na wykonaniu testów przedstawionych na Rysunku 1.

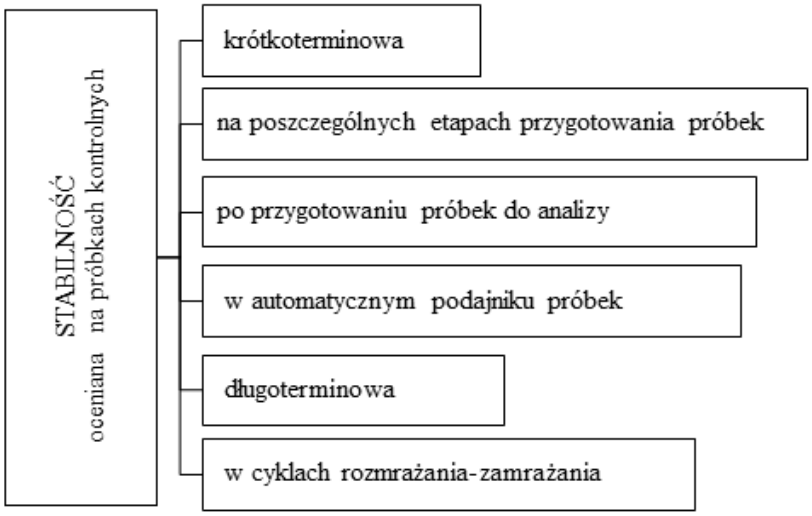


Rys. 1. Parametry walidacyjne metod bioanalitycznych związków niskocząsteczkowych.

Konsultacje społeczne po opublikowaniu projektu wytycznej ICH M10, które odbyły się w okresie 14.03-01.09.2019 [6], wskazały na brak jednomyślności w sprawie oceny parametrów walidacyjnych. Podkreśliły też potrzebę optymalizacji, a w niektórych przypadkach - doprecyzowania istniejących testów walidacyjnych [1]. W szczególności

zoptymalizowania wymagały: kolejność próbek w sekwencji analitycznej w teście oceniającym wpływ matrycy oraz zalecana liczba próbek kontrolnych (*ang. quality control* - QC) w teście stabilności.

Rekomendacje EMA [1], FDA [2] oraz projektu ICH M10 [7] różnią się w podejściu i zakresie prowadzonych testów walidacyjnych. EMA [1] nie zaleca konkretnej liczby próbek QC do przygotowania w teście stabilności (Rys. 2), podczas gdy FDA [2] i ICH [6] zalecają co najmniej trzy próbki QC na dwóch poziomach stężeń. Wiele instytucji stosuje w trakcie testów sprawdzających stabilność 5-6 próbek [8-12], niemniej do chwili ukazania się publikacji wchodzącej w skład niniejszej rozprawy, nie było żadnych danych naukowych pozwalających na racjonalny wybór liczby próbek QC w badaniu stabilności.



Rys. 2. Zakres badania stabilności w walidacji metody bioanalitycznej [1,2]

W przypadku badania wpływu matrycy, w momencie prowadzenia badań, wytyczna FDA nie opisywała sposobu wykonania testu wpływu matrycy, wytyczna EMA opisywała sposób przeprowadzenia testu (liczba próbek, rodzaj próbek) i określała $\%RSD_{MF}$ (zmienność normalizowanego wzorcem wewnętrznym czynnika matrycowego) jako parametr, który musi zostać poddany ocenie [1]. Nie wiadomo było natomiast czy kolejność analizowanych próbek (schemat blokowy lub naprzemienny) wpływa na wynik oceny $\%RSD_{MF}$. Aspekt ten jest szczególnie istotny, gdyż w wielu publikacjach [13-18] kolejność analizy próbek nie jest raportowana. W projekcie wytycznej ICH M10 [7] przedstawiono sposób przeprowadzenia testu za pomocą oceny precyzji pomiarów analitu w różnych źródłach matrycy. W konsultacjach społecznych [6] zgłaszane były krytyczne uwagi i postulaty o pozostaniu przy dotychczasowym sposobie oceny wpływu matrycy, innym niż przedstawiony w projekcie wytycznej. ICH nie uwzględniło postulatów z konsultacji społecznych w tym zakresie,

rekomendując przeprowadzanie testu precyzji z trzech próbek w kilku źródłach materiału biologicznego.

Innym problemem natury praktycznej, z którym borykają się instytucje prowadzące badania w systemie GLP, m.in. farmakokinetyczne i toksykologiczne (Sponsor badania, organizacja prowadząca badania na zlecenie – CRO) jest okres przechowywania próbek. Stabilność analitu w materiale biologicznym jest bardzo istotna dla wiarygodności wyniku w przypadku konieczności powtórzenia oznaczeń w próbkach z badania. Może mieć to miejsce w czasie weryfikacji dokumentacji w procesie rejestracji produktu leczniczego, będącego przedmiotem danego badania, nawet po kilku latach od zakończenia badania. Zgodnie z obowiązującymi wytycznymi WHO (2009, punkt 16.2) [19] i OECD o Dobrej Praktyce Laboratoryjnej (1997, punkty 6.6 i 10.1) [3] próbki powinny być przechowywane po badaniu tak długo, jak długo ich jakość pozwala na wiarygodną ocenę stężenia analitu. Długoterminowe przechowanie zamrożonych próbek generuje jednak koszty dla Sponsora. Sposobem na skrócenie czasu archiwizowania próbek jest stwierdzenie braku stabilności analitu [3]. Niemniej niewiele jest danych na temat stabilności analitów podczas długotrwałego przechowywania próbek z badań [9,20]. Podczas walidacji metody bioanalitycznej, sprawdzana jest stabilność analitu w próbkach QC, jedynie w warunkach przechowywania (czas i temperatura) próbek biologicznych - od momentu pobrania w klinice do momentu wykonania analiz. Próbki z badań są bardziej złożone niż próbki QC, np. poprzez obecność metabolitów I i II fazy biotransformacji [21], dlatego stabilność analitu w probkach QC i próbkach rzeczywistych może się różnić. Przeprowadzenie testu stabilności długoterminowej ma aspekt ekonomiczny dla Sponsora badania. Dostarcza bowiem danych weryfikujących zasadność ponoszenia kosztów przechowywania próbek osocza z danego badania.

Rozwój leków generycznych i opracowywanie nowych formulacji, np. bardziej dogodnych do stosowania dla pacjenta, wymusza opracowywanie nowych metod bioanalitycznych. W nurcie zielonej chemii dąży się do opracowywania metod przyjaznych dla środowiska [22], a także metod uwzględniających oprócz substancji czynnej, także jej metabolity [23,24]. Pomiary stężeń metabolitów mogą mieć kluczowe znaczenie także dla produktów leczniczych już stosowanych przez pacjentów. FDA odnotowała przypadki, w których klinicznie istotne metabolity nie zostały zidentyfikowane podczas badań przedklinicznych i podkreśliła potrzebę ich oznaczenia w badaniach klinicznych [25]. Oznaczenie poziomu metabolitów może pomóc w wyjaśnieniu zjawiska zmienności odpowiedzi pacjentów na leczenie. Jako modelowy lek, dla którego obserwowana jest duża

zmienność odpowiedzi na leczenie wybrano dutasteryd – syntetyczny analog testosteronu, specyficzny i selektywny inhibitor izoform 5α-reduktazy. Jest on stosowany w łagodnym rozroście gruczołu krokowego i objawach ze strony dolnych dróg moczowych [26-29]. Okres półtrwania dutasterydu wydłuża się wraz z wiekiem pacjenta i wynosi: 170 h u mężczyzn w wieku 20-49 lat, 260 h u mężczyzn w wieku 50-69 lat i 300 h u mężczyzn w wieku powyżej 70 lat [30]. Do głównych metabolitów dutasterydu należą 4′-hydroksydutasteryd, 6β-hydroksydutasteryd i 1,2-dihydrodutasteryd. Badania *in vitro* wykazały, że 4′-hydroksydutasteryd i 1,2-dihydrodutasteryd wykazują słabsze działanie hamujące na 5α-reduktazy niż dutasteryd, natomiast aktywność 6β-hydroksydutasterydu wobec enzymu jest porównywalna z aktywnością dutasterydu [28,31]. W literaturze brakowało metody oznaczania tych metabolitów w osoczu ludzkim [30,32-37].

Jedną z metod izolacji analitu, spełniającą założenia zielonej chemii, jest metoda ekstrakcji w punkcie zmętnienia (*ang. cloud-point extraction* – CPE). Metoda ta rzadko jest stosowana w połączeniu ze spektrometrią mas ze względu na obawy związane z supresją jonizacji przez używany podczas ekstrakcji surfaktant [38-41]. W szczególności nie ma metody wykorzystującej CPE, która pozwoliłaby na izolację substancji czynnych leków przeciwdepresyjnych z osocza i analizę metodą LC-MS.

2. Założenia i cel badań

Celem niniejszej pracy było opracowanie nowych metod bioanalitycznych oznaczania substancji czynnych leków i/lub ich metabolitów w osoczu ludzkim, optymalizacja wybranych testów walidacyjnych oraz ocena stabilności długoterminowej wybranych substancji czynnych leków w próbkach z badania.

Cel zrealizowany został poprzez:

- Opracowanie i walidację metody oznaczania 21 substancji czynnych leków przeciwdepresyjnych w osoczu ludzkim za pomocą metody ekstrakcji w punkcie zmętnienia połączonej z chromatografią cieczową i tandemową spektrometrią mas (**Publikacja 1**).
- Opracowanie i walidację metody oznaczania dutasterydu i jego metabolitów w osoczu ludzkim (**Publikacja 2**).
- Optymalizację liczby próbek kontroli jakości (QC) w teście stabilności podczas walidacji metody bioanalitycznej (**Publikacja 3**).
- Ocenę wpływu kolejności analizy próbek w teście wpływu matrycy podczas walidacji metody bioanalitycznej (**Publikacja 4**).
- Ocenę stabilności dutasterydu w osoczu ludzkim po trzech latach od momentu pobrania próbek w klinice (**Publikacja 2**, **Doniesienie zjazdowe 3**).
- Ocenę stabilności arypiprazolu w osoczu ludzkim po siedmiu latach od momentu pobrania próbek w klinice (**Doniesienie zjazdowe 2**).
- Analizę zmian wprowadzonych w wytycznej ICH M10, dotyczącej walidacji metod bioanalitycznych oraz analizy próbek z badania, w porównaniu z wytyczną FDA oraz EMA (**Publikacja 5**).

Piśmiennictwo

1. European Medicines Agency, Guideline on bioanalytical method validation, **2011**.
2. U.S. Department of Health and Human Services, Food and Drug Administration (FDA). Guidance for Industry. Bioanalytical Method Validation, **2018**.
3. OECD Principles on Good Laboratory Practice, OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring Number 1, **1997**.
4. European Medicines Agency, ICH guideline M4 (R4) on common technical document, **2021**.
5. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Final endorsed Concept Paper M10: Bioanalytical Method Validation. 7 October 2016 [dostęp 17.09.2023] https://database.ich.org/sites/default/files/M10_EWG_Concept_Paper.pdf.
6. ICH Public Events, US FDA and Health Canada Regional Public Consultation on ICH, Overcoming of ongoing ICH Topics [dostęp 17.09.2023] https://www.fda.gov/media/124548/download s.72.
7. European Medicines Agency, Draft ICH guideline M10 on bioanalytical method validation, **2019**.
8. Madej K., Paprotny Ł., Wianowska D., Kasprzyk J., Herman M., Piekoszewski W. A fully validated HPLC–UV method for determination of sulthiame in human serum/plasma samples. Biomedical Chromatography **2021**; 35:e5002.
9. Lee J.B., Zgair A., Kim T.H., Kim, M.G., Yoo, S.D., Fischer P.M., Gershkovich P. Simple and sensitive HPLC-UV method for determination of bexarotene in rat plasma. Journal of Chromatography B **2017**; 1040:73-80.
10. Jiang M., Wang L., Huang S., Xu L., Hu C., Jiang W. Pharmacokinetic study of 14-(3-Methylbenzyl)matrine and 14-(4-Methylbenzyl)matrine in rat plasma using liquid chromatography-tandem mass spectrometry. Plos One **2015**; 10:e0116010.
11. Lu W., Pan M., Ke H., Liang J., Liang W., Yu P., Zhang P., Wang Q. An LC-MS/MS method for the simultaneous determination of 18 antibacterial drugs in human plasma and its application in therapeutic drug monitoring. Frontiers in Pharmacology **2022**; 13: 1044234.
12. Sharma P., Guttikar S., Solanki G., Patel D.P., Shrivastav, P.S. Determination of (S)-(+)- and (R)-(-)-ibuprofen enantiomers in human plasma after chiral precolumn derivatization by reversed-phase LC–ESI-MS/MS. Bioanalysis **2012**; 4:2909-2927.
13. Cheng Y., Chen M., Zhang B., Lin H., Li X., Cai Y., Zhang H., Que W., Liu M., Qiu H. Rapid, simple, and economical LC–MS/MS method for simultaneous determination of ceftazidime and avibactam in human plasma and its application in therapeutic drug monitoring. Journal of Clinical Pharmacy and Therapeutics **2022**; 47:1426-1437.
14. Desfontaine V., Guinchard S., Marques S., Vocat A., Moulfi F., Versace F., Huser-Pitteloud J., Ivanyuk A., Bardinet C., Makarov V., Ryabova O., André P., Prod'Hom S., Chtioui H., Buclin T., Cole S.T., Decosterda L. Optimized LC-MS/MS quantification of tuberculosis drug candidate macozinone (PBTZ169), its dearomatized Meisenheimer Complex and other metabolites, in human plasma and urine. Journal of Chromatography B **2023**; 1215:123555.
15. Islam M.M., Kub E.F., Rajaratnam V., Mirza S.P. The development and validation of sensitive LC-MS/MS method for quantitative bioanalysis of carmofur in mouse plasma and its application to pharmacokinetic study. Journal of Chromatography B **2022**; 1212:123516.
16. Lakeev A.P., Yanovskaya E.A., Yanovsky V.A., Andropov M.O., Frelikh G.A., Yu. Chukicheva I., Kutchin A.V. LC-MS/MS method for the determination of a semi-synthetic phenolic antioxidant 2,6-diisobornyl-4-methylphenol in rats after different administration routes. Journal of Chromatography B **2022**; 1213:123537.
17. Nguyen T.-T.-L., Kim J.W., Choi H.-I., Maeng H.-J., Koo T.-S. Development of an LC-MS/MS

method for ARV-110, a PROTAC molecule, and applications to pharmacokinetic studies. Molecules **2022**; 27:1977.
18. Thomann J., Ley L., Klaiber A., Liechti M.E., Duthaler U. Development and validation of an LC-MS/MS method for the quantification of mescaline and major metabolites in human plasma. Journal of Pharmaceutical and Biomedical Analysis **2022**; 220:114980.
19. World Health Organization, Good Clinical Laboratory Practice (GCLP), **2009**.
20. Krens S.D., van der Meulen E., Jansman F.G.A., Burger D.M., van Erp N.P. Quantification of cobimetinib, cabozantinib, dabrafenib, niraparib, olaparib, vemurafenib, regorafenib and its metabolite regorafenib M2 in human plasma by UPLC–MS/MS. Biomedical Chromatography **2020**; 34: e4758.
21. Li W., Zhang J., Tse F.L.S. Strategies in quantitative LC-MS/MS analysis of unstable small molecules in biological matrices. Biomedical Chromatography **2011**; 25:258-277.
22. Gałuszka A., Migaszewski Z., Namieśnik J. The 12 principles of green analytical chemistry and the significance mnemonic of green analytical practices. Trends in Analytical Chemistry **2013**; 50:78-84.
23. Garofolo F., Savoie N., Bergeron A., Amestoy C., Lefebvre M., Caillé L., Furtado, M., Menard C. Importance of metabolite testing in regulated bioanalysis. Bioanalysis **2010**; 2:1171-1174.
24. Timmerman P., Blech S., White S., Green M., Delatour C., McDougall S., Mannens G., Smeraglia J., Williams S., Young G. Best practices for metabolite quantification in drug development: updated recommendation from the European Bioanalysis Forum. Bioanalysis **2016**; 8:1297-1305.
25. U.S. Department of Health and Human Services, Food and Drug Administration (FDA). Guidance for Industry: Safety Testing of Drug Metabolites, **2020**.
26. Aggarwal S., Thareja S., Verma A., Bhardwaj T.R., Kumar M. An overview on 5α-reductase inhibitors. Steroids **2010**; 75:109-153.
27. Gisleskog P.O., Hermann D., Hammarlund-Udenaes M., Karlsson M.O. The pharmacokinetic modelling of GI198745 (dutasteride), a compound with parallel linear and nonlinear elimination. British Journal of Clinical Pharmacology **1999**; 47:53-58.
28. Evans H.C., Goa K.L. Dutasteride. Drugs & Aging **2003**; 20:905-916.
29. Roehrborn C.G., Marks L.S., Fenter T., Freedman S., Tuttle J., Gittleman M., Morrill B., Wolford E.T. Efficacy and safety of dutasteride in the four-year treatment of men with benign prostatic hyperplasia. Urology **2004**; 63:709-715.
30. Prasaja B., Harahap Y., Lusthom W., Yumi L., Sofiana A., Sandra M., Safira F., Chilmi U. Impact of truncated area on point estimate and intra-subject variability in bioequivalence of dutasteride with long half-life. Drug Research **2018**; 68:238-240.
31. Keam S.J., Scott L.J. Dutasteride. Drugs **2008**; 68:463-485.
32. Contractor P., Kurani H., Guttikar S., Shrivastav P.S. Reliable and sensitive determination of dutasteride in human plasma by liquid chromatography–tandem mass spectrometry. Biomedical Chromatography **2013**; 27: 1168-1176.
33. Ramakrishna N.V.S., Vishwottam K.N., Puran S., Koteshwara M., Manoj S., Santosh M. Selective and rapid liquid chromatography–tandem mass spectrometry assay of dutasteride in human plasma. Journal of Chromatography B **2004**; 809:117-124.
34. Gomes N., Pudage A., Joshi S., Vaidya V., Parekh S., Tamhankar A. Rapid and sensitive LC–MS–MS method for the simultaneous estimation of alfuzosin and dutasteride in human plasma. Chromatographia **2009**; 69: 9-18.
35. Fossler M.J., Collins D.A., Ino H., Sarai N., Ravindranath R., Bowen C.L., Burns O. Evaluation of bioequivalence of five 0.1 mg dutasteride capsules compared to one 0.5 mg dutasteride capsule: a randomized study in healthy male volunteers. Journal of drug assessment **2015**; 4: 24-29.

36. Kang M.J., Cho, H., Yeom D., Choi Y., Choi Y. A method to monitor dutasteride in rat plasma using liquid-liquid extraction and multiple reaction monitoring: comparisons and validation. Mass Spectrometry Letters **2014**; 5:79-83.
37. Upreti R., Naredo G., Faqehi A.M.M., Hughes K.A., Stewart L.H., Walker B.R., Homer N.Z.M., Andrew R. Simultaneous pharmacokinetic and pharmacodynamic analysis of 5α-reductase inhibitors and androgens by liquid chromatography tandem mass spectrometry. Talanta **2015**; 131:728-735.
38. Makukha O.G., Ivashchenko L.A., Zaporozhets O.A., Doroschuk V.O. Cloud point extraction combined with HPLC–MS for the determination of nimesulide in biological samples. Chemical Papers **2019**; 73:693-699.
39. Giebułtowicz J., Kojro G., Buś-Kwaśnik K., Rudzki P.J., Marszałek R., Leś A., Wroczyński P. Cloud-point extraction is compatible with liquid chromatography coupled to electrospray ionization mass spectrometry for the determination of bisoprolol in human plasma. Journal of Chromatography A **2015**; 1423:39-46.
40. Kojro G., Rudzki P.J., Pisklak D.M., Giebułtowicz J. Matrix effect screening for cloud-point extraction combined with liquid chromatography coupled to mass spectrometry: Bioanalysis of pharmaceuticals. Journal of Chromatography A **2019**; 1591:44-54.
41. Ates E., Mittendorf K., Senyuva H. LC/MS method using cloud point extraction for the determination of permitted and illegal food colors in liquid, semiliquid, and solid food matrixes: single-laboratory validation. Journal of AOAC International **2019**; 94:1853-1862.

3. Kopie opublikowanych prac wchodzących w skład rozprawy

Publikacja 1 (oryginalna)

Gniazdowska E., Korytowska N., Kłudka G., Giebułtowicz J. Determination of antidepressants in human plasma by modified cloud-point extraction coupled with mass spectrometry. Pharmaceuticals. 2020; 13:1-25. IF = 5,863; MEiN = 100.

pharmaceuticals

MDPI

Article

Determination of Antidepressants in Human Plasma by Modified Cloud-Point Extraction Coupled with Mass Spectrometry

Elżbieta Gniazdowska [1,2], Natalia Korytowska [3], Grzegorz Kłudka [3] and Joanna Giebułtowicz [3,*]

1 Łukasiewicz Research Network, Industrial Chemistry Institute, 8 Rydygiera, 01-793 Warsaw, Poland; elzbieta.gniazdowska@wum.edu.pl
2 Department of Bioanalysis and Drugs Analysis, Doctoral School, Medical University of Warsaw, 61 Żwirki i Wigury, 02-091 Warsaw, Poland
3 Department of Bioanalysis and Drugs Analysis, Faculty of Pharmacy, Medical University of Warsaw, 1 Banacha, 02-097 Warsaw, Poland; nkorytowska@wum.edu.pl (N.K.); grzegorz.kludka@gmail.com (G.K.)
* Correspondence: joanna.giebultowicz@wum.edu.pl

Received: 5 October 2020; Accepted: 7 December 2020; Published: 12 December 2020

Abstract: Cloud-point extraction (CPE) is rarely combined with liquid chromatography coupled to mass spectrometry (LC–MS) in drug determination due to the matrix effect (ME). However, we have recently shown that ME is not a limiting factor in CPE. Low extraction efficiency may be improved by salt addition, but none of the salts used in CPE are suitable for LC–MS. It is the first time that the influences of a volatile salt—ammonium acetate (AA)—on the CPE extraction efficiency and ME have been studied. Our modification of CPE included also the use of ethanol instead of acetonitrile to reduce the sample viscosity and make the method more environmentally friendly. We developed and validated CPE–LC–MS for the simultaneous determination of 21 antidepressants in plasma that can be useful for clinical and forensic toxicology. The selected parameters included Triton X-114 concentration (1.5 and 6%, *w/v*), concentration of AA (0, 10, 20 and 30%, *w/v*), and pH (3.5, 6.8 and 10.2). The addition of 10% of AA increased recovery twice. For 20 and 30% (*w/v*) of AA, three phases were formed that prolonged the extraction process. The developed CPE method (6% Triton X-114, 10% AA, pH 10.2) was successfully validated through LC–MS/MS simultaneous determination of 21 antidepressants in human plasma. The linearity was in the range of 10–750 ng/mL ($r^2 > 0.990$).

Keywords: sample preparation; LC–MS/MS; antidepressant; bioanalytical methods

1. Introduction

Cloud-point extraction (CPE) is a modification of liquid–liquid extraction (LLE) that is more friendly to the environment and users, mainly due to lower consumption of organic solvents according to the global trend of "green chemistry." CPE has also advantages over solid phase extraction (SPE), such as faster and cheaper optimization processes and no need for expensive equipment [1]. In CPE, the surfactant (such as Triton X-114) in a concentration above the critical micelle concentration (CMC) can exist as a homogeneous isotropic liquid, which separates into two isotropic phases with different concentrations of surfactant. Sample ingredients are separated into the surfactant micelle-rich phase (hydrophobic components when nonionic surfactant is used) and the micelle-poor phase (hydrophilic components) [2].

Nowadays, the most reliable technique of pharmaceutical determination is liquid chromatography coupled to mass spectrometry (LC–MS). However, there are only a very few papers reporting LC–MS coupled with CPE in drug determination, i.e., in the determination of memantine in rat plasma [3],

and bisoprolol [4], antazoline [1], abacavir, efavirenz, lamivudine, and belfinavir in human plasma [5]. The disadvantage of the CPE–LC–MS, contrary to LLE–LC–MS, is the lower extraction efficiency (recovery). Recovery for bisoprolol was 46−61% using CPE and 74−86% using LLE [4]. In CPE methods coupled with other techniques than mass spectrometry, non-volatile salts such as NaCl [6–9], Na_2SO_4 [10], $Na_2B_4O_7$ [11], and $(NH_4)_2SO_4$ [12] are used to improve the efficiency of extraction. However, these salts are incompatible with LC–MS due to possible matrix effect (ME) and deposition in the ion source. Moreover, they crystallize in the capillary lumen resulting in the additional need for cleaning and maintenance of LC–MS. The alternative approach is the usage of volatile salts such as ammonium acetate (AA) and formate. These salts are the commonly used modifiers of a mobile phase in positive ionization mode in LC–MS. However, there are no reports on their application in CPE–LC–MS.

Determination of antidepressants in biological samples is necessary for the effective and safe treatment of depression, a common mental disorder. According to World Health Organization (WHO), more than 264 million people of all ages suffer from depression globally. This disease is one of the most serious public health problems [13]. Moreover, the treatment of depression is a complex issue, sometimes being associated with dysphoric, mixed, agitated, or psychotic states that can increase suicidal risk [14]. Moreover, antidepressants are frequently used together with other legal or illegal drugs and can result in a synergy of symptoms and intoxication. The drugs can be also used in intentional poisoning [15,16]. The antemortem screening analysis can be useful in cases of suspected poisoning or vehicle accidents in clinical or forensic toxicology investigations. The screening method should allow one to rapidly determine and quantify the wide spectrum of compounds. Thus, the method of simultaneous determination of antidepressants from different classes is needed.

There are many LC methods for the determination of antidepressants using various detectors, e.g., UV–Vis, photodiode-array (PDA), diode-array (DAD), and fluorescence detector. Recently, MS has been also extensively employed in the analysis of complex biological samples especially [17]. Since detectors based on UV–visible spectrometry are universal, less expensive, and less complicated than LC–MS, they are commonly used in routine laboratories. They frequently provide a satisfactory limit of quantitation for antidepressants in plasma (even 5 ng/mL for some compounds) [18], due to aromatic rings (e.g., phenyl group and naphthalene group) and other major UV chromophores present in their structures. Those detectors are also applied in multi-methods, permitting one to simultaneously determine up to 11 antidepressants in plasma (LC–PDA) [19]. However, the conventional detectors are less selective than LC–MS, which is crucial in forensic and clinical toxicology when in samples many legal and illegal compounds may occur. Thus, LC–MS remains the gold standard, especially in legal medicine. Since the method has a drawback of matrix effects and ion suppression issues, the careful validation needs to be performed [17].

LC–MS methods of antidepressant determination use electrospray ionization in positive ion mode (ESI+) [20–28], atmospheric pressure chemical ionization in positive ion mode (APCI+) [29], or QTOF MS in positive ionization mode with a DuoSpray ion source [30]. Samples were prepared using LLE [20,26,29], microextraction by packed sorbent (MEPS) [28], protein precipitation (PP) [25,30], SPE [22], on-line SPE [21,23], capillary restricted-access media (RAM) [27], and online-restricted access molecularly imprinted solid-phase extraction (RAMIP-BSA) [24]. All methods require the use of environmentally harmful organic solvents. As an alternative, we aim to develop the environmentally friendly CPE that can be coupled with mass spectrometry. The novel CPE protocol provides better recoveries than the previous studies reporting the use of CPE–LC–MS [1,4].

This study aimed to examine the effects of one volatile salt AA's addition on recovery and the matrix effect of the CPE–LC–MS method. To make the extraction method more environmentally friendly, we used ethanol instead of methanol or acetonitrile to reduce the sample viscosity at the last point of sample preparation. We have developed the method for the simultaneous determination of 21 major antidepressants. The compounds were from four different classes—i.e., non-selective monoamine reuptake inhibitors (N06AA): amitriptyline, clomipramine, desipramine, doxepin, imipramine, maprotiline,

nortriptyline, opipramol, protriptyline, and trimipramine; selective serotonin reuptake inhibitors (SSRIs)(N06AB): citalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline; monoamine oxidase A inhibitors (N06AG): moclobemide; and other antidepressants (N06AX): mianserin, mirtazapine, tianeptine, trazodone, and venlafaxine. The selected pharmaceuticals have different structures and chemical properties, and logP and pKa range from 1.5 to 5.2 and 6.0 to 10.5, respectively. The analyzed parameters for the simultaneous determination of the compounds were the concentration of non-ionic surfactant Triton X-114, sample pH, and AA concentration. Finally, validation parameters were compared with several reported methods.

2. Results and Discussion

2.1. Development of Cloud-Point Extraction

The analytical method was developed for determination of such antidepressants as amitriptyline (AMI), citalopram (CIT), clomipramine (CLO), desipramine (DES), doxepin (DOX), fluoxetine (FLX), fluvoxamine (FLV), imipramine (IMI), maprotiline (MAP), mianserin (MIA), mirtazapine (MIR), moclobemide (MOC), nortriptyline (NOR), opipramol (OPI), paroxetine (PAR), protriptyline (PRO), sertraline (SER), tianeptine (TIA), trazodone (TRA), trimipramine (TRI), and venlafaxine (VEN) in human plasma by LC–MS/MS (Figure 1).

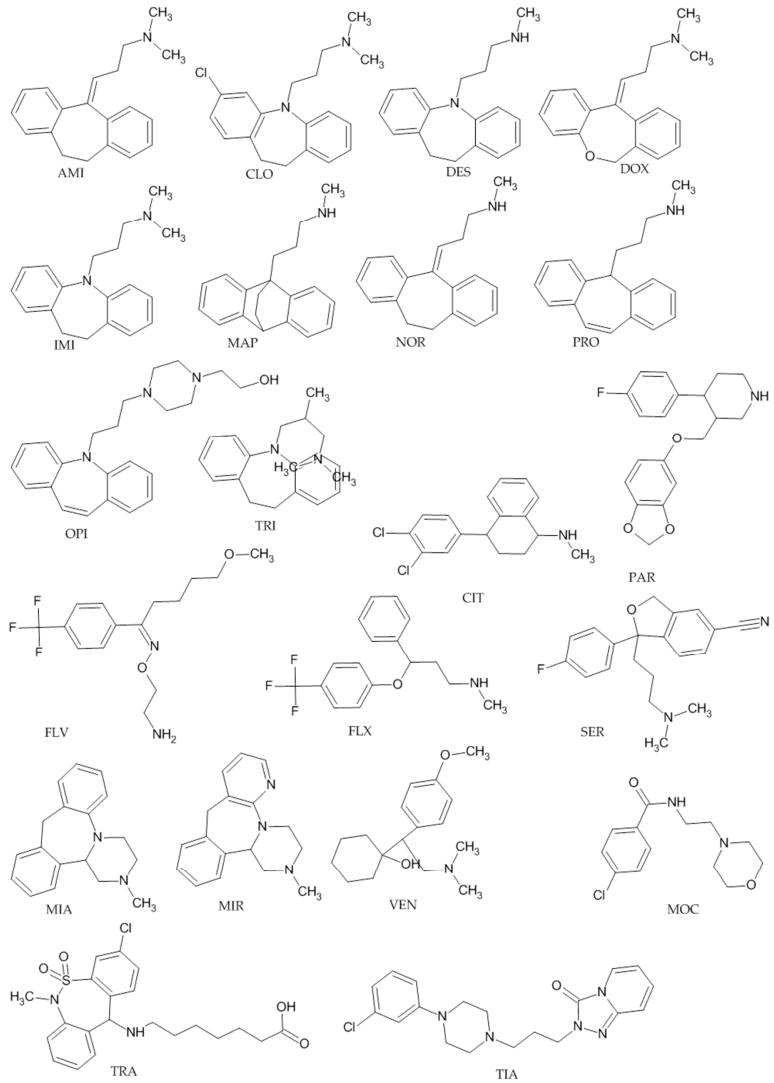


Figure 1. Chemical structures of antidepressants studied in the experiment.

The initial factors of extraction included pH 6.8, temperature 60°C, and 20% of AA. To reach pH 6.8, only water was used. Thus, pH 6.8 was selected to reduce the time of the process, reduce the cost, and diminish the risk of contamination. AA concentration was selected as a medium value from all tested in the experiment. The level of antidepressants corresponded to a plasma concentration of 100 ng/mL.

Many factors affect CPE efficiency. In this paper, the influences of surfactant concentration (Triton X-114), volatile salt (AA—0, 10, 20, and 30% (*w/v*)), and extraction pH (about 3.5, 6.8, 10.2) was tested to select the optimal conditions for the simultaneous determination of 21 antidepressants. Other parameters, i.e., volume of reagents and equilibrium time, were used as described previously [2,31]. However, in the current study higher temperature was selected (60 °C). The optimal temperature for CPE is 15–20 °C greater than the cloud point of the surfactant, which in the case of Triton X-114 is 23 °C, and was reviewed to be 40–60 °C [32]. With an increase in temperature, the efficacy of the extraction increases, and the volume of the surfactant-rich phase decreases due to the disruption of the hydrogen bonds and the dehydration of the phase [32].

The optimal conditions (concentration of Triton-X-114 and AA, pH sample) were defined as the ones showing the highest mean recovery and the lowest absolute ME (ME_A) (Equation (1)) [33], for simultaneously analyzing all 21 antidepressants. The influences of selected parameters are presented in Figures 2–5 and summarized in Figure 6. The chromatography separation was not optimized. We used the gradient elution mode, eluent flow rate, and analytical column commonly used in our analysis [31].

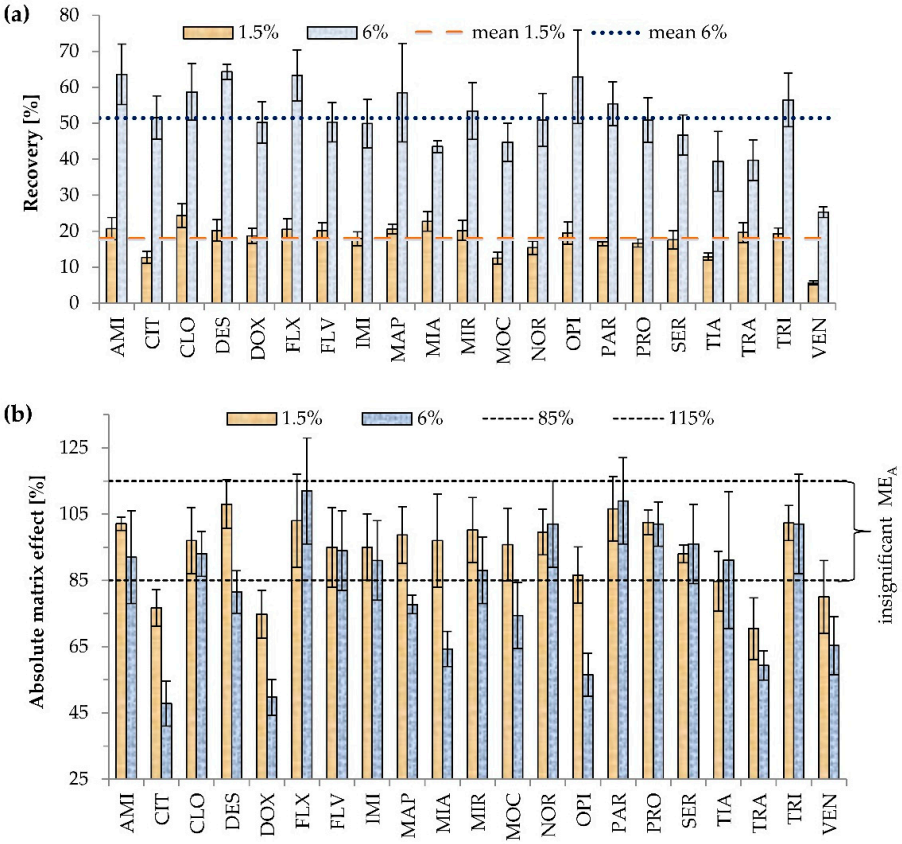


Figure 2. Effects of Triton X-114 concentration on (**a**) recovery ($p < 0.001$), (**b**) absolute matrix effect (ME_A) ($p = 0.182$). Extraction conditions: equilibrium temperature—60°C; equilibrium time—20 min; sample pH 6.8; ammonium acetate content—20% (*w/v*). Antidepressants level corresponds to a plasma concentration of 100 ng/mL Higher mean recovery was observed with higher Triton X-114 concentration (the experiment was repeated three times) ($p < 0.001$). Results are presented as means and standard deviations.

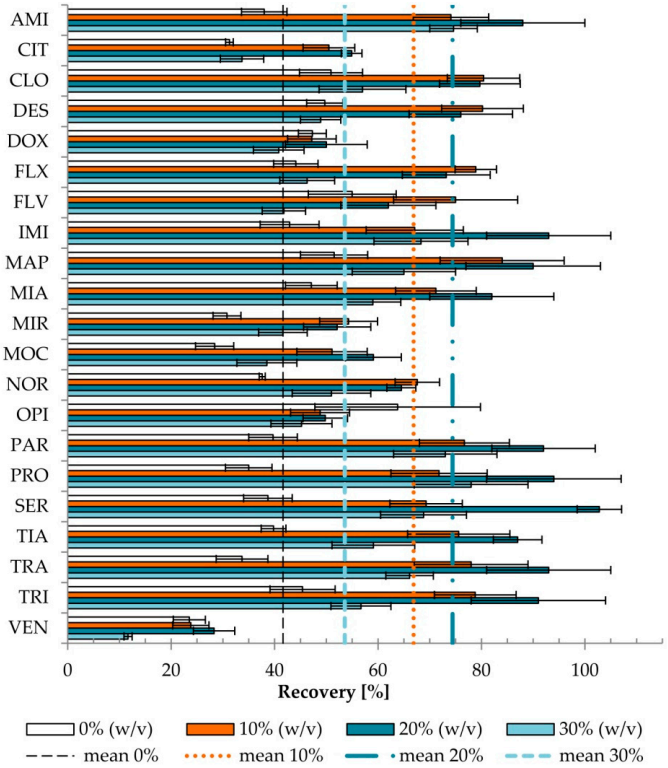


Figure 3. The effect of ammonium acetate concentration (0, 10, 20, and 30% (*w/v*)) on recovery ($p < 0.001$). Extraction conditions: equilibrium temperature—60 °C; equilibrium time—20 min; sample pH, 6.8; concentration of Triton X-114–6% (*w/v*). Antidepressants level corresponds to a plasma concentration of 100 ng/mL. Results are presented as means and standard deviations. Mean recoveries for 0% AA (dashed line), 10% AA (dotted line), 20% AA (dotted-dashed line), and 30% AA (bold dashed) are shown.The highest increases of recovery, as a result of AA addition, were observed for AMI, PAR, PRO, TIA, and TRA, whereas the weakest effect was recorded for both FLV and MIA. No increase in recovery for VEN or decrease for OPI was observed. The increase of recovery (10% *vs.* 0 % (*w/v*)) was correlated ($r_s = 0.465$, $p = 0.034$) with the calculated octanol–water partition coefficient (cLogP).

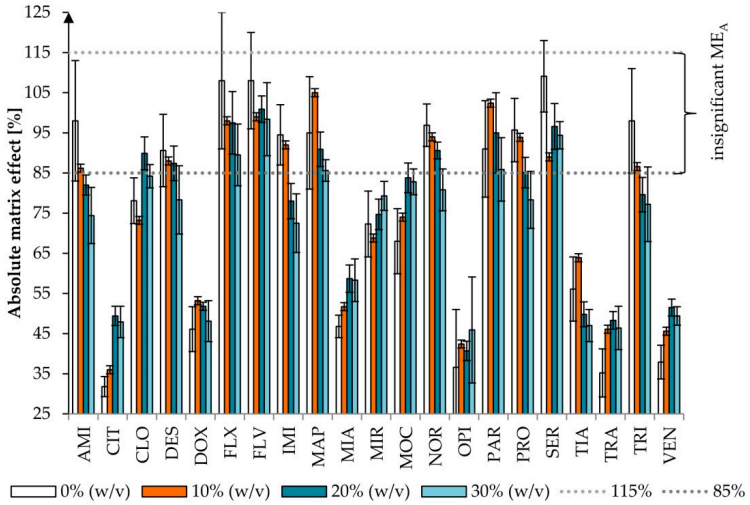


Figure 4. Effect of the addition of ammonium acetate on the absolute matrix effect (ME_A) ($p = 0.198$). Extraction conditions: equilibrium temperature—60°C; equilibrium time—20 min; sample pH—6.8; concentration of Triton X-114—6% (*w/v*). Antidepressants level corresponds to a plasma concentration of 100 ng/mL. Results are presented as means and standard deviations. ME_A between 85% (dotted line) and 115% (dotted line) was considered insignificant.

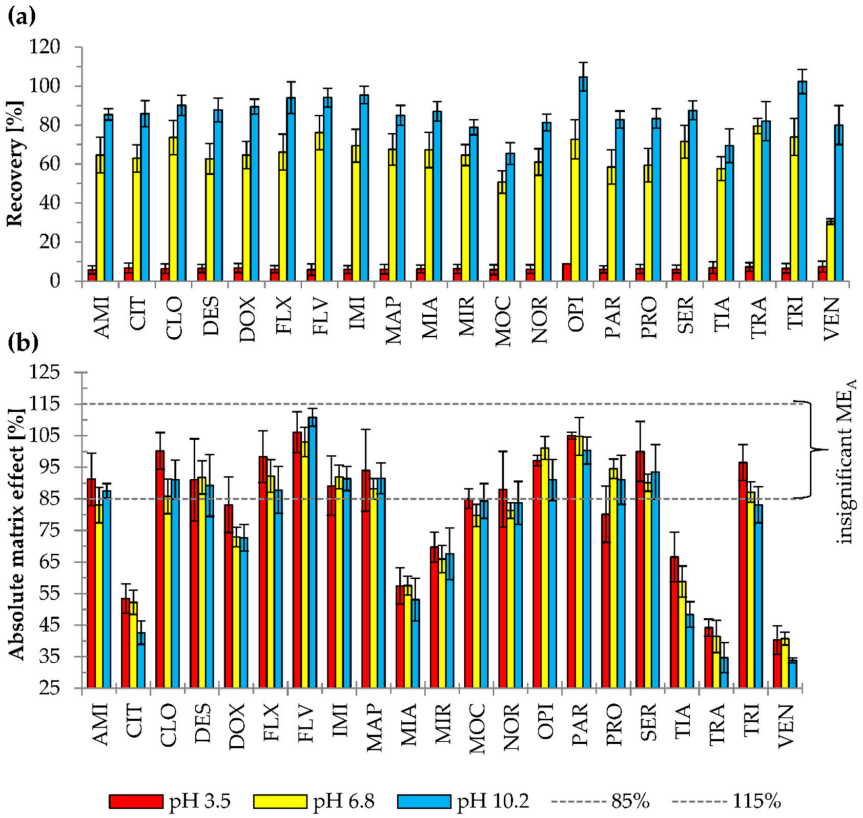


Figure 5. The effects of different pH of a sample on (**a**) recovery ($p < 0.001$), (**b**) absolute matrix effect (ME_A) ($p = 0.514$). Results are presented as means and standard deviations. ME_A between 85% (dashed line) and 115% (dashed line) was considered as insignificant. Extraction conditions: Triton X-114 at 6% (*w*/*v*); ammonium acetate at 10% (*w*/*v*); equilibrium temperature—60°C; equilibrium time—20 min. Antidepressants level corresponds to plasma concentration of 100 ng/mL.

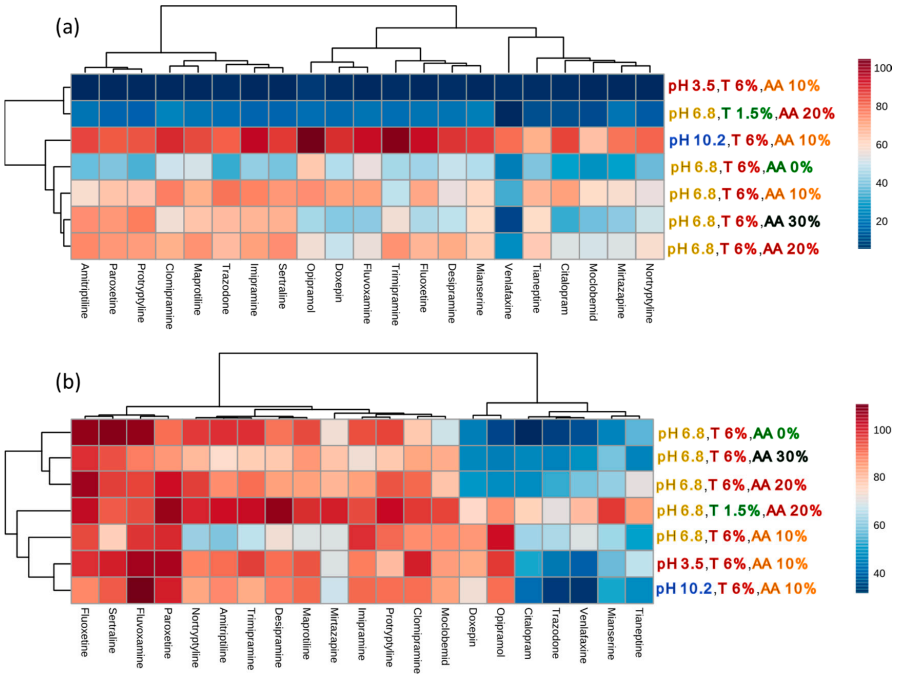


Figure 6. The effects of cloud-point extraction conditions on (**a**) the recoveries and (**b**) the absolute matrix effects of 21 antidepressants determined using liquid chromatography coupled with mass spectrometry. T—Triton concentration (*w*/*v*), AA—ammonium acetate concentration (*w*/*v*).

2.1.1. The Effects of Triton X-114

The concentration of Triton X-114, used as the surfactant in CPE, was very carefully selected. This was because the overly low concentration of Triton X-114 decreases recovery and prevents effective separation of the aqueous and micellar phases. On the other hand, too high concentration of surfactant is associated with a high volume of the micelle-rich phase, and the unwanted dilution of the analyte [5,10,32]. The range of tested Triton X-114 concentrations, added to samples in multi-compound determination, varied from 1.5 to 9% (*w*/*v*) [1,2,4,5,8,32], with the most frequently used concentration being 4% [32]. In this study, two variants of Triton concentration (1.5 and 6%) were studied. The effects of the surfactant concentration on recovery and ME_A are shown in Figure 2.

The volume of the micellar rich phase was about 100 µL, and the initial volume of the sample was 1 mL, so the analytes were concentrated about 10 times. Over twofold increases in recovery at higher Triton X-114 concentration were noted for CLO, DOX, FLV, IMI, MAP, MIR, SER, and TRA; over threefold increases for AMI, CIT, DES, FLX, MOC, NOR, OPI, PAR, PRO, and TIA; and the highest for VEN (4.5 times). A lower than twofold increase was noted only for MIA. Higher recoveries at 6% Triton were expected based on the partition coefficient equation. The partition coefficient is characteristic for the compound and the extraction conditions (e.g., type of surfactant, pH). It is calculated as the ratio of antidepressant concentration in the surfactant to that in the aqueous phase [34]. Thus, with the increase of the surfactant rich phase volume, the mass of the extracted compound increases as well.

Significant ME_A values were observed for four (CIT, DOX, TRA, and VEN) and nine antidepressants (CIT, DES, DOX, MAP, MIA, MOC, OPI, TRA, and VEN) out of 21 analyzed at 1.5 and 6% Triton X-114, respectively. In contrast to 6%, at 1.5% Triton X-114 concentration, ME_A was not observed for DES, MAP, MIA, MOC, and OPI. Higher surfactant concentration is related to a higher risk of ME_A, as reported previously [31]. The possible reason is the interference of surfactant with droplet evaporation or higher recovery not only of analytes, but also impurities of samples that interfere with the analyte ionization. However, in the current study, the differences in the occurrence of ME_A for all 21 antidepressants between the two surfactant concentrations were not statistically significant ($p = 0.182$). The concentration of 6% (*w*/*v*) Triton X-114 was selected as optimal considering higher mean recovery for 21 antidepressants and comparable ME_A.

2.1.2. The Effects of Salt Addition

Salts affect the extraction efficiency by decreasing or increasing the analyte concentration in the aqueous phase and CMC. Effects of salt addition depend on the concentrations of salts and the type of surfactant used. For the nonionic surfactants, such as Triton X-114, the value of CMC decreases with an increasing concentration of the electrolyte [35–40]. The anions (i.e., Cl^- and SO_4^{2-}) are likely to decrease self-association of water molecules, whereas the cation (i.e., Na^+) may decrease the cloud point by dehydration of the polyoxyethylene chain [39,41]. Electrolytes support demulsification, which is needed to limit the amount of impurities in the final surfactant-rich phase [40]. The other important effects of electrolytes are the decrease in the cloud point temperature and promotion of phase separation by increasing the density of aqueous phase [8,41]. However, overly large amounts of salts can cause the formation of three phases, as observed in our study [8].

The most frequently used concentration of different salts is 4–6% [32], increasing the recovery of the analyte by 10–20%. The opposite effect was observed if the salt concentration ranged from 7 to 10%. Improvement of extraction efficiency associated with salt addition was probably due to the salting-out effect, which reduces the amount of water to dissolve the analyte. While the overly high concentration of salt will competitively carry substances into the protein deposition, it can lower the concentration of drug in the solution and will have an impact on the recovery [41]. Even if the addition of salt increases the recovery, non-volatile salts contaminate the ion source in LC–MS. Thus, we tested the addition of the volatile salt (AA) in three variants: 10, 20, and 30% (*w*/*v*) (sample pH 6.8, 6% (*w*/*v*)

of Triton X-114). The influences of various concentrations of salt added on the recovery and ME_A were evaluated (Figures 3 and 4).

There was a statistically significant difference ($p < 0.001$) in recovery depending on the concentration of AA used in the tested conditions (sample pH 6.8, 6% Triton X-114). The differences occurred between all sample variants. The addition of 10% (*w/v*) AA increased recovery 1.64 times on average (range 0.87–2.31), while 20% (*w/v*) AA increased recovery 1.85 times on average (range 0.88–2.76), compared with the sample without the addition of AA.

The addition of 30% (*w/v*) AA resulted in lower recovery than the addition of 10% or 20% (*w/v*) of AA. Moreover, for 20 and 30% (*w/v*) of AA, three phases were observed (from the bottom of the tube: an aqueous phase, a micelle-rich phase, an aqueous phase), unlike for 0 and 10% (*w/v*) of AA, for which only two phases were formed (lower layer—micelle-rich phase; and aqueous surfactant—lean phase above). To separate the micelle-rich phase, an upper surfactant-lean phase was decanted. In the cases of 20 and 30% (*w/v*) of AA, the microsyringe should be used to remove the lower aqueous surfactant-lean phase from the bottom of the test tube. Thus, although the mean recovery was higher for 20% (*w/v*) AA, the addition of 10% (*w/v*) AA was selected as the most convenient concentration. Similar observations of the influence of electrolytes on the location of the micellar phases in the tube were reported [35].

ME_A can be observed as signal suppression (below 100%) or an enhancement (above 100%) in the presence of a matrix. Significant ME_A <85% or >115% was observed for 10, 10, 12, and 16 out of 21 studied drugs for 0, 10, 20, and 30% (*w/v*) of AA, respectively. The differences were not statistically significant (χ^2 test, $p = 0.198$), nor were they in statistical analysis performed only for the compounds with significant ME_A ($p = 0.244$). In all variants of AA, insignificant ME_A was observed for five compounds: FLX, FLV, MAP, PAR, and SER. For 0 and 10% (*w/v*) of AA, that was additionally observed for AMI, IMI, and TRI, whereas for 0, 10, and 20% (*w/v*) of AA, that was the case for DES, NOR, and PRO.

The effect of salt adding (sodium chloride) on the recovery of only one antidepressant venlafaxine in the CPE procedure was reported for the concentration range 0.1–0.5 M. The concentration of NaCl 0.3 M was chosen as optimal [8]. In this work, AA was used first time in CPE procedure combined with LC–MS not only for venlafaxine determination but also other 20 antidepressants. As the optimal concentration of salt, 1.3 M AA (10% (*w/v*) of AA) was selected.

2.1.3. The Effects of pH

Depending on the sample pH, analytes can be either charged or uncharged. That also depends on the chemical properties of said analytes. The interaction of the analyte with the micellar aggregate formed by a nonionic surfactant is weaker for ionic than the neutral form. Thus, the highest recovery can be observed at pH where all analytes are uncharged and partitioned into the rich-micellar phase of Triton X-114 or another nonionic surfactant [37,42].

The effect of sample pH was examined using 6% (*w/v*) Triton X-114 and 10% (*w/v*) AA at pH 3.5, 6.8, and 10.2 (Figure 5). The mean recoveries were 6.4, 64.7, and 86.2% for acidic, neutral, and alkaline conditions, respectively. The majority of antidepressants studied have basic and lipophilic properties. In alkaline pH, more particles occur in molecular form; thus, higher affinity to nonionic micelles and higher extraction recovery were observed. At acidic pH, the majority of the antidepressants were in ionic form and had their lowest affinity for nonionic micelles. That resulted in the loss of some of the analytes during phase separation and low extraction efficiency. Moreover, the separation of two phases—the aqueous and micelle-rich ones—in the acidic and neutral pHs, was not as pronounced as in alkaline pH.

Statistical analysis ($p < 0.001$) indicated significant differences in recovery of the extraction performed at various pH values. However, no relationship between the pH of the extraction environment and the number of compounds exhibiting significant ME_A was observed (test χ^2; $p = 0.514$). Statistical analysis carried out for compounds with significant ME_A (ANOVA, $p = 0.013$) showed statistically significant differences in ME_A between pHs. The results of the post hoc test revealed that these differences occurred between the alkaline and acidic environment ($p = 0.004$). The mean ME_A was 6.1% lower for samples with a pH of 3.5 than those of pH 10.2. However, it should be emphasized that in acidic conditions, the basic compounds have less affinity to nonionic surfactant micelles, which resulted in the lower analytical signal and lower recovery. Moreover, the separation into two phases was not distinct in acidic pH or neutral pH, which caused the loss of analytes during phases separation. Thus, pH 10.2 was selected as optimal to use in the final method.

Madej and Persona [2] developed a CPE to extract basic compounds (pKa 9.0–9.5) at pH 12, with Triton X-114 at 3.25% (*w/v*), without salt addition. The recoveries were 26.2, 81.4, 77.3, and 98.5% for paracetamol, amitriptyline, clomipramine, and promazine, respectively [2]. In this paper, the number of isolated compounds from plasma in the basic environment (pH 10.2) was much higher ($n = 21$ *vs.* $n = 4$) and the pKa range was wider (pKa 6.0–10.5). A higher mean recovery (86.2%) was observed than in the reported experiment (70.9%). Compared to the recovery of particular compounds, higher recoveries were achieved for AMI (81.4 *vs.* 85.4%) and CLO (77.3% *vs.* 90.1%). OPI was extracted as a neutral compound at pH 6, which resulted in 21.8% recovery, while in this study at pH 10.2, the recovery was 102.5% [2]. Thus, the extraction at pH 10.2 and the addition of AA allow for efficient isolation of antidepressants from biological matrices by the CPE method. Additionally, the extraction environment does not significantly affect the number of compounds exhibiting a matrix effect.

2.1.4. Principal Component Analysis (PCA)

Some compounds revealed significant ME_A in all tested conditions (CIT, DOX, TRA, VEN). All of them showed a significant surfactant effect (the ratio of peak area of the analyte in a solvent with the surfactant to that without the surfactant) previously [31]. That means that the ME_A observed is not related to impurities extracted from plasma, but the presence of Triton in the sample. PCA was used to determine which molecular descriptors (polar volume, pKa, cLogP, dipole moment, number of hydrogen bond acceptors (HBA), number of hydrogen bond donors (HBD), total volume, molecular mass, total area, polar surface area (PSA)) influenced the ME_A in optimal conditions [31]. Retention time and predicted water solubility were also added to the analysis. Three principal components (PC) accounted for 82.4% of the total variation. PC1 (44.7% of the total variation) was correlated mainly with the number of HBA (−0.91), PSA (−0.90), total area (−0.85), molecular mass (−0.85), and total volume (−0.83). In turn, PC2 (27.1% of the total variation) was correlated with the retention time (−0.90), pKa (−0.77) and solubility (0.94), and PC3 (10.6% of the total variation) with the dipole moment (0.78) and HBD (0.58). The best predictors of ME_A occurrence were PC1 (PSA, total area, total volume, molecular mass, and the number of HBA) and PC2 (retention time, pKa, and predicted solubility) (Figure 7).

Low polar volume; few HBA; low solubility; and high values of retention time, pKa, and cLogP indicated that the ME_A should not be expected. Compounds with lower lipophilicity (low cLogP and high polar volume) and low pKa were prone to significant ME_A [31].

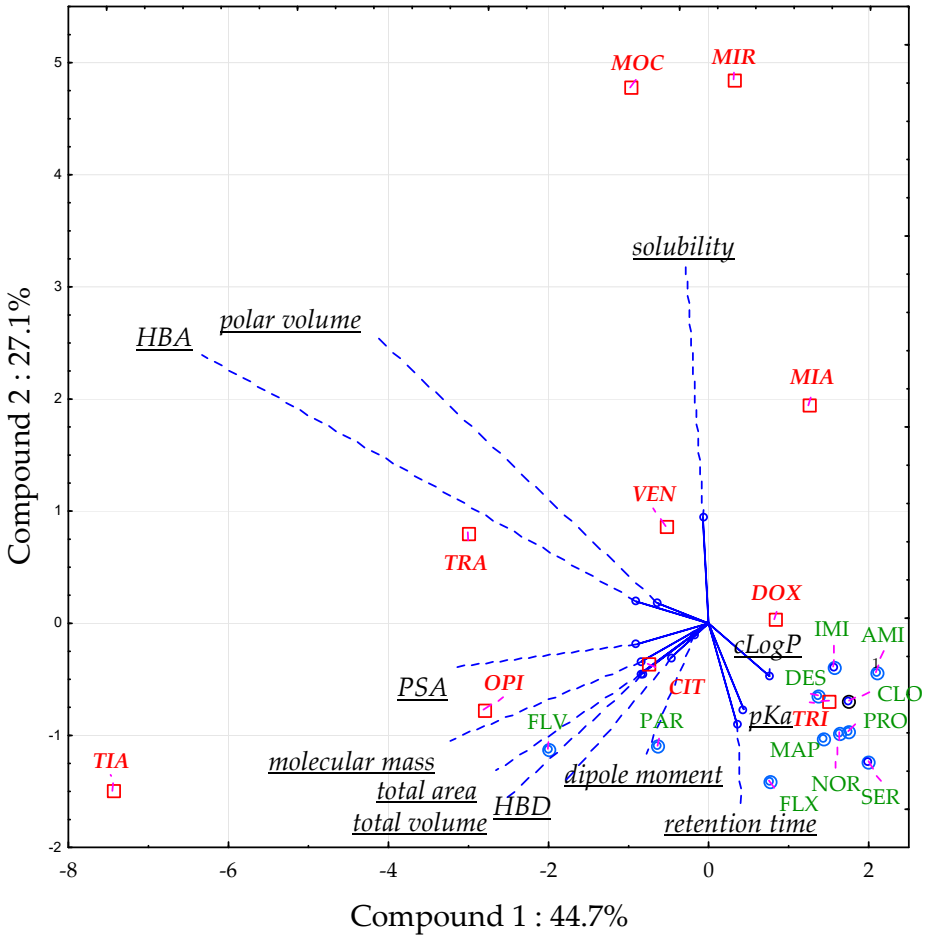


Figure 7. Distribution of the analyzed compounds on a score plot (principal component 2 *vs.* component 1) in a principal component analysis (PCA). The results are presented for the optimal CPE variant (pH 10.2; 6% Triton X-114, 10% (*w/v*) ammonium acetate; equilibrium temperature 60°C; equilibrium time 20 min). HBA—number of hydrogen bond acceptors, HBD—number of hydrogen bond donors, PSA—polar surface area. Compounds labelled with green text exhibited insignificant ME_A, whereas compounds labeled with red—significant ME_A.

2.2. Analytical Performance of the Proposed Method

After development, the analytical method's performance was evaluated in terms of linearity, accuracy, precision, the lower limit of quantification (LLOQ = 10 ng/mL), and stability (autosampler, short-term, freeze and thaw). The interference from metabolites was not studied, since it occurs rarely in mass spectrometry. Due to different masses, metabolites were differentiated from parent compounds using LC–MS (Table A1). In some cases, European Medicines Agency (EMA) guidelines recommend the evaluation of the possibility of back-conversion of a metabolite into the parent analyte during the successive steps of the analysis. However, it is performed only when relevant (i.e., in case of potentially unstable metabolites—e.g., acidic metabolites to esters, unstable N-oxides, or glucuronide metabolites, lactone-ring structures) [43]. It is not an issue in the case of antidepressants.

2.2.1. Linearity

Calibration standards of eight concentrations of all analytes were prepared in blank plasma. The curves (n = 6) were obtained by weighted linear regression analysis with w = 1/x. The regression parameters for all analytes were described by the equation: y = ax + b. The values of a, b, and r^2 for all analytes are presented in Table A2. Good linearities covered the range of 10–750 ng/mL with a coefficient of determination (r^2) > 0.990 were obtained for all analytes regarding the peak area ratio of every analyte to internal standard (IS)versus the nominal concentration.

The linearity range of the method corresponds to therapeutic concentrations of determined antidepressants in human plasma and is similar to the reported ranges [20–22,28,29].

2.2.2. Limit of Quantification, Precision, and Accuracy

Intra-run and between-run accuracy and precision of the method for LLOQ (10 ng/mL) and QC (quality control; 16, 375, and 625 ng/mL) samples for all analytes were determined. For each LLOQ, a signal-to-noise ratio (S/N) higher than five (from 7.0 to 76.1) was observed. The accuracy was within the acceptance criteria of 85–115 and 80–120% for each QC sample and LLOQ, respectively. The precision was within the acceptance criteria, < 15 and < 20% for QC and LLOQ respectively. Precision was in the range 0.6–14.0%, and accuracy was from 85 to 114% (Table 1). Chromatograms of the analyzed antidepressants extracted from blank plasma and high QC are presented in Figure A1.

The results were repeatable and reproducible and met the criteria of the EMA [43] and the U.S. Food and Drug Administration [44] in the guidelines on bioanalytical method validation. The obtained precision is comparable to the reported values for the other extraction methods of isolating the antidepressants from plasma [20,21].

2.2.3. Stability

All analytes and IS were stable under all tested conditions: autosampler stability (stability in the extract, 48 h at 4°C); and stability in human plasma, i.e., short-term stability (3 h at room temperature), and freeze-thaw stability (3 cycles at −20 °C). The QC (16 and 625 ng/mL) samples showed no significant changes in comparison to nominal concentrations. In all cases, accuracy and precision were found to be within the acceptable limits of ± 15% in both cases, as shown in Table A3.

2.2.4. Matrix Effect and Recovery

The significant matrix effect was observed for eight and seven out of 21 antidepressants, for 16 and 625 ng/mL, respectively. However, since the effect was compensated by the addition of the internal standard, it did not affect the method's accuracy and precision. It was proven by the variability of relative matrix factor (MF). The calculated coefficient of variation (CV) of the IS-normalized MF did not exceed 15% for all tested compounds (Table A4). The mean CV values were 10.2% (5.7%–15.0%) and 7.1% (2.6%–13.9%) for the concentrations of 16 and 625 ng/mL, respectively. The maximum mean CV equal to 15.0% was obtained for MIA and VEN for low QC (Table A4). The hemolysis and lipemia did not influence the method reliability.

We compared the mean ME_A with the values reported using other extraction techniques. The values were 122% (96–156%) and 110% (101–125%) for LLE [20], and 93.5% (47.5–115.6%) and 88.1% (48.0–107.8%) for CPE for 17 compounds at lower and higher concentrations, respectively. For five compounds extracted using SPE, the values were 102% (88–121%) and 102% (99–108%) [22] compared to 99.6% (58.2–115.6%) and 92% (54.2–107.8%) for CPE at lower and higher concentrations, respectively. The mean matrix effect of 89% (82-94%) for 12 compounds extracted using on-line SPE (1000 ng/mL) [21] was observed, whereas for CPE (625 ng/mL) was 93.2% (44.5–107.8%). Thus, CPE has a comparable mean ME_A to the other extraction methods, such as LLE or SPE. However, some compounds revealed significant ion suppression.

Table 1. Precision and accuracy intra-run (n = 5) and between-run (n = 15) in human plasma for lower limit of quantification (LLOQ), low QC, medium QC, and high QC.

		LLOQ 10 ng/mL		Low 16 ng/mL		Medium QC 375 ng/mL		High QC 625 ng/mL	
Compound		**Precision [%]**	**Accuracy [%]**	**Precision [%]**	**Accuracy [%]**	**Precision [%]**	**Accuracy [%]**	**Precision [%]**	**Accuracy [%]**
Amitriptyline	intra-run	1.5	92	5.5	107	5.9	86	5.4	98
	between-run	8.2	104	6.4	103	2.3	88	1.5	99
Citalopram	intra- run	6.1	93	4.8	90	7.7	89	6.1	109
	between-run	2.6	93	3.4	90	5.3	86	10	99
Clomipramine	intra- run	6.7	88	3.7	98	10	104	9.1	105
	between-run	9	100	5.4	98	3.3	101	3	108
Desipramine	intra- run	5.1	106	5.6	101	11	92	5.6	95
	between-run	14	102	6.9	99	3	89	4.1	99
Doxepin	intra-run	3.4	104	6.8	99	7.6	110	8.4	113
	between-run	6.2	110	3	96	2.5	103	5	109
Fluoxetine	intra-run	6.7	108	5.4	109	8.1	91	0.8	97
	between-run	0.8	109	0.6	108	0.3	91	0.4	98
Fluvoxamine	intra-run	5.9	108	9.6	102	7.8	108	5.6	109
	between-run	11	85	4.3	95	1.3	100	9	112
Imipramine	intra-run	4.3	94	3.3	99	7.7	85	6.7	104
	between-run	9.3	89	13	92	4.3	89	0.3	112
Maprotiline	intra-run	2.7	96	6.1	89	12	92	7.1	104
	between-run	7.9	97	5.2	88	1.2	93	0.7	105
Mianserin	intra-run	3.8	113	3.9	101	8.8	114	11	93
	between-run	2.9	112	3.5	96	14	97	11	105
Mirtazapine	intra-run	6.2	97	13	101	11	95	5	100
	between-run	12	102	6.2	104	2.8	93	7.2	107
Moclobemide	intra-run	2.4	102	4.3	85	11	90	11	98
	between-run	11	110	7.9	89	4.4	86	1.6	96
Nortriptyline	intra-run	8.7	93	6.9	102	8.1	102	4.1	110
	between-run	9.6	95	14	99	6.7	109	0.2	110
Opipramol	intra-run	5.6	93	5.2	90	3.2	92	9.2	105
	between-run	10	101	3.6	87	3.5	89	4.7	110
Paroxetine	intra-run	7.5	105	7.4	101	12	100	11	91
	between-run	4.5	106	2.9	102	0.8	99	6.7	97

Table 1. *Cont.*

Compound		LLOQ 10 ng/mL Precision [%]	LLOQ 10 ng/mL Accuracy [%]	Low 16 ng/mL Precision [%]	Low 16 ng/mL Accuracy [%]	Medium QC 375 ng/mL Precision [%]	Medium QC 375 ng/mL Accuracy [%]	High QC 625 ng/mL Precision [%]	High QC 625 ng/mL Accuracy [%]
Protriptyline	intra-run	3.7	94	4.8	97	11	90	7.3	94
	between-run	9.5	104	8.5	95	2.1	88	6.8	100
Sertraline	intra-run	4.4	109	2	104	3.2	107	5.3	105
	between-run	6.1	109	2.5	101	7	100	1.5	107
Tianeptine	intra-run	2	103	3.2	93	11	93	9	111
	between-run	3.2	106	2.9	97	1.8	95	5.8	105
Trazodone	intra-run	2.4	111	4.1	88	6.1	89	5.6	105
	between-run	9	103	3.3	92	1.6	91	2.7	102
Trimipramine	intra-run	6.1	85	3.7	98	6	88	5.3	100
	between-run	11	100	3.7	101	0.2	87	1.9	102
Venlafaxine	intra-run	5.3	106	2.4	100	9.7	92	3.3	103
	between-run	3.4	102	3	104	6	87	4.8	98

The efficiency of the extraction is determined by recovery. Recovery higher than 80% was noted for 85% of the test compounds (Figure A2). Thirteen out of 21 compounds, AMI, CIT, CLO, DES, DOX, FLX, FLV, IMI, MAP, MIA, OPI, SER, and TRI, showed satisfactory (85 to 115%) recovery. The lowest extraction efficiency was obtained jointly for MOC (65.4%) and TIA (69.4%).

In comparison to the classic LLE developed by Fernández et al. (2012) including AMI, CIT, CLO, DES, DOX, FLX, FLV, IMI, MAP, MIA, MIR, MOC, NOR, PAR, SER, TRA, and VEN [20], mean recovery obtained for the CPE method in our study was 10.6% higher. The SPE extraction for SSRIs such as CIT, FLX, FLV, PAR, and SER provided recovery within 71–85% (mean 80%) [22], while the developed CPE extraction of these compounds was 83–94% (mean 89%). In another modification of the SPE method for AMI, CIT, CLO, DES, FLX, FLV, IMI, NOR, PAR, TRA, and VEN, the recovery was 99.6–99.9% (mean 99.8%) [21]. The CPE method developed guarantees the recovery of these compounds at the level of 80.0–95.4%, on average 87.2% (Table 2).

Table 2. Comparison of the reported recoveries of antidepressants using various methods. The arrows indicate the differences in recoveries in comparison to this study.

Study	This study	del Fernández et al. 2012 [20]			Madej and Persona 2013 [2]		Qin et al. 2008 [8]		Ansermot et al. 2012 [22]		de Castro et al. 2007 [21]	
Extraction	**CPE**	**LLE**			**CPE**		**CPE**		**SPE**		**on-Line SPE**	
Analytical Method	LC/MS	LC/MS			LC/DAD		LC/FL		LC/MS		LC/MS	
QC		Low	High									
Amitryptyline	85.4	70	67	↑	70	↑	-		-		99.9	↓
Citalopram	85.8	85	81	↑	-		-		83	↑	99.9	↓
Clomipramine	90.1	64	66	↑	-		-		-		99.7	↓
Desipramine	87.7	72	74	↑	-		-		-		99.8	↓
Doxepine	89.4	70	60	↑	-		-		-			
Fluoxetine	94.0	74	72	↑	-		-		82	↑	99.9	↓
Fluvoxamine	94.1	70	71	↑	-		-		-		99.7	↓
Imipramine	95.4	76	78	↑	-		-		-		99.9	↓
Maprotyline	85.0	77	74	↑	-		-		-			
Mianserine	87.0	86	83	↑	-		-		-			
Mirtazapine	78.9	83	84	↓	-		-		-			
Moclobemide	65.4	71	73	↓	-		-		-			
Nortryptyline	81.3	71	73	↑	-		-		85	↓	99.6	↓
Opipramol	102.5	-	-		25	↑	-		-			
Paroxetine	82.8	69	73	↑	-		-		74	↑	99.8	↓
Protryptiline	83.3	-	-		-		-		-			
Sertraline	87.5	63	70	↑	-		-		80	↑	99.9	↓
Tianeptine	69.4	-	-		-		-		-			
Trazodone	82.0	87	88	↓	-		-		-		99.9	↓
Trimipramine	102.3	-	-		-		-		-			
Venlafaxine	80.0	83	81	↓	-		89	↓	-		99.8	↓
Mean	86.2	75	75		48		89		81		99.8	
Range	65.4–102.5	63–87	60–88		25–70				74–85		99.6–99.9	

3. Materials and Methods

3.1. Materials

Four lots of human plasma with EDTA as an anticoagulant were obtained from the Regional Blood Donation and Blood Therapy Centre (Warsaw, Poland). Besides, plasma with visible hemolysis and plasma with visible lipemia were obtained from the Public Central Teaching Hospital (Warsaw, Poland).

3.2. Reference Standards and Chemicals

Reference standards (n = 21, purity ≥ 98%) (AMI, CIT, CLO, DES, DOX, FLX, FLV, IMI, MAP, MIA, MIR, MOC, NOR, OPI, PAR, PRO, SER, TIA, TRA, TRI, and VEN) were purchased from Sigma-Aldrich (St. Louis, MO, US = Merck KGaA, Darmstadt, Germany). The isotope-labeled standard (purity ≥ 98%) FLX-d5 and SER-d3 were purchased from Toronto Research Chemicals, VEN-d6 (0.1 mg/mL in methanol) from LoGiCal (LGC, Luckenwalde, Germany). Ammonia solution 25% and ethyl alcohol 96% were obtained from Avantor Performance Materials (Gliwice, Poland). Methanol and acetic acid

glacial (anhydrous for analysis, EMSURE®) were purchased from Merck KGaA (Darmstadt, Germany). Ammonium acetate was obtained from Chempur (Piekary Śląskie, Poland). Surfactant Triton™X-114 was obtained from Sigma-Aldrich Co (Merck KGaA, Darmstadt, Germany).

3.3. Chromatographic and Mass Spectrometric Conditions

Instrumental analysis was performed on an Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, US), equipped with an autosampler, degasser, and binary pump coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer QTRAP 4000 (ABSciex, Framingham, MA, USA). The Turbo Ion Spray source was operated in the positive mode. The ion spray voltage and source temperatures were 5000 V and 600 °C, respectively. The curtain gas, ion source gas 1, ion source gas 2, and collision gas were set at 345 kPa, 207 kPa, 276 kPa, and "high" instrument units, respectively [4]. The target compounds were optimized and analyzed in the Multiple Reaction Monitoring (MRM) mode (Table 3).

Table 3. The optimized parameters of MRM mode of antidepressant determination in ESI+.

Drug Name	Parent Ion [*m/z*] [M+H$^+$]	Daughter Ion [*m/z*]	DP [V]	CE [V]	CXP [V]
Amitriptyline	278	233	91	25	20
Citalopram	325	109	86	39	8
Clomipramine	315	86	81	29	6
Desipramine	267	72	81	31	12
Doxepin	280	107	71	33	6
Fluoxetine	310	44	56	37	6
Fluoxetine-d5	315	44	71	41	6
Fluvoxamine	319	71	71	33	4
Imipramine	281	86	76	25	6
Maprotiline	278	250	86	27	16
Mianserin	265	208	96	31	12
Mirtazapine	266	195	46	37	10
Moclobemide	269	182	76	27	10
Nortriptyline	264	233	71	23	14
Opipramol	364	171	91	31	14
Paroxetine	330	192	51	31	14
Protriptyline	264	191	96	41	14
Sertraline	306	159	51	35	12
Sertraline-d3	309	275	56	17	16
Tianeptine	437	292	61	25	8
Trazodone	372	176	81	37	10
Trimipramine	295	100	71	25	6
Venlafaxine	278	58	56	47	10
Venlafaxine-d6	284	64	66	49	10

Chromatographic separation was achieved with a Kinetex C18 column (100 mm × 4.6 mm, 5µm, Phenomenex, Milford, US) and gradient elution: (%B) 0 min 20%, 1 min 20%, 3 min 95%, 9 min 95% [4,31]. Phase A consisted of HPLC grade water with 0.1% (*v/v*) formic acid, whereas phase B was methanol and 0.1% (*v/v*) formic acid. The flow rate was 0.75 mL/min. Total time of chromatographic analysis was 11 min, including 2 min for re-equilibration. The injection volume was 10 µL.

3.4. Preparation of Solutions

The surfactant Triton X-114 solutions were prepared at 5 and 20% (*w/v*) and were used to obtain final surfactant concentrations of 1.5 and 6%, respectively. The standard stock solutions of antidepressants were made by dissolving 10 mg of the reference standard in 10 mL of methanol and were stored at −39 °C. The standard working solutions of 25 µg/mL for the validation and 500 ng/mL for development

were prepared by mixing equal volumes of 21 different stock solutions and the appropriate volumes of water.

The internal standard stock solutions SER-d3, FLX-d5 (1 mg/mL), and VEN-d6 (0.1 mg/mL) were prepared in methanol. Each internal standard working solution (500 ng/mL) was prepared by mixing the internal standard stock solution with an appropriate volume of water.

FLX-d5 was an internal standard for AMI, DES, DOX, FLX, FLV, IMI, MAP, MIR, OPI, PAR, PRO, and TRI; SER-d3 was an internal standard for CLO, MIA, MOC, NOR, and SER, and VEN-d6 was an internal standard for CIT, TIA, TRA, and VEN.

3.5. Sample Preparation

The experiment consisted of the method development and validation part. All the experiments were performed in human plasma with EDTA as an anticoagulant. Tested parameters are shown in Table 4 and Figure A3. The method of extraction was the method of Kojro (2019) [31] with our modifications of Triton X-114 concentration (step a), the addition of AA (step b), and pH (step c), which was selected during the development part.

Table 4. Variants of samples tested during CPE development and validation—different variants of final concentration of surfactant Triton X-114, concentration of ammonium acetate (AA), and sample pH. Samples were incubated in a water bath for 20 min at 60 °C.

Part 1. Development—Changes in Each Part			
Variant	**Triton X-114 in Sample [% (*w*/*v*)]**	**Sample pH**	**AA [% (*w*/*v*)]**
Concentration of surfactant (a)	(a1) 1.5 (a2) 6	6.8 [1]	20
Concentration of salt (b)	6	6.8 [1]	(b1) 0 (b2) 10 (b3) 20 (b4) 30
Sample pH (c)	6	(c1) 3.5 [2] (c2) 6.8 [1] (c3) 10.2 [3]	10
Part 2. Validation method on sample C			
Optimal method	6	10.2 [3]	10

[1] water; [2] by acetic acid 100%; [3] by ammonia solution 25%.

3.5.1. CPE Procedure—Development

The development was carried out in one source of plasma in 3 kinds of sample fortified by antidepressant standard-mix (50 µL of 500 ng/mL aqueous solution without the isotope-labeled standard) for all 21 analytes at a concentration corresponding to 100 ng/mL in plasma in 5 repetitions: sample A (fortified lower phase after diluting with ethanol), sample B (fortified neat ethanol), and sample C (fortified plasma before extraction). Samples A and B were used to calculate the absolute matrix effect ME_A [33], also called matrix factor [43] (Equation (1) in Section 3.6). The final concentrations of analytes in sample A and sample B were all the same. Samples A and C were used to assess recovery (Equation (3) in Section 3.6).

In the first step of development, Triton X-114 at concentrations 1.5 and 6% (*w*/*v*) was tested. In the second step, the concentrations of extraction salt AA—0, 10, 20, and 30 % (*w*/*v*)—were examined; and in the third step—pHs of 3.5, 6.8, and 10.2 were tested. At every step of development, samples A, B, and C were prepared with various modifications of the studied parameters, as described in Table 4 and Figure A3.

Sample A: An aliquot of 250 µL of plasma was placed in a 1.5 mL Eppendorf tube. Then, 50 µL of water was added. The sample was vortexed for 30 s. Afterwards, 300 µL of the surfactant solution,

Triton X-114 (5% or 20% to obtain the final concentration of 1.5 or 6%); 400 µL of 100% acetic acid, water, or 25% ammonia solution; and 0, 100, 200, or 300 mg AA (to obtain concentration of 0, 10, 20 or 30% (*w/v*)) were added. The sample was vortexed at low speed for 5 min and incubated in a water bath for 20 min at 60 °C. After that, the phase separation was accelerated by centrifugation (10,000× *g*, 5 min, 25 °C). The surfactant-lean aqueous phase was decanted to obtain the surfactant-rich phase, which was diluted with an aliquot of 500 µL of ethanol and incubated for 5 min at –80 °C. Then, the sample was centrifuged (10,000 g, 5 min, 25 °C); 200 µL of supernatant was diluted with 650 µL water and mixed together with 50 µL 500 ng/mL aqueous solution mix of the antidepressants standard without internal standards.

Sample B: 200 µL of 75%-ethanol, 650 µL water, and 50 µL 500 ng/mL aqueous solution of standard-mix were added.

Sample C: Like sample A with modifications: (1) instead of 50 µL water, 50 µL of a 500 ng/mL aqueous solution of antidepressant standard-mix was added to 250 µL plasma; (2) finally, to the vial, 700 µL of water was added instead of 650 µL water and 50 µL of antidepressants standard-mix solution.

3.5.2. CPE Procedure—Validation

An aliquot of 250 µL of human plasma fortified with a solution of mixed standards of 21 antidepressants in appropriate concentrations from QC samples and calibration samples was placed in a 1.5 mL Eppendorf tube. Then, 50 µL of 500 ng/mL aqueous internal standard solution was added. The sample was vortexed for 30 s. Afterward, 300 µL of the surfactant 20% Triton X-114, 400 µL of the 25% ammonia solution, and 100 mg AA (to final concentration of 10% (*w/v*)) were added. The sample was vortexed at low speed for 5 min and incubated in a water bath for 20 min at 60 °C. The phases were centrifuged and separated (10,000 g, 5 min, 25 °C); the surfactant-lean phase was decanted. The surfactant-rich phase was mixed with a 500 µL aliquot of ethanol and incubated for 5 min at −80 °C. Then, the sample was centrifuged (10,000 g, 5 min, 25 °C). An aliquot of 200 µL of supernatant was diluted with 700 µL of water and analyzed.

3.5.3. Preparation of Calibration Standards and Quality Samples for Validation Optimal CPE Conditions

The calibration standards contained all analytes at eight concentrations ranging from 10 to 750 ng/mL. The quality control samples were prepared at concentrations of 16, 375, and 625 ng/mL. All calibration standards and the quality control samples were prepared via the appropriate dilutions of blank human plasma spiked with all analytes in working solutions and were stored at ≤−39 °C. The precision was calculated as the ratio of standard deviation/mean (%). The accuracy was calculated as the determined value divided by the nominal value and expressed in percent.

Samples A and B for the matrix effect and samples A and C for the recovery test were prepared at two concentrations for all analyte standards and for IS at the working concentration in blank human plasma samples derived from six sources.

3.6. Matrix Effect and Recovery Calculation

ME calculated as ME_A [33], also called matrix factor (MF) (Equation (1)) [43], was regarded as insignificant if included between 85 and 115%. In development, the samples for matrix effect were prepared from blank human plasma from one source at a level corresponding to a plasma concentration of 100 ng/mL, so the matrix factors were calculated as ME_A. The preparation of samples A, B, and C was described in Section 3.5.1. In validation, the MF were calculated as the ratios of the instrument response for substances in sample A and sample B at two concentrations (16 and 625 ng/mL) for all analyte standards and for IS in the working concentration in 6 different sources, including haemolyzed and hyperlipidaemic plasma. The CV of the IS-normalized matrix factor (Equation (2)) should not exceed 15%.

Recovery was calculated by dividing the analytical signal pre-extraction spiked sample plasma (sample C) by post-extraction spiked sample plasma (sample A) (Equation (3)). Sample C concentrations were calculated on account of the volume of the rich-micellar phase.

$$\mathrm{ME_A} = \mathrm{MF} = \frac{\text{peak area of the analyte in a matrix (sample A)}}{\text{peak area of the analyte in a solvent (sample B)}} \times 100\% \quad (1)$$

$$\text{IS} - \text{normalised MF} = \frac{\text{MFanalyte}}{\mathrm{MF_{IS}}} \quad (2)$$

During development, recovery was calculated according to Equation (3).

$$\text{Recovery} = \frac{\text{peak area of the analyte in variant sample C}}{\text{peak area of the analyte invariant sample A}} \times 100\% \quad (3)$$

3.7. Statistical Analysis

The statistical analysis of the results was performed with the STATISTICA version 12 for Windows (TIBCO Software Inc., Palo Alto, CA, USA). The normal distribution of the results was evaluated by the Shapiro–Wilk test. For the normal distribution, the t-Student test and ANOVA were used.

The one-way ANOVA followed by the LSD post hoc test for multiple comparisons of the dependent group was applied. Differences between two nominal variables were tested using the chi-square test (χ^2). A *p*-value below 0.05 was considered significant.

PCA was used to find molecular descriptors that divided the studied group into prone and not prone to the ME.

4. Conclusions

The use of a volatile salt (1.3 M–10% (*w/v*) of AA) is a promising modification of the CPE procedure for LC–MS applications, since the mean recovery of studied antidepressants increased almost two-fold. The procedure can be applied to other groups of analytes to develop reliable and environmentally friendly screening methods. In the future, other volatile salts such as ammonium formate can be used for comparisons. Non-volatile salts can be applied in CPE-LC/MS when the discharge of the initial part of the chromatographic course is possible. The modified CPE method is an alternative for LLE and SPE for sample preparation prior to LC–MS, giving comparable recovery and matrix effect. Using ethanol instead of methanol or acetonitrile in CPE to reduce the sample viscosity at the last point of sample preparation makes the method more friendly to the environment. The selected conditions of CPE extraction of antidepressants were as follows: 6% (*w/v*) Triton X-114, 10% (*w/v*) AA, and pH 10.2. The developed CPE–LC–MS method was proven to be precise, accurate, and reliable, and can be applied for the simultaneous determination of various antidepressants in human plasma in clinical and forensic toxicology.

Limitation of the study: The method was not applied to clinical samples. Not all parameters that can influence the method performance were tested, e.g., extraction temperature, equilibration time, types of volatile salts, and anticoagulants used in blood sample collection.

Author Contributions: Conceptualization, J.G; methodology, J.G.; software, G.K. and N.K.; validation, J.G., N.K., and G.K.; formal analysis, G.K.; investigation, J.G., N.K., G.K., and E.G.; resources, J.G.; data curation, J.G, E.G., and N.K.; writing—original draft preparation, E.G.; writing—review, and editing. N.K., J.G; visualization, E.G.; supervision, J.G.; project administration, J.G.; funding acquisition, J.G. and E.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science and Higher Education contract number: DWD/3/6/2019 dated 21.11.2019. The APC was funded by the Medical University of Warsaw.

Acknowledgments: The authors are grateful to Ryszard Marszałek for technical assistance in LC–MS/MS analyses.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

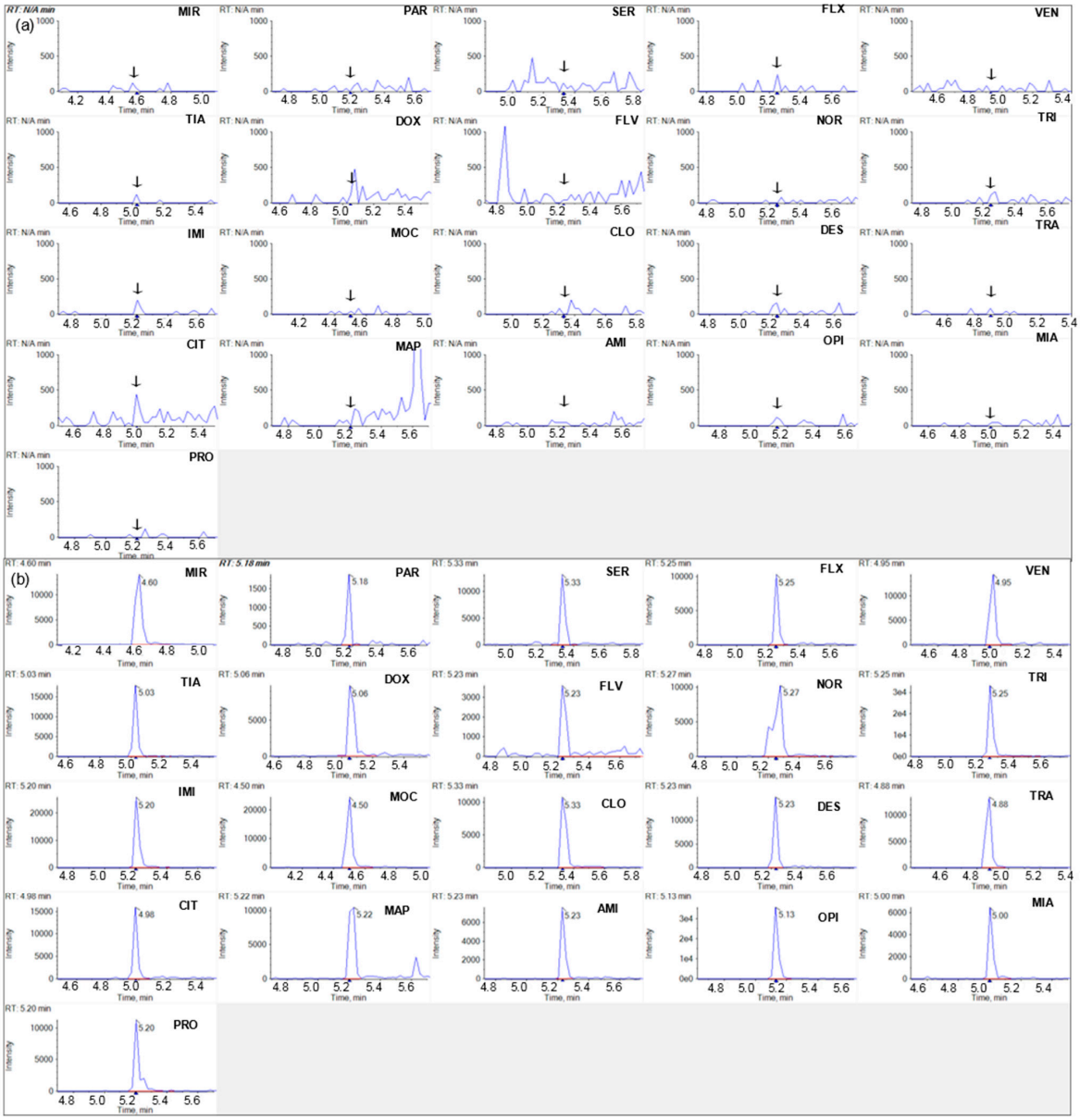


Figure A1. Multiple reaction monitoring (MRM) chromatogram peaks of the analyzed antidepressants extracted from (**a**) blank plasma; (**b**) quality control at a concentration of 625 ng/mL using cloud-point extraction. The MS parameters of MRM mode are presented in Table 3.

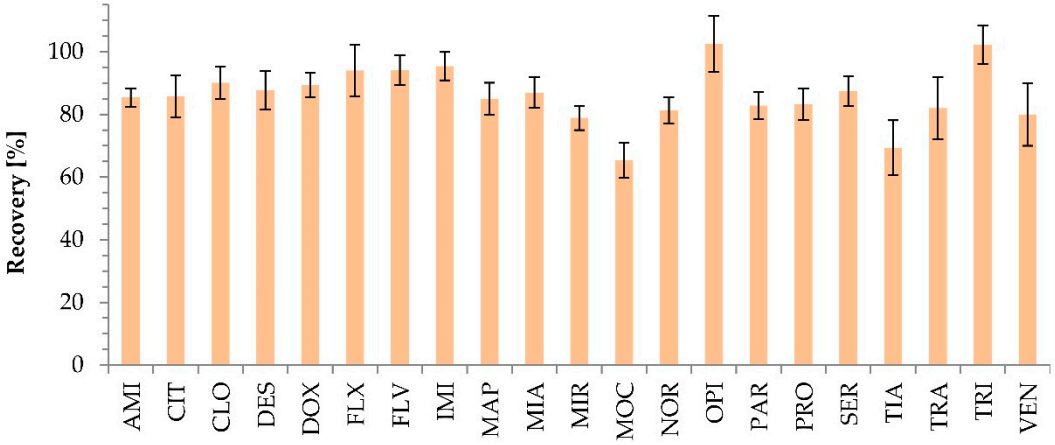


Figure A2. Recovery in the selected CPE method conditions (pH 10.2, 6% Triton X-114, 10% (*w*/*v*) ammonium acetate) determined for 6 different sources of plasma.

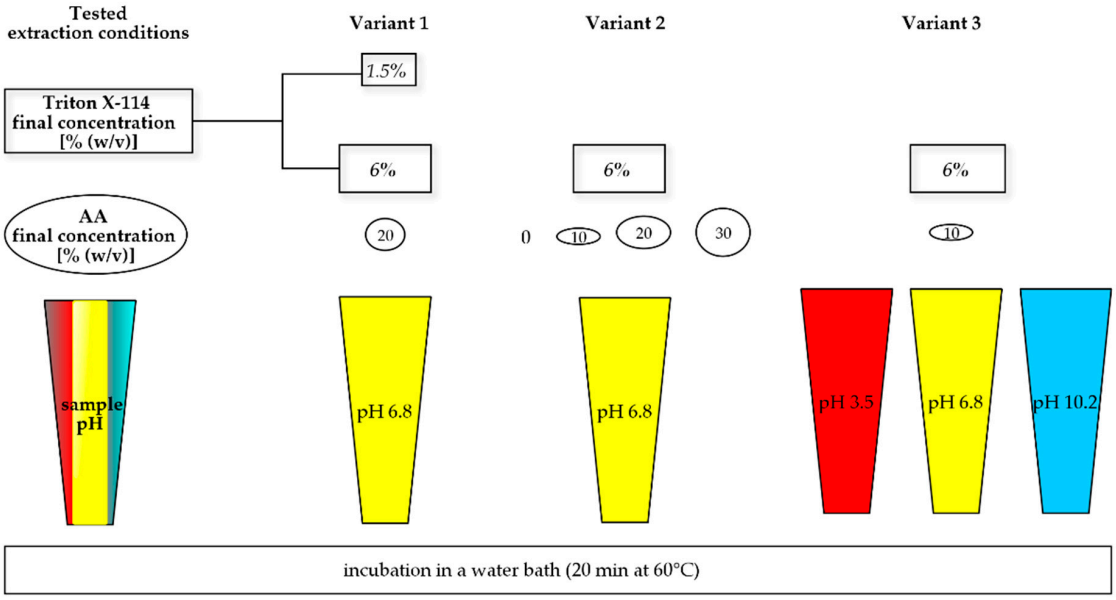


Figure A3. Variants of samples tested during CPE development.

Table A1. Comparison of MRM transitions of the analyzed antidepressants with those of their metabolites determined in other papers using LC–MS/MS.

Drug Name	Parent ion [*m*/*z*] [M+H$^+$]	Daughter ion [*m*/*z*]	Metabolite	Parent ion [*m*/*z*] [M+H$^+$]	Daughter ion [*m*/*z*]	Source	Biofluid
Amitriptyline	278	233	Nortriptyline	264	90	[25]	human plasma
Citalopram	325	109	Desmethylcitalopram	311	109 262	[45,46]	human plasma whole blood
			Citalopram *N*-oxide	341	262 109		human plasma whole blood
Clomipramine	315	86	Norclomipramine	301	72	[47]	human plasma
			Demetylclomipramine	302	243 271	[48]	whole blood
			Hydroxydesipramine	282	252		
Doxepin	280	107	Nordoxepin	266	107	[49]	human plasma
Fluoxetine	310	44	Norfluoxetine	296	134	[47]	human plasma
Imipramine	281	86	Desipramine	267	72	This study	human plasma
Maprotiline	278	250	*N*-desmethylmaprotiline	264	117 169	[46]	human whole blood
Mirtazapine	266	195	Desmethyl mirtazapine	252	195	[50]	human bile
			8-Hydroxydesmethylmirtazapine	282	211		

Table A1. *Cont.*

Drug Name	Parent ion [*m*/*z*] [M+H$^+$]	Daughter ion [*m*/*z*]	Metabolite	Parent ion [*m*/*z*] [M+H$^+$]	Daughter ion [*m*/*z*]	Source	Biofluid
Nortriptyline	264	233	10-hydroxynortriptyline	280.1	262.2	[51]	human plasma
Paroxetine	330	192	4-hydroxy-3-methoxy	332	192	[52]	human plasma
Sertraline	306	158	*N*-Desmethyl sertraline	292.32	275.1	[46]	human whole blood
Tianeptine	437	292	MC5	409	292 228	[53]	rat plasma
Trazodone	372	176	m-Chlorophenylpiperazine	197	118	[49]	human plasma
Trimipramine	295	100	Desmethyltrimipramine	281	86	[54]	human hair
			2-Hydroxytrimipramine	311	100		
			2-Hydroxydesmethyltrimipramine	297	86		
Venlafaxine	278	58	O-Desmethyl venlafaxine	264	58 246	[46]	whole blood

There is no reported MRM methods for metabolites of other analyzed antidepressants.

Table A2. The parameters of the calibration curve for all 21 analytes (n = 6).

Compound	R^2	Linear Equation y = ax + b	Standard Deviation a	Standard Deviation b
Amitriptyline	0.998	y = 0.001731x + 0.0100	0.000062	0.0016
Citalopram	0.993	y = 0.001895x + 0.0016	0.000052	0.0011
Clomipramine	0.997	y= 0.000728x + 0.002747	0.000017	0.000940
Desipramine	0.996	y = 0.002608 ± 0.0008	0.000051	0.0015
Doxepin	0.994	y= 0.001895x + 0.0119	0.000064	0.0075
Fluoxetine	0.997	y = 0.002061x+ 0.0030	0.000075	0.0030
Fluvoxamine	0.994	y = 0.000679x + 0.0079	0.000030	0.0032
Imipramine	0.997	y = 0.00395x + 0.0054	0.00012	0.0057
Maprotiline	0.995	y = 0.002716x + 0.0159	0.000050	0.0031
Mianserin	0.996	y = 0.0002657x + (−0.000142)	0.0000077	0.000265
Mirtazapine	0.997	y = 0.003092x + (−0.0013)	0.000081	0.0050
Moclobemide	0.990	y = 0.0026x + (−0.0014)	0.0011	0.0019
Nortriptyline	0.996	y = 0.001011x + 0.000020	0.000027	0.000936
Opipramol	0.997	y = 0.0260x + (−0.010)	0.0044	0.017
Paroxetine	0.996	y = 0.0001912x + 0.0050	0.0000063	0.0027
Protriptyline	0.997	y = 0.002013x + 0.0050	0.000048	0.0040
Sertraline	0.998	y = 0.000591x + 0.0035	0.000012	0.0015
Tianeptine	0.995	y = 0.002512x + 0.0026	0.000084	0.0032
Trazodone	0.996	y = 0.002249x + (−0.0026)	0.000095	0.0025
Trimipramine	0.998	y = 0.00573x + 0.0033	0.00023	0.0046
Venlafaxine	0.999	y = 0.002268x + 0.0014	0.000086	0.0032

The uncertainty (standard deviation) was rounded up to two significant figures. The mean was rounded to have the same number of decimal places as the uncertainty.

Table A3. Sample stability—autosampler, short-term, and freeze and thaw stability; mean accuracy [%] (n = 5) for low and high QC.

Test Name	Autosampler Stability (48h at 4°C)				Short-Term Stability (3h at Room Temperature)				Freeze and Thaw Stability (−20°C)			
Drug Name	16 ng/mL	CV (%)	625 ng/mL	CV (%)	16 ng/mL	CV (%)	625 ng/mL	CV (%)	16 ng/mL	CV (%)	625 ng/mL	CV (%)
Amitriptyline	112.2	13	98.1	10	96	9	91.9	4	103.2	8	88.4	13
Citalopram	92.9	14	91.7	12	96.9	6	102.6	4	89.5	2	96.3	8
Clomipramine	102.8	1	96.3	4	101.2	4	98.3	5	102	9	97.2	4
Desipramine	108.7	6	99.1	13	92.7	6	93.1	3	101.5	5	92.3	5
Doxepin	110.3	9	90.6	11	94.1	3	91.1	2	91.3	7	86.5	12
Fluoxetine	104.9	14	98.8	9	97.7	5	94.2	1	110	7	90.9	8
Fluvoxamine	106.7	9	102.5	5	98.3	11	100	7	91.5	13	98.9	5
Imipramine	110.6	12	98.4	8	97.1	13	92.4	3	95.9	3	91.9	6
Maprotiline	104	9	106.2	6	113.3	2	98.4	7	110.5	3	96.4	7

Table A3. *Cont.*

Test Name	Autosampler Stability (48h at 4°C)				Short-Term Stability (3h at Room Temperature)				Freeze and Thaw Stability (−20°C)			
Drug Name	**16 ng/mL**	**CV (%)**	**625 ng/mL**	**CV (%)**	**16 ng/mL**	**CV (%)**	**625 ng/mL**	**CV (%)**	**16 ng/mL**	**CV (%)**	**625 ng/mL**	**CV (%)**
Mianserin	106.9	13	91.1	6	95.2	3	99.4	4	91.5	2	91.3	6
Mirtazapine	107.2	10	92.4	9	92.7	11	94.6	6	99.7	13	88.3	12
Moclobemide	108	9	90.8	10	99.3	8	98.1	7	99.6	9	91.1	4
Nortriptyline	112.6	6	105.5	9	110.5	10	100.3	5	113.3	6	97.6	8
Opipramol	101.5	14	91.7	10	102.2	3	91.5	4	99.5	5	89.1	8
Paroxetine	109.3	6	94.2	6	108.5	8	101.6	9	107.1	9	91.3	13
Protriptyline	111.4	4	101.1	9	102.1	5	91.9	4	103.2	3	88	7
Sertraline	99.6	11	99.4	12	96.9	6	100.3	6	109.2	9	99.2	3
Tianeptine	96	10	90.4	12	93.5	7	102.7	6	93	6	100.8	9
Trazodone	97.2	5	96.6	12	99.7	7	104.7	5	92.1	8	100.3	8
Trimipramine	110	12	95.9	6	89.6	4	102.6	2	101.7	11	90.3	4
Venlafaxine	101.2	8	100.4	7	95.8	7	104.7	9	97.9	12	99.8	8

Table A4. Matrix effect values of tested compounds for low and high QC.

QC Samples	Low QC (16 ng/mL)			High QC (625 ng/mL)		
Drug Name	**MF Analyte**	**IS-Normalized MF**	**CV IS-Norm. MF [%]**	**MF Analyte**	**Is-Normalized MF**	**CV IS-Norm. MF [%]**
Amitriptyline	98.7	92.5	10.0	94.8	94.6	6.1
Citalopram	58.2	123.3	14.6	54.2	129.8	12.1
Clomipramine	108.3	106.2	9.3	95.1	99.1	2.7
Desipramine	95.6	90.2	14.6	95.4	95.5	8.9
Doxepin	79.2	73.3	13.7	73.9	73.7	6.5
Fluoxetine	106.3	99.1	9.3	96.6	96.5	10.0
Fluvoxamine	108.8	101.4	7.9	107.8	107.8	7.7
Imipramine	100.1	93.0	6.7	94.0	93.8	4.4
Maprotiline	102.1	95.7	9.4	97.7	97.5	2.6
Mianserin	64.8	63.1	15.0	58.7	61.0	8.9
Mirtazapine	72.6	68.1	14.8	70.4	70.4	3.3
Moclobemide	88.9	87.1	6.8	89.0	93.2	5.3
Nortriptyline	94.2	92.2	5.7	92.6	96.8	3.6
Opipramol	107.9	101.4	10.9	102.4	102.4	7.7
Paroxetine	115.6	111.8	6.7	107.1	107.1	7.6
Protriptyline	104.3	97.8	9.2	97.7	97.6	5.1
Sertraline	109.1	106.9	7.2	94.4	98.5	2.8
Tianeptine	73.8	142.1	6.3	64.3	153.9	13.9
Trazodone	53.2	108.0	12.4	48.0	108.1	11.9
Trimipramine	101.7	95.0	7.7	88.7	88.5	6.8
Venlafaxine	47.5	96.5	15.0	44.5	105.5	11.6

References

1. Giebułtowicz, J.; Kojro, G.; Piotrowski, R.; Kułakowski, P.; Wroczyński, P. Cloud-point extraction is compatible with liquid chromatography coupled to electrospray ionization mass spectrometry for the determination of antazoline in human plasma. *J. Pharm. Biomed.* **2016**, *128*. [CrossRef]
2. Madej, K.; Persona, K. Drug screening in human plasma by cloud-point extraction and HPLC. *J. Open Chem.* **2013**, *11*, 94. [CrossRef]
3. Wentao, L.; Bi, K.; Liu, X.; Zhao, J.; Chen, X. Cloud-Point Extraction Combined with LC–MS for Analysis of Memantine in Rat Plasma. *Chromatographia* **2009**, *69*, 837–842. [CrossRef]
4. Giebułtowicz, J.; Kojro, G.; Buś - Kwaśnik, K.; Rudzki, P.; Marszałek, R.; Leś, A.; Wroczyński, P. Cloud-point extraction is compatible with liquid chromatography coupled to electrospray ionization mass spectrometry for the determination of bisoprolol in human plasma. *J. Chromatogr. A* **2015**, *1423*. [CrossRef] [PubMed]
5. Hunzicker, G.A.; Hein, G.J.; Hernández, S.R.; Altamirano, J.C. Cloud point extraction for analysis of antiretrovirals in human plasma by UFLC-ESI-MS/MS. *Anal. Chem. Res.* **2015**, *6*, 1–8. [CrossRef]

6. Rukhadze, M.D.; Tsagareli, S.K.; Sidamonidze, N.S.; Meyer, V.R. Cloud-point extraction for the determination of the free fraction of antiepileptic drugs in blood plasma and saliva. *Anal. Biochem.* **2000**, *287*, 279–283. [CrossRef]
7. Tabrizi, A.B.; Naini, S.; Parnian, K.; Mohammadi, S.; Emami zad, F.; Anvarian, S.P.; Abdollahi, A. Determination of triamterene in human plasma and urine after its cloud point extraction. *Quím. Nova* **2014**, *37*, 1182–1187. [CrossRef]
8. Qin, X.Y.; Meng, J.; Li, X.Y.; Zhou, J.; Sun, X.L.; Wen, A.D. Determination of venlafaxine in human plasma by high-performance liquid chromatography using cloud-point extraction and spectrofluorimetric detection. *J. Chromatogr. B.* **2008**, *872*, 38–42. [CrossRef]
9. Zhao, Q.; Ding, J.; Jin, H.; Ding, L.; Ren, N.-Q. A Green Method Using a Micellar System for Determination of Andrographolide and Dehydroandrographolide in Human Plasma. *J. Chromatogr. Sci.* **2012**, *51*. [CrossRef]
10. Filik, H.; Şener, İ.; Cekiç, S.; Kilic, E.; Apak, R. Spectrophotometric determination of paracetamol in urine with tetrahydroxycalix[4]arene as a coupling reagent and preconcentration with Triton X-114 using cloud point extraction. *Chem. Pharm. Bull.* **2006**, *54*, 891–896. [CrossRef]
11. Wang, C.C.; Luconi, M.O.; Masi, A.N.; Fernández, L. Determination of terazosin by cloud point extraction-fluorimetric combined methodology. *Talanta* **2007**, *72*, 1779–1785. [CrossRef] [PubMed]
12. Zhao, J.; Wang, J.; Yang, Y.; Lu, Y. The Determination of Nitrate and Nitrite in Human Urine and Blood by High-Performance Liquid Chromatography and Cloud-Point Extraction. *J. Chromatogr. Sci.* **2015**, *53*, 1169–1177. [CrossRef] [PubMed]
13. World Health Organization. Depression. Available online: https://www.who.int/news-room/fact-sheets/detail/depression (accessed on 8 August 2020).
14. Methling, M.; Krumbiegel, F.; Hartwig, S.; Parr, M.K.; Tsokos, M. Toxicological findings in suicides—frequency of antidepressant and antipsychotic substances. *Forensic Sci. Med. Pathol.* **2019**, *15*, 23–30. [CrossRef] [PubMed]
15. Pompili, M.; Serafini, G.; Innamorati, M.; Ambrosi, E.; Giordano, G.; Girardi, P.; Tatarelli, R.; Lester, D. Antidepressants and Suicide Risk: A Comprehensive Overview. *Pharmaceuticals* **2010**, *3*, 2861–2883. [CrossRef] [PubMed]
16. Hawton, K.; Bergen, H.; Simkin, S.; Cooper, J.; Waters, K.; Gunnell, D.; Kapur, N. Toxicity of antidepressants: Rates of suicide relative to prescribing and non-fatal overdose. *Br. J. Psychiatry* **2010**, *196*, 354–358. [CrossRef] [PubMed]
17. Jia, E.; Bartlett, M. Recent Advances in Liquid Chromatographic Methods for the Determination of Selective Serotonin Reuptake Inhibitors (SSRI) and Serotonin Norepinephrine Reuptake Inhibitors (SNRI). *Biomed. Chromatogr.* **2019**, *34*. [CrossRef]
18. Vaghar-Lahijani, G.; Aberoomand-Azar, P.; Saber-Tehrani, M.; Soleimani, M. Application of ionic liquid-based ultrasonic-assisted microextraction coupled with HPLC for determination of citalopram and nortriptyline in human plasma. *J. Liq. Chromatogr. Relat.* **2017**, *40*, 1–7. [CrossRef]
19. Duverneuil, C.; de la Grandmaison, G.L.; de Mazancourt, P.; Alvarez, J.-C. A high-performance liquid chromatography method with photodiode-array UV detection for therapeutic drug monitoring of the nontricyclic antidepressant drugs. *Ther. Drug Monit.* **2003**, *25*, 565–573. [CrossRef]
20. Del Mar Ramírez Fernández, M.; Wille Sm Fau-Samyn, N.; Samyn, N. Quantitative method validation for the analysis of 27 antidepressants and metabolites in plasma with ultraperformance liquid chromatography-tandem mass spectrometry. *Ther. Drug Monit.* **2012**, *34*, 11–24. [CrossRef]
21. De Castro, A.; Fernandez, M.d.M.R.; Laloup, M.; Samyn, N.; De Boeck, G.; Wood, M.; Maes, V.; López-Rivadulla, M. High-throughput on-line solid-phase extraction–liquid chromatography–tandem mass spectrometry method for the simultaneous analysis of 14 antidepressants and their metabolites in plasma. *J. Chromatogr. A* **2007**, *1160*, 3–12. [CrossRef]
22. Ansermot, N.; Brawand-Amey, M.; Eap, C.B. Simultaneous quantification of selective serotonin reuptake inhibitors and metabolites in human plasma by liquid chromatography—Electrospray mass spectrometry for therapeutic drug monitoring. *J. Chromatogr. B* **2012**, *885–886*, 117–130. [CrossRef] [PubMed]
23. Petrides, A.K.; Moskowitz, J.; Johnson-Davis, K.L.; Jannetto, P.J.; Langman, L.J.; Clarke, W.; Marzinke, M.A. The development and validation of a turbulent flow-liquid chromatography-tandem mass spectrometric method for the simultaneous quantification of citalopram, sertraline, bupropion and hydroxybupropion in serum. *Clin. Biochem.* **2014**, *47*, 73–79. [CrossRef] [PubMed]

24. Santos, M.G.; Tavares, I.M.; Barbosa, A.F.; Bettini, J.; Figueiredo, E.C. Analysis of tricyclic antidepressants in human plasma using online-restricted access molecularly imprinted solid phase extraction followed by direct mass spectrometry identification/quantification. *Talanta* **2017**, *163*, 8–16. [CrossRef] [PubMed]
25. Berm, E.J.J.; Paardekooper, J.; Brummel-Mulder, E.; Hak, E.; Wilffert, B.; Maring, J.G. A simple dried blood spot method for therapeutic drug monitoring of the tricyclic antidepressants amitriptyline, nortriptyline, imipramine, clomipramine, and their active metabolites using LC-MS/MS. *Talanta* **2015**, *134*, 165–172. [CrossRef] [PubMed]
26. Nielsen, M.K.; Johansen, S.S. Simultaneous determination of 25 common pharmaceuticals in whole blood using ultra-performance liquid chromatography-tandem mass spectrometry. *J. Anal. Toxicol.* **2012**, *36*, 497–506. [CrossRef] [PubMed]
27. Santos-Neto, A.J.; Bergquist, J.; Lanças, F.M.; Sjöberg, P.J.R. Simultaneous analysis of five antidepressant drugs using direct injection of biofluids in a capillary restricted-access media-liquid chromatography-tandem mass spectrometry system. *J. Chromatogr. A* **2008**, *1189*, 514–522. [CrossRef] [PubMed]
28. De Souza, I.D.; Domingues, D.S.; Queiroz, M.E.C. Hybrid silica monolith for microextraction by packed sorbent to determine drugs from plasma samples by liquid chromatography–tandem mass spectrometry. *Talanta* **2015**, *140*, 166–175. [CrossRef]
29. Remane, D.; Meyer, M.R.; Wissenbach, D.K.; Maurer, H.H. Full validation and application of an ultra high performance liquid chromatographic-tandem mass spectrometric procedure for target screening and quantification of 34 antidepressants in human blood plasma as part of a comprehensive multi-analyte approach. *Anal. Bioanal. Chem.* **2011**, *400*, 2093. [CrossRef]
30. Roemmelt, A.T.; Steuer, A.E.; Kraemer, T. Liquid Chromatography, In Combination with a Quadrupole Time-of-Flight Instrument, with Sequential Window Acquisition of All Theoretical Fragment-Ion Spectra Acquisition: Validated Quantification of 39 Antidepressants in Whole Blood As Part of a Simultaneous Screening and Quantification Procedure. *Anal. Chem.* **2015**, *87*, 9294–9301. [CrossRef]
31. Kojro, G.; Rudzki, P.J.; Pisklak, D.M.; Giebułtowicz, J. Matrix effect screening for cloud-point extraction combined with liquid chromatography coupled to mass spectrometry: Bioanalysis of pharmaceuticals. *J. Chromatogr. A* **2019**, *1591*, 44–54. [CrossRef]
32. Kojro, G.; Wroczyński, P. Cloud Point Extraction in the Determination of Drugs in Biological Matrices. *J. Chromatogr. Sci.* **2020**, *58*, 151–162. [CrossRef] [PubMed]
33. Matuszewski, B.K.; Constanzer, M.L.; Chavez-Eng, C.M. Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC−MS/MS. *Anal. Chem.* **2003**, *75*, 3019–3030. [CrossRef] [PubMed]
34. More, P.R.; Arya, S.S. A novel, green cloud point extraction and separation of phenols and flavonoids from pomegranate peel: An optimization study using RCCD. *J. Environ. Chem. Eng.* **2019**, *7*, 103306. [CrossRef]
35. Koshy, L.; Saiyad, A.H.; Rakshit, A.K. The effects of various foreign substances on the cloud point of Triton X 100 and Triton X 114. *Colloid Polym. Sci.* **1996**, *274*, 582–587. [CrossRef]
36. Quina, F.H.; Hinze, W.L. Surfactant-Mediated Cloud Point Extractions: An Environmentally Benign Alternative Separation Approach. *Ind. Eng. Chem. Res* **1999**, *38*, 4150–4168. [CrossRef]
37. Madej, K. Microwave-assisted and cloud-point extraction in determination of drugs and other bioactive compounds. *Trends Anal. Chem.* **2009**, *28*, 436–446. [CrossRef]
38. Sayem Alam, M.; Mandal, A.B. The clouding phenomena of mixed surfactant (non-ionic Triton X-114+cationic gemini 16-5-16) solutions: Influence of inorganic and organic additives on the cloud point. *J. Mol. Liq.* **2015**, *212*, 237–244. [CrossRef]
39. Pourreza, N.; Elhami, S. Spectrophtometric determination of malachite green in fish farming water samples after cloud point extraction using nonionic surfactant Triton X-100. *Anal. Chim. Acta* **2007**, *596*, 62–65. [CrossRef]
40. Kori, S. Cloud point extraction coupled with back extraction: A green methodology in analytical chemistry. *Forensic Sci. Res.* **2019**, 1–15. [CrossRef]
41. Zhang, H.; Choi, H.-K. Analysis of meloxicam by high-performance liquid chromatography with cloud-point extraction. *Anal. Bioanal. Chem.* **2008**, *392*, 947–953. [CrossRef]
42. Han, F.; Yin, R.; Shi, X.-l.; Jia, Q.; Liu, H.-z.; Yao, H.-m.; Xu, L.; Li, S.-m. Cloud point extraction-HPLC method for determination and pharmacokinetic study of flurbiprofen in rat plasma after oral and transdermal administration. *J. Chromatogr. B* **2008**, *868*, 64–69. [CrossRef] [PubMed]

43. Guideline on bioanalytical method validation. Committee for Medicinal Products for Human Use (CHMP), EMEA/CHMP/EWP/192217/2009, EuropeanMedicines Agency. 2011. Available online: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf (accessed on 5 October 2020).
44. Guidance for Industry: Bioanalytical Method Validation. Food and Drug Administration. Center for Drug Evaluation and Research and Center for Veterinary Medicine. 2001. Available online: http://academy.gmp-compliance.org/guidemgr/files/4252FNL.PDF (accessed on 5 October 2020).
45. Jiang, T.; Rong, Z.; Peng, L.; Chen, B.; Xie, Y.; Chen, C.; Sun, J.; Xu, Y.; Lu, Y.; Chen, H. Simultaneous determination of citalopram and its metabolite in human plasma by LC–MS/MS applied to pharmacokinetic study. *J. Chromatogr. B* **2010**, *878*, 615–619. [CrossRef] [PubMed]
46. Zhu, R.; Dong, X.; Zhang, D.; Liu, X.; Ye, Y.; Jiang, Y. Simultaneous Quantification of 38 Psychotropic Drugs and Relevant Metabolites in Blood using LC–MS-MS. *J. Anal. Toxicol.* **2020**. [CrossRef] [PubMed]
47. De Castro, A.; Concheiro, M.; Quintela, O.; Cruz, A.; López-Rivadulla, M. LC–MS/MS method for the determination of nine antidepressants and some of their main metabolites in oral fluid and plasma: Study of correlation between venlafaxine concentrations in both matrices. *J. Pharm. Biomed.* **2008**, *48*, 183–193. [CrossRef]
48. Titier, K.; Castaing, N.; Le-Déodic, M.; Le-bars, D.; Moore, N.; Molimard, M. Quantification of Tricyclic Antidepressants and Monoamine Oxidase Inhibitors by High-Performance Liquid Chromatography-Tandem Mass Spectrometry in Whole Blood. *J. Anal. Toxicol.* **2007**, *31*, 200–207. [CrossRef]
49. Patel, B.N.; Sharma, N.; Sanyal, M.; Shrivastav, P.S. High throughput and sensitive determination of trazodone and its primary metabolite, m-chlorophenylpiperazine, in human plasma by liquid chromatography–tandem mass spectrometry. *J. Chromatogr. B* **2008**, *871*, 44–54. [CrossRef]
50. Wenzel, S.; Aderjan, R.; Mattern, R.; Pedal, I.; Skopp, G. Tissue distribution of mirtazapine and desmethylmirtazapine in a case of mirtazapine poisoning. *Forensic Sci. Int.* **2006**, *156*, 229–236. [CrossRef]
51. Hotha, K.K.; Ravindranath, L.K.; Veera, K.N.J.; Kumar, K.K.; Reddy, Y.R.; Jagadeesh, B.; Rao, D.V.N.; Bharathi, D.V.; Mullangi, R. Development and validation of a highly sensitive LC-MS/MS method for simultaneous quantitation of nortriptyline and 10-hydroxynortriptyline in human plasma: Application to a human pharmacokinetic study. *Biomed. Chromatogr.* **2010**, *24*, 1113–1119. [CrossRef]
52. Segura, M.; Ortuño, J.; Farré, M.; Pacifici, R.; Pichini, S.; Joglar, J.; Segura, J.; Torre, R.d.l. Quantitative determination of paroxetine and its 4-hydroxy-3-methoxy metabolite in plasma by high-performance liquid chromatography/electrospray ion trap mass spectrometry: Application to pharmacokinetic studies. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1455–1461. [CrossRef]
53. Szafarz, M.; Wencel, A.; Pociecha, K.; Fedak, F.A.; Wlaź, P.; Wyska, E. Pharmacokinetic study of tianeptine and its active metabolite MC5 in rats following different routes of administration using a novel liquid chromatography tandem mass spectrometry analytical method. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2018**, *391*, 185–196. [CrossRef]
54. Doherty, B.; Rodriguez, V.; Leslie, J.C.; McClean, S.; Smyth, W.F. An electrospray ionisation tandem mass spectrometric investigation of selected psychoactive pharmaceuticals and its application in drug and metabolite profiling by liquid chromatography/electrospray ionisation tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2031–2038. [CrossRef] [PubMed]

Publikacja 2 (oryginalna)

Gniazdowska E., Kaza M., Buś-Kwaśnik K., Giebułtowicz J. LC-MS/MS determination of dutasteride and its major metabolites in human plasma. Journal of Pharmaceutical and Biomedical Analysis. 2021; 206:114362. IF= 3,571; MEiN = 100.

Journal of Pharmaceutical and Biomedical Analysis 206 (2021) 114362

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

LC-MS/MS determination of dutasteride and its major metabolites in human plasma

Elżbieta Gniazdowska[a,b], Michał Kaza[b,*], Katarzyna Buś-Kwaśnik[b], Joanna Giebułtowicz[c,*]

[a] Department of Bioanalysis and Drugs Analysis, Doctoral School, Medical University of Warsaw, 61 Żwirki i Wigury, 02-091 Warsaw, Poland
[b] Łukasiewicz Research Network, Industrial Chemistry Institute, 8 Rydygiera, 01-793 Warsaw, Poland
[c] Department of Bioanalysis and Drugs Analysis, Faculty of Pharmacy, Medical University of Warsaw, 1 Banacha, 02-097 Warsaw, Poland

ARTICLE INFO

Article history:
Received 22 March 2021
Received in revised form 4 August 2021
Accepted 19 August 2021
Available online 3 September 2021

Keywords:
1,2-dihydrodutasteride
4′-hydroxydutasteride
6β-hydroxydutasteride
Dutasteride
Sample preparation
Incurred sample stability

ABSTRACT

Dutasteride is a specific and selective inhibitor of both 5α-reductase isoforms used mainly in benign prostatic hyperplasia and lower urinary tract symptoms. Although the drug is extensively metabolized in humans, data on the concentrations of its main metabolites are lacking. There is also a lack of data on dutasteride stability in frozen plasma samples. Our method was used to determine dutasteride and its active metabolites: 4′-hydroxydutasteride, 6β-hydroxydutasteride, and 1,2-dihydrodutasteride in plasma after a single administration of 0.5 mg of dutasteride. We also assessed the long-term stability (two years in the freezer) of dutasteride in clinical samples. The developed method covered the range of 0.1–3.5 ng/mL for dutasteride and 0.08–1.2 ng/mL for 1,2-dihydrodutasteride, 4′-hydroxydutasteride, 6β-hydroxydutasteride. It was proved to be reliable as it met all validation criteria required by the European Medicine Agency for bioanalytical methods. 4′-hydroxydutasteride and 1,2-dihydrodutasteride concentrations in plasma were higher than 6β-hydroxydutasteride. Dutasteride was stable in the freezer for up to 2 years in clinical samples. Thus within 1014 days of storage (below − 65 °C), samples can be reanalyzed without the risk of unreliable results.

1. Introduction

Dutasteride (DUT) is a synthetic analogue of testosterone and a specific and selective inhibitor of both 5α-reductase isoforms of type 1 and 2, which convert testosterone to dihydrotestosterone. The drug is administered in benign prostatic hyperplasia and lower urinary tract symptoms [1–3]. DUT actions improve flow rate and decrease the prostate's total and transition zone volume in patients with benign prostatic hyperplasia [4]. There is also a study on DUT utility in treating breast cancer [5].

DUT is extensively metabolized in humans by cytochrome P-450 3A4 and 3A5. After oral administration at a daily dose of 0.5 mg, only 5.4% of DUT and < 1% of DUT is excreted unchanged in the feces and urine. The major metabolites include monohydroxy metabolites (4′-hydroxydutasteride (4OH), 6β-hydroxydutasteride (6OH), and 1,2-dihydrodutasteride (DHD)) (Fig. 1). In vitro studies showed that 4OH and DHD are less potent than DUT against both 5α-reductase isoforms, whereas the activity of 6OH is comparable to that of DUT [3,6]. DUT has a long half-life increasing with patient age. The half-life is 170 h in men aged 20–49 years, 260 h in men aged 50–69 years, and 300 h in men older than 70 years old [7]. Measurements of the metabolite levels might be crucial in understanding this phenomenon and the variability of the patient's response to the treatment. The Food and Drug Administration (FDA) noted the cases for which clinically relevant metabolites have not been identified during preclinical studies and emphasized the need for their determination in human studies [8]. Data on 6OH, 4OH, and DHD plasma concentrations are needed in this context, as they are currently missing.

The only data on 4OH metabolite levels are from the clinical pharmacology and biopharmaceutics review of DUAGEN but concern the steady-state [9]. Seo et al. recently reported the level of 6OH metabolite in rats after intravenous administration of DUT at the dose 2.5 mg/kg b.w. and after oral administration at 5 mg/kg b.w. [10]. However, based on kg body weight (kg b.w.), the dosage was higher than in humans. Combined with the interspecies difference in drug metabolism, it is impossible to assess the level of this metabolite in humans [11]. Understanding the levels of the DUT metabolites after a single administration to humans will fill in the missing data and possibly help elucidate the processes that influence treatment. Moreover, drug metabolites with a higher half-life time than parent drugs can be used in doping control analysis. Dutasteride was

* Corresponding authors.
E-mail addresses: michal.kaza@gmail.com (M. Kaza), joanna.giebultowicz@wum.edu.pl (J. Giebułtowicz).

https://doi.org/10.1016/j.jpba.2021.114362
0731-7085/

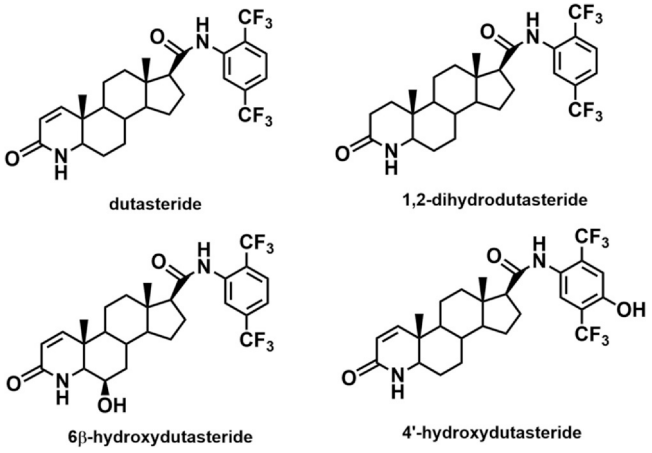


Fig. 1. Chemical structure of dutasteride (DUT), 1,2-dihydrodutasteride (DHD), 4′-hydroxydutasteride (4OH), and 6β-hydroxydutasteride (6OH).

included as masking agents in the World Anti-Doping Agency's (WADA) prohibited list in 2005 [12].

Reliability of measurements in bioanalysis of clinical samples is ensured by using analytical methods validated following the requirements of the European Medicines Agency (EMA) and FDA [13–15]. The assessment of the analyte stability in a biological sample under certain conditions (storage temperature and time from the sampling to the analysis) is as critical as the other validation parameters, e.g., accuracy and precision or the influence of the matrix. According to the World Health Organization's Guideline on Good Clinical Laboratory Practice [16] section 16.2 and the Organization for Economic Cooperation and Development Guideline on Good Laboratory Practice [17] sections 6.6 and 10.1, clinical samples from a clinical trial should be stored ten years at minimum or as long as the substance is stable in the matrix. The DUT stability data is missing. It was reported only on spiked, non-clinical samples and for storage time as short as 59 days [18,19] (Table A1). There is a lack of data on the long-term stability of DUT in samples collected from clinical trial participants.

There are numerous methods of DUT determination in plasma and serum [18–24]. Due to the low concentrations of DUT in plasma following an oral therapeutic dose, they are mainly based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). However, the linearity range adequate for bioequivalence study after a single administration of 0.5 mg of DUT was described only in two of them [7,22]. Furthermore, the reported methods aimed to determine DUT only. The simultaneous determination of DUT and 6OH was reported for DUT administered intravenously and orally to rats [10]. It was based on fluorescence detection and covered the range from 10 to 1000 ng/mL, so it is unsuitable for the determination following the oral therapeutic dose [10]. We aimed to validate a bioanalytical method of simultaneous analysis of DUT and its three major metabolites: 6OH, 4OH, DHD in human plasma using the LC-MS/MS technique. That approach of simultaneous detection of drugs and metabolites was recently frequently applied [25–27]. The method was applied to determine DUT and its metabolites in plasma samples from volunteers following a single oral administration of 0.5 mg of DUT. Additionally, we aimed to assess the long-term stability (1014 days in the freezer) of DUT in clinical samples.

2. Materials and methods

2.1. Materials

Reference standards (purity ≥ 98%) of DUT was purchased from Alsachim (Luckenwalde, Germany), whereas 1,2-dihydrodutasteride (DHD) (98%), 4′-hydroxydutasteride (4OH) (98%), and 6β-hydroxydutasteride (6OH) (99.91%) from Toronto Research Chemicals (Toronto, Canada). The isotope-labeled standard (purity ≥ 99%) dutasteride-$^{13}C_6$ (internal standard, IS) was purchased from TLC Pharmaceutical Standards (Ontario, Canada). Acetonitrile, methanol, hexane and formic acid were purchased from Merck KGaA (Darmstadt, Germany). Ammonium formate, 25% ammonia solution (*aq*) and NaOH were obtained from Chempur (Piekary Śląskie, Poland). Human plasma with CPD (Citrate, Phosphate, Dextrose) as an anticoagulant was obtained from the Regional Blood Donation and Blood Therapy Centre (Warsaw, Poland).

2.2. Chromatographic and mass spectrometric conditions

Instrumental analysis was performed on Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, US), equipped with an autosampler, degasser, binary pump coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer QTRAP 4000 (ABSciex, Framingham, MA, US). The Turbo Ion Spray source was operated in the positive mode. The ion spray voltage and source temperatures were 5500 V and 600 °C, respectively. The curtain gas, ion source gas 1, ion source gas 2, and collision gas (all high purity nitrogen) were set at 50 kPa, 40 kPa, 40 kPa, and "high" instrument units. The target compounds were analyzed in the Multiple Reaction Monitoring (MRM). We selected the following transitions: DUT – *m/z* 529 > 461, IS – *m/z* 535 > 467, 4OH – *m/z* 545 > 477, 6OH – *m/z* 545 > 270, DHD – *m/z* 531 > 239. The optimized MS parameters, i.e., declustering potential, collision energy, collision cell exit potential, are presented in Table A2.

Chromatographic separation was achieved with a BionaCore C18 4.6 × 100 mm, 2.7 µm, Superficially Porous Particles (Bionacom, Great Britain) and gradient elution with a flow rate of 0.5 mL/min. Mobile phase A consisted of 5 mM ammonium formate and formic acid (1000:1, *v*/*v*); mobile phase B was acetonitrile. The gradient (%B) was as follows: 0 min 70%, 2.5 min 70%, 4 min 100% and 7 min 100%. The re-equilibration of the column to the initial conditions lasted 2 min. The column temperature was set at 35 ± 1 °C, whereas the autosampler temperature was at 4 °C. The injection volume was 10 µL.

2.3. Standard solution, calibration and quality control samples preparation

The standard stock solutions of DUT (1.0 mg/mL), DHD, 4OH, 6OH and internal standard $^{13}C_6$-DUT (0.1 mg/mL) were made in methanol and were stored at −20 °C. The standard working solutions were prepared in 80% methanol to obtain the final plasma concentration in calibration standards of 0.1, 0.2, 0.3, 0.6, 1.2, 2.0, 2.8 and 3.5 ng/mL for DUT and 0.08, 0.2, 0.35, 0.5, 0.65, 0.8, 1.0 and 1.2 ng/mL for all metabolites. Quality control samples were prepared at low (0.2 ng/mL of DUT and 0.2 ng/mL of metabolites), medium (1.2 ng/mL of DUT, 0.65 ng/mL of metabolites) and high level (2.8 ng/mL of DUT, 1.0 ng/mL of metabolites). The internal standard working solution was in concentration of 20 ng/mL. All calibration standards and quality control samples were prepared on plasma.

2.4. Sample preparation

To an aliquot of 500 µL of human plasma, 25 µL of internal standard solution (20 ng/mL) and 50 µL of 0.001% ammonia were added, and vortexed for 5 s; as extrahent 1.0 mL of methyl tert-butyl ether was used. The mixture was mixed on a Vibrax mixer for 5 min at 1000 rpm and centrifuged for 5 min at 3500 rpm. The aqueous phase was frozen, and the organic phase was transferred to a test tube. The organic solvent was evaporated under the stream of nitrogen. The dry residue was dissolved in 150 µL of 80% acetonitrile (*v*/*v*), vortex mixed for 5 s, and analyzed.

2.5. Method optimization

Method optimization consisted of three steps and was a modification of the Contractor's method [18]. Two extrahents were tested: methyl tert-butyl ether with n-hexane (80:20, *v*/*v*) (2.5 mL) and methyl tert-butyl ether alone (2.5 mL). Then, for methyl tert-butyl ether different volumes of extrahent were tested (2.5, 2. 0, 1.5, 1.0 mL). The compounds were isolated from 0.5 mL of plasma, 0.5% formic acid was added as pH modifier (50 µL). The influence of pH on sample recovery was tested by the addition of NaOH (1, 0.5, and 0.25 M) or ammonia (1% and 0.001%) instead of 0.5% formic acid. The compounds were isolated from 0.5 mL of plasma with 1 mL of tert-butyl ether. Finally, two analytical columns were tested: Bionacore C18 4.6 × 100 mm, 2.7 µm and the Zorbax 100 × 3.0 mm, 3.5 µm.

2.6. Validation tests

The lower limit of quantification (LLOQ) was determined as 0.1 ng/mL for DUT, and 0.08 ng/mL for metabolites with a minimum of five replicates within and between sequences. The signal of the DUT and the metabolites should be five times higher than the baseline noise. The acceptance criterion for within-sequence and between-sequence accuracy at each concentration should not exceed 80–120%, and the precision value should not exceed 20%. The method precision and accuracy were determined for three concentration levels: QC_{low}, $QC_{medium,}$ and QC_{high}. The acceptance criterion for within-sequence and between-sequence precision for each concentration should not exceed 85–115%. The precision value should not exceed 15%.

The samples for matrix effect (calculated as matrix factor, MF) [28] were prepared from blank human plasma. MF was calculated as the ratios of the instrument response for substances in sample A (extracted plasma spiked post-extraction) and sample B (the analytes in neat solvent) at three concentrations for DUT, DHD, 4OH, 6OH (QC_{low}, $QC_{medium,}$ and QC_{high}) and for IS at the working concentration in six different sources, including haemolyzed and hyperlipidaemic plasma. The CV of the IS-normalized matrix factor should not exceed 15%. To visualize the absolute matrix effect for the analytes, we performed a steady post-column infusion of the analytes at a concentration of QC_{low} and injected the extracted blank plasma on the column. Recoveries were calculated using the same concentrations and matrices as MF. They were calculated as the ratios of the instrument response for substances in sample C (plasma spiked before extraction) to sample A (extracted plasma spiked post-extraction).

We selected the range of the calibration curve ($n = 6$) from 0.1 to 3.5 ng/mL for DUT and from 0.08 to 1.2 ng/mL for DHD, 4OH, 6OH. DUT concentrations for the linearity test were assessed based on our previous study (not published). The range of the calibration curve for metabolites was selected based on concentrations of 4OH published in the report on clinical pharmacology and biopharmaceutics review (s) of DUAGEN (GlaxoSmithKline) [9].

Dilution integrity was tested by spiking the blank plasma with an analyte concentration five times above the highest concentration on the calibration curve and diluting this sample with a blank matrix ($n = 5$). Accuracy should be within 85–115% and precision within ± 15%.

All stability tests were made at two concentration levels (QC_{low} and QC_{high}). The short-term stability of DUT and metabolites in plasma was determined by analysis of samples after storage for four hours at room temperature, whereas long-term stability for 32 days at − 65 °C. The freeze and thaw stability of the analytes in plasma was determined in the process of three freeze-thaw cycles at − 65 °C storage and 25 °C thawings at least 12 h after freezing. All results were compared to concentrations determined in freshly prepared QC_{low} and QC_{high} that were run in the same analytical sequence as the stability QCs. The autosampler stability in the extract was determined directly after sample preparation and 24 h after storage in an autosampler (4 °C) each time using the freshly prepared calibration curve. The acceptable stability was 85–115%.

2.7. Method application

The method was applied to determine the metabolite concentration in plasma of three volunteers, one from period I and two from period II of the clinical trial after administration of Avodart

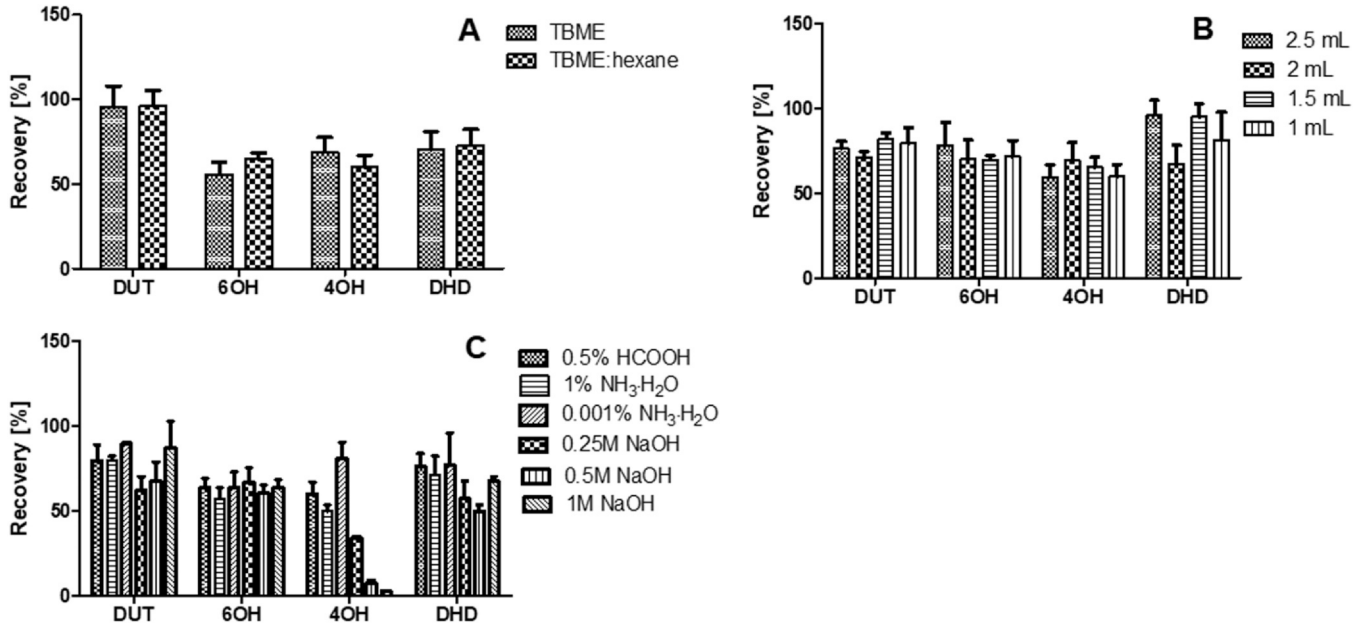


Fig. 2. Results of the method optimization for dutasteride (DUT) (2.8 ng/mL), 1,2-dihydrodutasteride (DHD), 4′-hydroxydutasteride (4OH) and 6β-hydroxydutasteride (6OH) (1.2 ng/mL). **(A)** Extrahent selection: methyl tert-butyl ether (TBME) or methyl tert-butyl ether/hexane (8:2, v/v) (TBME/hexane) (2.5 mL). **(B)** Selection of the methyl tert-butyl ether volume – 2.5 mL, 2 mL, 1.5 mL, 1 mL. Extraction conditions for (**A**) and (**B**): 0.5 mL of plasma, 50 µL of 0.5% formic acid (HCOOH). **(C)** Selection of the pH modifier (at the volume of 50 µL). Extraction conditions: 0.5 mL of plasma, 2.5 mL of methyl tert-butyl ether.

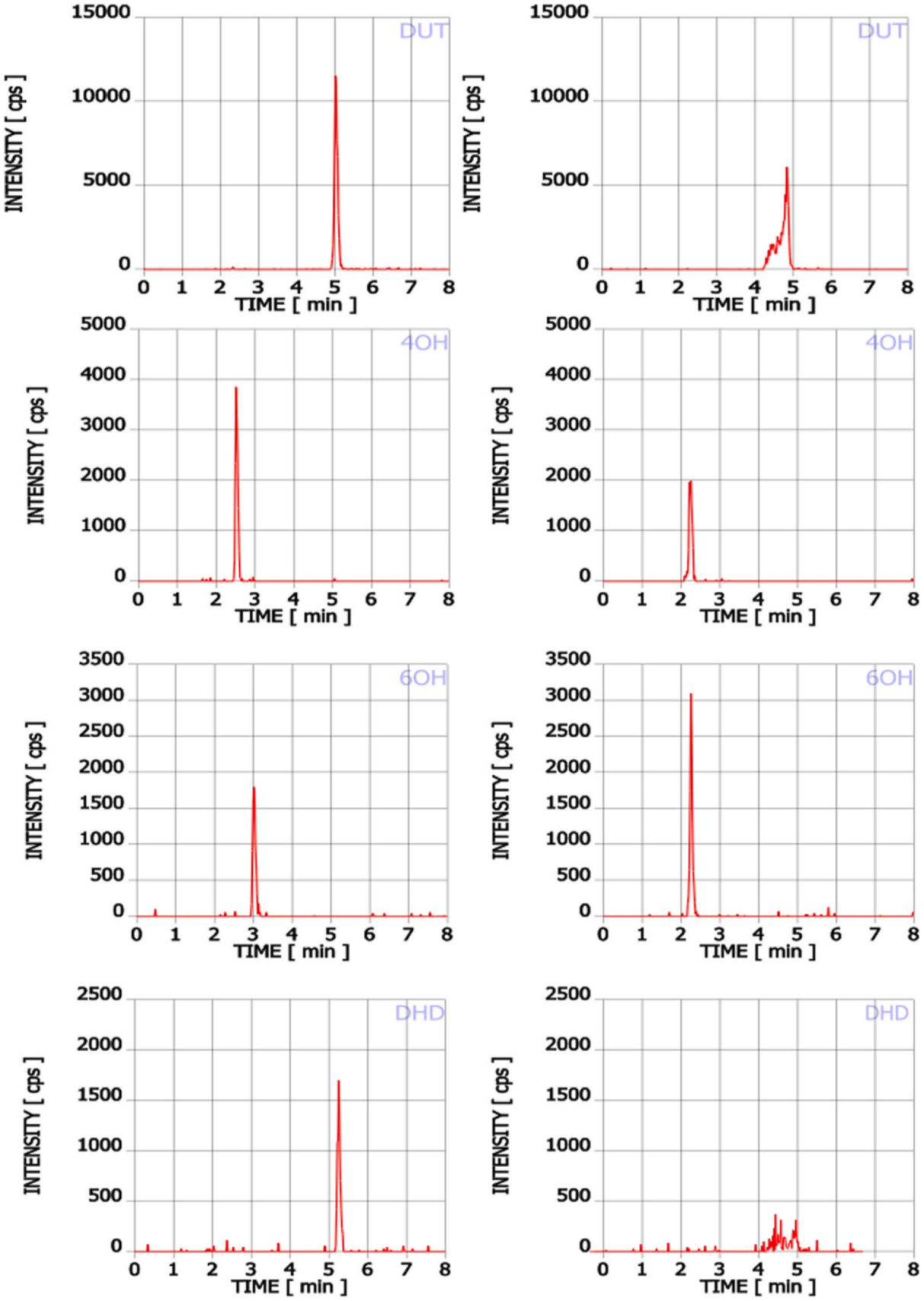


Fig. 3. Chromatogram for dutasteride (DUT) and metabolites: 4′-hydroxydutasteride (4OH), 6β-hydroxydutasteride (6OH) and 1,2-dihydrodutasteride (DHD) on Bionacore C18 4.6 × 100 mm, 2.7 µm (left) and Zorbax 100 × 3.0 mm, 3.5 µm (right) at the concentration of upper limit of quantitation (3.5 ng/mL for DUT, 1.2 ng/mL for metabolites).

(0.5 mg, DUT soft gelatin capsule, GlaxoSmithKline, Research Triangle Park, NC, USA). The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Regional Medical Chamber in Warsaw (EudraCT: 2017-000716-41, No. DUT-BIO-01-17). The pharmacokinetic parameters were calculated using the non-compartmental analysis tool of PKSolver, a freely available menu-driven add-in program for Microsoft Excel written in Visual Basic for Applications (VBA) [29]. The area under the plasma concentration versus time curve (AUC) was calculated by the linear trapezoidal method. The apparent terminal elimination rate constant, λz, was obtained by linear regression of the log-linear terminal phase of the concentration-time profile using at least three non-zero declining concentrations in the terminal phase with a correlation coefficient of > 0.8. The terminal half-life value ($t_{1/2}$) was calculated using the equation (ln2) × λz. Additionally, we determined the long-term stability of DUT in clinical material. The reanalysis of the clinical trial samples, stored below − 65 °C, was done after two years. The results of DUT concentration in reanalysis (P2) were compared with the first results from the determination in the clinical trial (P1) by using incurred sample reanalysis (ISR) described in the EMA guideline [14,28] and recommended by Lowes et al. [30] as incurred sample stability (ISS). Samples are regarded as stable if the % difference (Eq. (1)) has not exceeded 20% for at least 67% of the samples [14].

$$\%\ \boldsymbol{difference} = \frac{\boldsymbol{P_2 - P_1}}{\bar{\boldsymbol{P}}} \cdot \boldsymbol{100}\% \quad (1)$$

Where P_1 – the first result, P_2 – reanalysis result, $\bar{P}$ – arithmetic mean of P_1 and P_2.

3. Results and discussion

3.1. Optimization method

The method development started from the modification of the Contractor's method [18]. In the first step, we compared two extrahents: methyl tert-butyl ether/n-hexane (80:20, *v*/*v*) and methyl tert-butyl ether alone (2.5 mL) (Fig. 2A). Both were acidified with 0.5% formic acid. The mean recoveries were similar, so the methyl tert-butyl ether was selected, due to the shorter reagents' preparation step. Next, various volumes of methyl tert-butyl ether (2.5, 2. 0, 1.5, 1.0 mL) with 0.5% formic acid were tested (Fig. 2B). The best recoveries were obtained for 1.5 mL of extrahent. However, since the differences were not significant from the analytical point of view, we selected 1.0 mL of methyl tert-butyl ether as a more environment-friendly variant. The highest influence on the recovery had the pH. It was assessed by sample extraction with a solvent with NaOH (1, 0.5, and 0.25 M), 0.5% formic acid, and ammonia (1% and 0.001%) (Fig. 2C). Recoveries obtained for NaOH in all studied concentrations were much lower than for other studied pH modifiers. The best results were obtained with the addition of 0.001% ammonia, which was applied in the final method. In the last step, the shape of the peaks on two columns was compared. Bionacore C18 4.6 × 100 mm, 2.7 µm column gave sharper and higher (except 6OH) peaks that enable to obtain the lower limit of detection than Zorbax 100 × 3.0 mm, 3.5 µm, thus was selected for the final method (Fig. 3). Differences were especially prominent for DUT and DHD.

The performance of our method was evaluated for linearity, accuracy, precision, matrix effect, recovery, the lower limit of quantification, and stability (autosampler, short-term, long-term, freeze, and thaw). All validation criteria were fulfilled.

3.2. Linearity

The developed LC-MS/MS method with good linearity covered the range of 0.1–3.5 ng/mL for DUT and 0.08–1.2 ng/mL for DHD, 4OH, 6OH with a coefficient of determination (r^2) > 0.980 that was obtained for all analytes regarding the peak area ratio of every analyte to the internal standard (IS) versus the nominal concentration. The weighted linear regression 1/x was selected as optimal. The regression parameters for all analytes were described by the equation: y = ax + b. The values of a, b, and r^2 for all analytes are presented in Table 1.

The previous bioanalytical methods for dutasteride determination after a single administration of 0.5 mg covered a wider range of linearity, from 0.1 ng/mL to 25 ng/mL [18,19], it did not need to be as wide in our case. The pharmacokinetic literature data indicated that the maximum concentration value would not exceed 4 ng/mL.

Table 1
The parameters of the calibration curve for dutasteride (DUT) and its metabolites: 4′-hydroxydutasteride (4OH), 6β-hydroxydutasteride (6OH) and 1,2-dihydrodutasteride (DHD) (*n* = 6).

Analyte	r^2	y = ax + b	Standard deviation		Significance of *b*
			a	b	
DUT	0.9950	y = 1.1977x + 0.232	0.269	0.0185	Yes
4OH	0.9834	y = 0.7617x − 0.0040	0.0450	0.0184	No
DHD	0.9815	y = 0.6631x − 0.0016	0.276	0.0113	No
6OH	0.9851	y = 0.9322x − 0.0038	0.0357	0.0153	No

Table 2
Precision and accuracy intra-run (*n* = 5) and between-run (*n* = 15) in human plasma for the lower limit of quantification (LLOQ) and quality control (QC) samples at low, medium, and high concentration of dutasteride (DUT) and its metabolites: 4′-hydroxydutasteride (4OH), 6β-hydroxydutasteride (6OH) and 1,2-dihydrodutasteride (DHD).

Compound	Intra/between-run	LLOQ		QC_{low}		QC_{medium}		QC_{high}	
		Precision [%]	Accuracy [%]	Precision [%]	Accuracy [%]	Precision [%]	Accuracy [%]	Precision [%]	Accuracy [%]
DUT	intra	9.1–17.5	96.2–103.1	7.4–12.3	95.2–105.9	6.9–8.2	95.8–105.2	3.5–6.5	100.7–105.7
	between	13.7	103.1	10.3	99.7	8.2	100.6	5.5	103.3
4OH	intra	12.0–17.7	93.6–114.3	3.6–7.2	98.3–104.1	3.0–6.3	96.0–109.6	5.3–7.3	93.9–104.9
	between	16.9	111.8	6.31	100.4	7.80	101.7	8.2	97.6
6OH	intra	8.4–17.4	94.1–104.2	5.0–7.9	90.3–107.9	5.2–10.0	92.5–97.6	6.5–8.6	94.5–105.4
	between	13.5	99.2	10.1	99.5	7.50	95.7	9.53	97.8
DHD	intra	7.6–12.0	98.2–106.9	7.7–11.8	97.2–103.7	2.45–4.3	89.7–100.7	8.6–13.6	92.9–98.3
	between	16.1	97.7	9.24	99.8	4.2	93.9	10.42	96.3

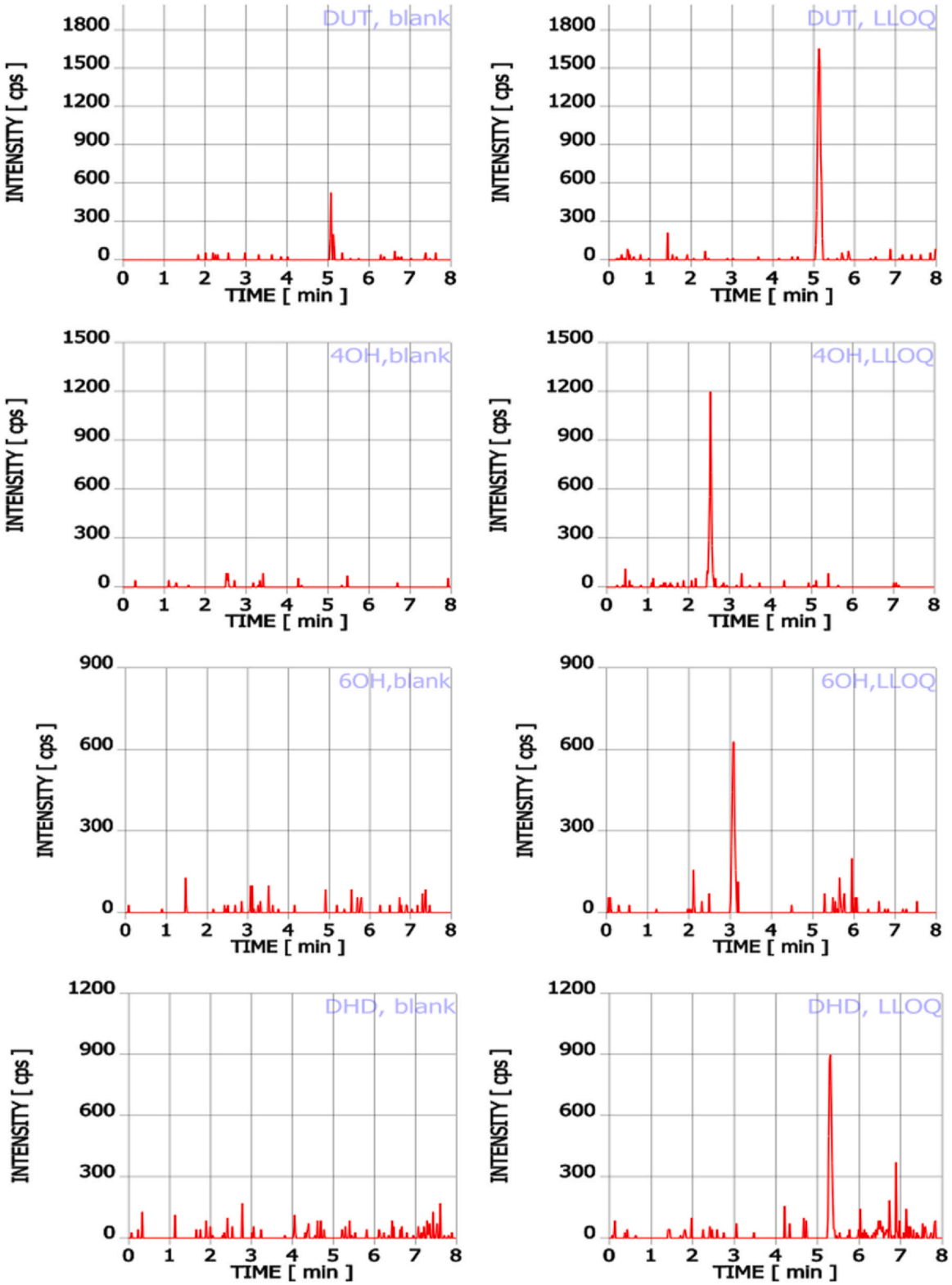


Fig. 4. Multiple reaction monitoring (MRM) chromatogram peaks of dutasteride (DUT) and metabolites: 4′-hydroxydutasteride (4OH), 6β-hydroxydutasteride (6OH) and 1,2-dihydrodutasteride (DHD) in blank plasma and spiked plasma at the lower limit of quantitation (0.1 ng/mL for DUT, 0.08 ng/mL for metabolites).

Originally, the method was planned in the range of 0.1–7.5 ng/mL. However, the method was revalidated with linearity range narrowed until 3.5 ng/mL, because the determined concentrations of the reference drug were relatively low (mean value 1.415 ng/mL and maximum value not exceeding 3.0 ng/mL), and wide range with an upper limit of quantitation at 7.5 ng/mL was not justified in this case. Moreover, what is more important, this range made it possible to meet the requirements of the European Medicine Agency regarding the AUC, i.e. to determine more than 80% of the entire pharmacokinetic profile. During the analysis of samples from volunteers, it is needed to have at least three QCs within the range of concentrations determined in the samples. Such a narrow calibration curve allowed

us to meet the requirement without the need for preparation of the additional QC. Because the metabolite concentrations were lower than dutasteride their linearity range was narrowed down to 1.2 ng/mL.

3.3. The lower limit of quantification, precision, and accuracy

Intra-run and between-run accuracy and precision of the method for LLOQ and QC samples for all analytes met the acceptance criteria (see Section 3.5). For each LLOQ, a signal-to-noise ratio (s/n) higher than five was observed: s/n = 9.5 for DHD, s/n = 15 for 6OH, s/n = 25 for 4OH, s/n = 43 for DUT. Between-run DUT precision was in the range 5.5–13.7%, and accuracy was from 99.7% to 103.3% (Table 2). The obtained accuracy and precision for DUT are comparable to the reported values in other papers [19,20]. Chromatograms of the DUT and metabolites extracted from blank plasma and spiked plasma at LLOQ are presented in Fig. 4. The acceptance criteria for accuracy was 85–115% for QC and 80–120% for LLOQ, whereas for precision was 15% for QC and 20% for LLOQ.

3.4. Matrix effect and recovery calculation

The coefficient of variation of internal standard (IS) normalized matrix factor ($MF_{IS\text{-}norm}$) at three concentrations of all metabolites and DUT did not exceed the acceptance criterion of 15% ranging from 4.1% to 14.1% ($n = 6$ at each QC level). The recovery of DUT and metabolites were constant at all concentrations. The recovery of IS was independent of the concentration of the analyte (Table 3). The hemolysis and lipemia did not influence the method's reliability.

Fig. A1 visualizes the absolute matrix effect of DUT, DHD, 4OH, and 6OH. As we can see, the signal from the analytes is stable till 5.5 min, which proves the lack of the absolute matrix effect, probably due to satisfactory sample purification during the extraction process. Relatively high variation of $MF_{IS\text{-}norm}$ can be explained by the method precision, which could be better if isotopically labeled analogues (currently not commercially available) of each analyte would be used. Fig. 2 shows the multiple reaction monitoring (MRM) chromatogram peaks of DUT, DHD, 4OH, and 6OH in blank plasma and spiked plasma at the lower limit of quantitation (0.1 ng/mL for DUT, 0.08 ng/mL for metabolites).

Table 3
Matrix effect and recovery for dutasteride (DUT) and its metabolites: 4′-hydroxydutasteride (4OH), 6β-hydroxydutasteride (6OH), and 1,2-dihydrodutasteride (DHD) at low, medium, and high concentrations ($n = 6$ at each level). Internal standard (IS) was a dutasteride$^{13}C_6$.

Analyte	QC	$MF_{IS\text{-}norm}$		Recovery [%]			
		[%]	CV [%]	Analyte	CV [%]	IS	CV [%]
DUT	low	102.2	14.1	95.0	13.5	75.9	3.9
	medium	97.3	8.0	80.7	4.2	85.9	14.1
	high	104.3	4.1	81.8	9.9	83.1	9.8
4OH	low	90.6	8.6	79.3	11.9	79.4	10.8
	medium	97.1	8.3	79.3	12.0	85.9	14.1
	high	101.8	9.5	81.3	5.7	83.1	9.8
DHD	low	108.2	9.1	78.6	16.2	75.9	3.9
	medium	101.5	8.5	78.6	10.2	85.9	14.1
	high	103.7	8.7	79.7	7.2	83.1	9.8
6OH	low	94.7	12.1	80.2	15.0	75.9	6.3
	medium	98.2	6.7	85.5	5.0	85.9	14.0
	high	98.2	9.1	86.5	6.6	83.1	11.0

IS – internal standard, $^{13}C_6$-dutasteride; QC-quality control; The acceptance criteria for CV of $MF_{IS\text{-}norm}$ was ≤ 15%.

3.5. Dilution integrity and stability

The dilution integrity was confirmed for 5-times diluted samples. The accuracy was determined as 100.3% (CV = 2.5%) for DUT, 108.3% (CV = 2.3%) for 4OH, 102.6% (CV = 5.7%) for 6OH and 96.4% for DHD (CV = 5.8%), respectively. All analytes were stable under all tested conditions: autosampler stability and stability in human plasma, i.e., short-term stability (4 h at room temperature), long-term stability (32 days at − 65 °C), and freeze-thaw stability. Both QC_{low} and QC_{high} samples showed no significant changes in comparison to nominal concentrations (Table 4). The acceptance criteria for stability was 85–115%.

3.6. Method application

3.6.1. Concentration in plasma of volunteers

The concentration of DUT in plasma ranged from < 0.1 ng/mL to 2.4 ng/mL, which corresponds well with the levels reported in other studies of single oral administration of 0.5 mg of DUT (Fig. 5) [7,20,22,31]. The highest mean concentration (2.08 ± 0.66 ng/mL) was observed after 1.92 ± 0.56 h. The $t_{1/2}$ was 55 ± 21 h. The AUC until the infinity was 68 ± 26 ng·h/mL. The corresponding values found in literature were equal within the uncertainty and were ranged from 44.54 ± 20.11 ng h/mL [32] to 81.06 ± 31.82 ng h/mL [18] for AUC, from 1.96 ng/mL [20] to 3.56 ± 0.92 ng/mL [18] for C_{max}, from 1.5 h (0.75–3.00 h) [32] to 3.0 h (2.0–6.0 h) [33] for t_{max} and from 56.27 ± 5.87 h [34] to 80.91 ± 36.79 h [18] for $t_{1/2}$. The only metabolite determined at a significant level (up to 0.36 ng/mL)

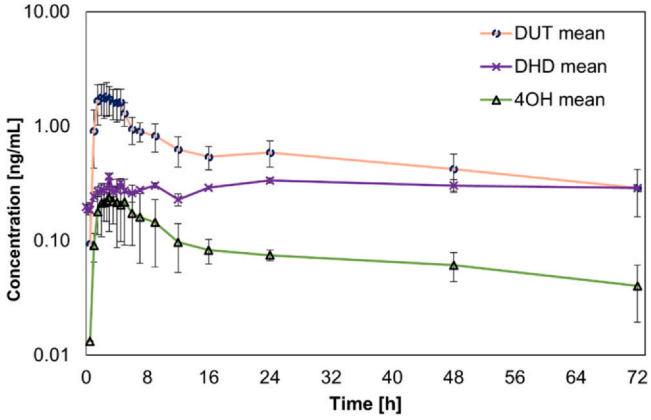


Fig. 5. Mean plasma concentration-time profile of dutasteride (DUT) and its major metabolites, 4-hydroxydutasteride (4OH) and 1,2-dihydrodutasteride (DHD) after oral administration of Avodart (0.5 mg of DUT in soft gelatin capsules) to three volunteers. Error bars represent the standard deviation.

Table 4
Stability of dutasteride (DUT) and their major metabolites: 4′-hydroxydutasteride (4OH), 6β-hydroxydutasteride (6OH), and 1,2-dihydrodutasteride (DHD).

Type of stability	Short-term [%]		Long-term [%]		Freeze-thaw [%]		In autosampler [%]	
Analyte	QC_{low} (CV%)	QC_{high} (CV%)	QC_{low} (CV%)	QC_{high} (CV%)	QC_{low} (CV%)	QC_{high} (CV%)	QC_{low} (CV%)	QC_{high} (CV%)
DUT	92.0 (14)	103.1 (4.5)	94.0 (4.4)	97.9 (7.7)	87.7 (8.1)	97.4 (5.3)	102 (12)	94.6 (6.9)
4OH	100.6 (5.6)	106.1(10)	109.0 (4.9)	106.0 (6.0)	93.7 (10)	90.0 (7.5)	94.6 (13)	97.4 (13)
6OH	94.2 (11)	104.6 (3.7)	106 (11)	106.0 (8.2)	90.6 (7.9)	88.2 (6.2)	100.8 (7.6)	105.6 (6.5)
DHD	95.2 (8.3)	96 (12)	105.5 (7.9)	105.0 (2.4)	101 (11)	97.4 (5.4)	103.3 (12)	95.4 (10)

was 4OH. The only reported data on the concentration of 4OH in human plasma are for steady-state [9]. The drug was administrated for healthy subjects at 0.5 mg for at least six months, not once as in our study. Thus, the concentration reported (7.9 ng/mL) were much higher than those in our study [9].

6OH was observed only in two samples and was very low (up to 0.17 ng/mL). There is no report on the concentration of the metabolite in human plasma. In rat plasma, the mean maximum level ($n = 4$) of 6OH was 103 ± 20 ng/mL, but the administrated doses were much higher (5 mg/kg b.w., orally) [10]. Another metabolite, DHD reached the concentration of 0.40 ng/mL at 3 h. The concentration of DHD in plasma was not detected for the first 2 h after drug administration in a volunteer from the period I of the clinical trial. On the contrary, for the volunteers from period II of the clinical trial, the DHD was detected in all data points. It can be caused by the long half-life of the metabolite and too short a wash-out period for the metabolite.

In the case of DUT and its metabolites, double peaks in concentration versus time curves were observed. In general, the possible reasons for the double peak phenomenon can be enterohepatic circulation, two different sites of absorption or an irregular pattern of gastric emptying [35].

3.6.2. Long-term stability of DUT

DUT was stable in the clinical study samples after more than two years of storage below − 65 °C (i.e. 1014 days). The results exceeding the permissible % difference value were less than 12% of all the results obtained for the DUT, as is shown in Fig. A2.

4. Conclusions

To the best of our knowledge, this is the first report in which a straightforward, sensitive, and validated LC-MS/MS method was developed for the simultaneous determination of dutasteride and its major metabolites 4′-hydroxydutasteride, 6β-hydroxydutasteride and 1,2-dihydrodutasteride in human plasma. The method was applied to determine the concentration of dutasteride and its metabolites in plasma after administration of Avodart (0.5 mg, dutasteride soft gelatin capsule) to healthy volunteers. The concentration of 4′-hydroxydutasteride and 1,2-dihydrodutasteride are much higher than 6β-hydroxydutasteride. The clinical samples can be stored for up to 1014 days without the dutasteride loss. Further studies on the stability should be performed, including a longer storage period to verify the possibility of shortening the time of archiving clinical trial samples.

Funding

The project was financially supported by the Ministry of Science and Higher Education: Contract no. DWD/3/6/2019 dated 21.11.2019 and The National Centre for Research and Development: Synthesis and New Manufacturing Technology of a Drug Containing a 5-α Reductase Inhibitor (INNOTECh-K2/IN2/65/182982/NCBR/13).

CRediT authorship contribution statement

M.K: Conceptualization, **K.B., E.G., M.K., and J.G:** Methodology, **E.G., and M.K:** Software, **M.K., K.B., E.G. and J.G:** Validation, **K.B., M.K. and J.G:** Formal analysis, **K.B. and E.G:** Investigation, **J.G., K.B:** Resources, **J.G., E.G and M.K:** Data curation, **J.G., E.G:** Writing – original draft preparation, **J.G., E.G. and M.K:** Writing – review & editing, **E.G:** Visualization, **J.G. and M.K:** Supervision, **J.G. and M.K:** Project administration, **E.G:** Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are grateful to F1 Pharma sp. z o.o., Jagiellonian Centre of Innovation and Trial Clinical Research s.c. for their cooperation in the project, Ryszard Marszałek for technical assistance in LC-MS/MS analyses, and Andrzej Kutner and Piotr J. Rudzki for the critical reviewing of the manuscript.

Conflict of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2021.114362.

References

[1] S. Aggarwal, S. Thareja, A. Verma, T.R. Bhardwaj, M. Kumar, An overview on 5α-reductase inhibitors, Steroids 75 (2) (2010) 109–153.
[2] P.O. Gisleskog, D. Hermann, M. Hammarlund-Udenaes, M.O. Karlsson, The pharmacokinetic modelling of GI198745 (dutasteride), a compound with parallel linear and nonlinear elimination, Br. J. Clin. Pharmacol. 47 (1) (1999) 53–58.
[3] H.C. Evans, K.L. Goa, Dutasteride, Drugs Aging 20 (12) (2003) 905–916.
[4] C.G. Roehrborn, L.S. Marks, T. Fenter, S. Freedman, J. Tuttle, M. Gittleman, B. Morrill, E.T. Wolford, Efficacy and safety of dutasteride in the four-year treatment of men with benign prostatic hyperplasia, Urology 63 (4) (2004) 709–715.
[5] J. Wiebe, L. Souter, G. Zhang, Dutasteride affects progesterone metabolizing enzyme activity/expression in human breast cell lines resulting in suppression of cell proliferation and detachment, J. Steroid Biochem. Mol. Biol. 100 (2006) 129–140.
[6] S.J. Keam, L.J. Scott, Dutasteride, Drugs 68 (4) (2008) 463–485.
[7] B. Prasaja, Y. Harahap, W. Lusthom, L. Yumi, A. Sofiana, M. Sandra, F. Safira, U. Chilmi, Impact of truncated area on point estimate and intra-subject variability in bioequivalence of dutasteride with long half-life, Drug Res. 68 (4) (2018) 238–240.
[8] Guidance for Industry: Safety Testing of Drug Metabolites, U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), 2020.
[9] Duagen (dutasteride) Soft gel Capsules, Clinical Pharmacology and Biopharmaceutics Review(s) Part 1, Center for Drug Evaluation and Research Application numer: 21-319, 2001, (access online:).
[10] S.-W. Seo, J.W. Park, D.-G. Han, J.-M. Kim, S. Kim, T. Park, K.-H. Kang, M.H. Yang, I.-S. Yoon, In vitro and in vivo assessment of metabolic drug interaction potential of dutasteride with ketoconazole, Pharmaceutics 11 (12) (2019) 673.
[11] T. Hutchinson, J. Madden, V. Naidoo, C. Walker, Comparative metabolism as a key driver of wildlife species sensitivity to human and veterinary pharmaceuticals, Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 369 (2014).
[12] E.M. Brun, A. Torres, R. Ventura, R. Puchades, Â. Maquieira, Enzyme-linked immunosorbent assays for doping control of 5α-reductase inhibitors finasteride and dutasteride, Anal. Chim. Acta 671 (1) (2010) 70–79.
[13] European Medicines Agency, Guideline on the investigation of bioequivalence (CPMP/EWP/QWP/1401/98/Rev. 1/Corr**), London, 2010.
[14] Guideline on bioanalytical method validation, Committee for Medicinal Products for Human Use (CHMP), EMEA/CHMP/EWP/192217/2009, European Medicines Agency, 2011.
[15] Guidance for Industry: Bioanalytical Method Validation, Food and Drug Administration, Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine (CVM), 2018.
[16] Good Clinical Laboratory Practice (GCLP), World Health Organization, World Health Organization, 2009.
[17] OECD Principles on Good Laboratory Practice, OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring Number 1, 1997.
[18] P. Contractor, H. Kurani, S. Guttikar, P.S. Shrivastav, Reliable and sensitive determination of dutasteride in human plasma by liquid chromatography–tandem mass spectrometry, Biomed. Chromatogr. 27 (9) (2013) 1168–1176.
[19] N.V.S. Ramakrishna, K.N. Vishwottam, S. Puran, M. Koteshwara, S. Manoj, M. Santosh, Selective and rapid liquid chromatography–tandem mass spectrometry assay of dutasteride in human plasma, J. Chromatogr. B 809 (1) (2004) 117–124.

Supplementary material

LC-MS/MS determination of dutasteride and its major metabolites in human plasma

Elżbieta GNIAZDOWSKA[1,2], Michał KAZA[2*], Katarzyna BUŚ-KWAŚNIK[2] and Joanna GIEBUŁTOWICZ[3]*

[1]Department of Bioanalysis and Drugs Analysis, Doctoral School, Medical University of Warsaw, 61 Żwirki i Wigury, 02-091Warsaw, Poland

[2]Łukasiewicz Research Network, Industrial Chemistry Institute, 8 Rydygiera, 01-793Warsaw, Poland;

[3]Department of Bioanalysis and Drugs Analysis, Faculty of Pharmacy, Medical University of Warsaw,1 Banacha, 02-097Warsaw, Poland;

*Correspondence:
Joanna Giebułtowicz, Department of Bioanalysis and Drugs Analysis, Faculty of Pharmacy, Medical University of Warsaw,1 Banacha, 02-097Warsaw, Poland;
joanna.giebultowicz@wum.edu.pl
Michał Kaza, Łukasiewicz Research Network, Industrial Chemistry Institute, 8 Rydygiera, 01-793 Warsaw, Poland; michal.kaza@gmail.com

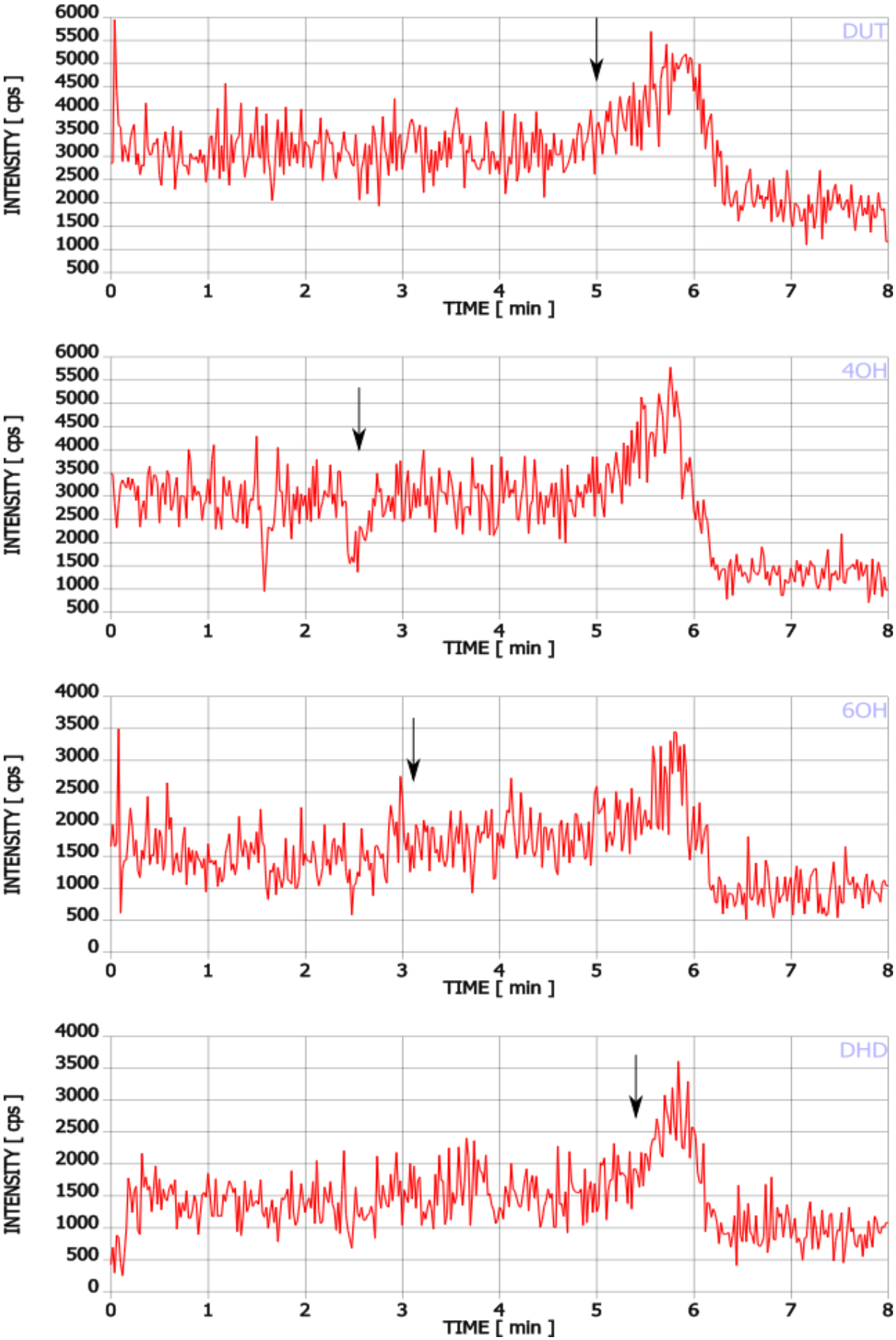


Figure A1. Chromatograms recorded during the evaluation of the matrix effect4by the steady post-column infusion and the injection of the extracted blank plasma sample for dutasteride (a), 1,2-dihydrodutasteride (b), 4'-hydroxydutasteride (c) and 6β-hydroxydutasteride (d).

Table A1. Previous bioanalytical methods for DUT.

Instrumental method	Matrix	Metabolite	Internal standard	Sample volume [µL]	Sample preparation [1]	LLOQ [ng/mL]	ULOQ [ng/mL]	Recovery	Long-term stability	Ref.	Application
LC-MS/MS	human plasma	6OH, 4OH, DHD	dutasteride $^{13}C_6$	500	LLE MTBE (1 mL) + 0.001% (*v/v*) NH_4OH	0.1	3.5	78.6-95.0%	2 years −65 ° C	The study	PK study with 0.5 mg of DUT (oral single dose) (healthy)
LC-MS/MS	human plasma	-	dutasteride $^{13}C_6$	300	LLE MTBE-n-hexane (80:20, *v/v*) (2.5 mL) + 0.5% (*v/v*) HCOOH	0.1	25	96.8%	59 days −20 ° C, and −72 ° C	[2]	BE study with 0.5 mg of DUT (oral single dose) (healthy)
LC-MS/MS	human serum	-	dutasteride $^{13}C_6$	100	LLE hexane-MTBE-DCM [2]	0.025	2.5	-	-	[3]	BE study with 5 capsules of 0.1 mg DUT in comparison with one capsule of 0.5 mg DUT
LC-MS/MS	human plasma	-	finasteride D_9	500	SPE Waters Oasis ® HLB cartridges	1.0	100	-	28 days -20 ℃	[4]	PK study with 0.5 mg of DUT (healthy volunteers) (three months)
LC-MS/MS	human plasma	-	Finasteride	900	LLE DEE–DCM (70:30, *v/v*) (6 mL) + 0.1M NaOH	2.0	20	55.2%	10 days −20 ° C	[5]	BE study with 0.5 mg of DUT and 0.4 mg of tamsulosin (healthy)
LC-MS/MS	human plasma	-	Finasteride	500	LLE DEE–DCM (70:30, v/v) (4 mL) + 0.1M NaOH	0.1	25	40.7%	10 days −72 ° C	[6]	PK study with 0.5 mg of DUT (oral single dose) (healthy)
LC-MS/MS	human plasma	-	Finasteride	500	SPE Phenomenex Strata-X cartridges	0.1	10	103.8%	33 days -20 ℃	[7]	BE study with 0.5 mg of DUT and 10 mg of alfuzosin (healthy)
LC-MS/MS	human plasma	-	Alprazolam	1000	LLE MTBE [2]	0.05	4	73–77.3%	-	[8]	BE study with 0.5 mg of DUT (healthy) to investigate the effect of using the truncated area under the curve (AUC0-72) on BE of DUT with a long half-life in point estimate and intra-subject variability.

Instrumental method	Matrix	Metabolite	Internal standard	Sample volume [µL]	Sample preparation [1]	LLOQ [ng/mL]	ULOQ [ng/mL]	Recovery	Long-term stability	Ref.	Application
LC-MS/MS	rat plasma	-	Finasteride	100	LLE MTBE-DCM (70:30, v/v) (0.6 mL) + 1.0 M NaOH	1	500	-	-	[9]	PK study for oral administration of a micronized drug, physical mixture, or solid in rat
LC-MS/MS	rat plasma	-	Finasteride	100	LLE MTBE-DCM (70:30, v/v) (0.6 mL) + 1.0 M NaOH	5	400	107.8%	-	[10]	PK - absorption studies of the drug suspension with the marketed product (Avodart®,GlaxoSmithKline) on rats at 2 mg/kg
HPLC-FL	rat plasma	6OH	Celecoxib	100	PP	10	1000	-	-	[11]	Interaction DUT (5 mg/kg) with ketoconazole (20 mg/kg) and PK study for 6OH and DUT in rat

Table A2. Multiple Reaction Monitoring parameters and retention time for dutasteride (DUT) and its metabolites: 4'-hydroxydutasteride (4OH), 6β-hydroxydutasteride (6OH) and 1,2-dihydrodutasteride (DHD).

	DUT	IS	4OH	6OH	DHD
DP [V]	116	161	161	136	156
CE [V]	49	49	49	45	55
CXP [V]	12	12	12	16	10
Retention time [min]	5.1	5.1	2.5	3.1	5.3

DP - declustering potential; CE - collision energy; CXP - collison cell exit potential

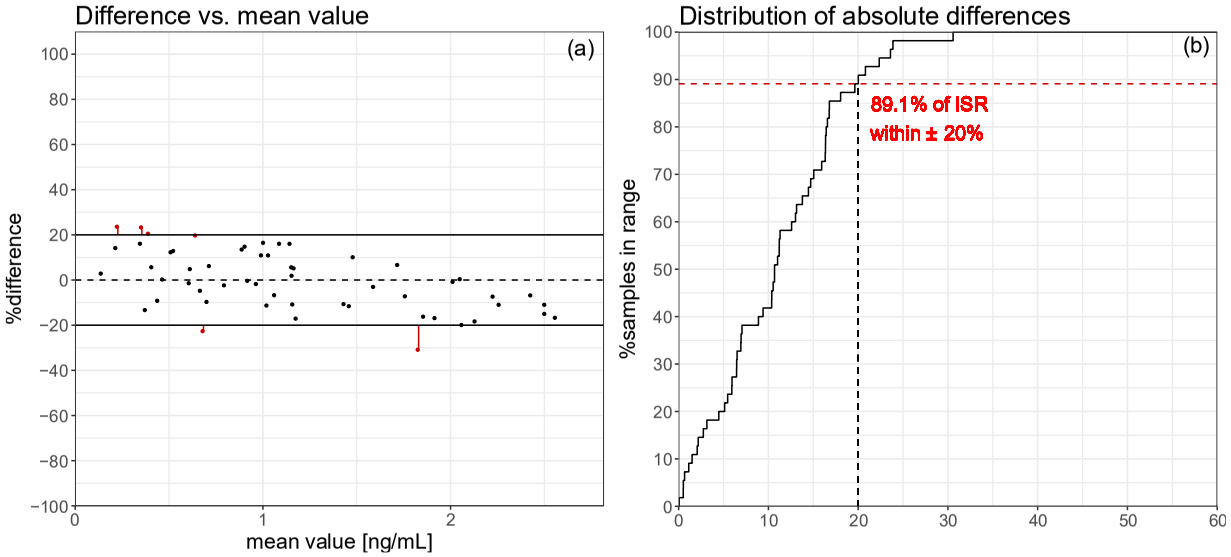


Figure A2. The stability of dutasteride (DUT) in clinical samples over 2 years of storage below -65°C. (**a**) The % of difference i.e (concentration in the reanalyzed sample - first result)/ mean concentration in first and reanalyzed samples (**b**) Distribution of the absolute differences between results for analyzed and reanalyzed samples. Samples are regarded as stable if % of difference did not exceed 20 % for at least 67% of the samples. The Figure was prepared using ISR tool [1].

References

[1] P.J. Rudzki, P. Biecek, M. Kaza, Comprehensive graphical presentation of data from incurred sample reanalysis, Bioanalysis 9(12) (2017) 947-956. https://doi.org/10.4155/bio-2017-0038.
[2] P. Contractor, H. Kurani, S. Guttikar, P.S. Shrivastav, Reliable and sensitive determination of dutasteride in human plasma by liquid chromatography–tandem mass spectrometry, Biomedical Chromatography 27(9) (2013) 1168-1176. https://doi.org/https://doi.org/10.1002/bmc.2923.
[3] M.J. Fossler, D.A. Collins, H. Ino, N. Sarai, R. Ravindranath, C.L. Bowen, O. Burns, Evaluation of bioequivalence of five 0.1 mg dutasteride capsules compared to one 0.5 mg dutasteride capsule: a randomized study in healthy male volunteers, Journal of drug assessment 4(1) (2015) 24-29. https://doi.org/10.3109/21556660.2015.1067219.
[4] R. Upreti, G. Naredo, A.M. Faqehi, K.A. Hughes, L.H. Stewart, B.R. Walker, N.Z. Homer, R. Andrew, Simultaneous pharmacokinetic and pharmacodynamic analysis of 5alpha-reductase inhibitors and androgens by liquid chromatography tandem mass spectrometry, Talanta 131 (2015) 728-35. https://doi.org/10.1016/j.talanta.2014.07.087.

[5] S. Agarwal, K.V. Gowda, A.K. Sarkar, D. Ghosh, U. Bhaumik, T.K. Chattaraj, T.K. Pal, Simultaneous Determination of Tamsulosin and Dutasteride in Human Plasma by LC–MS–MS, Chromatographia 67(11-12) (2008) 893-903. https://doi.org/10.1365/s10337-008-0613-7.
[6] N.V.S. Ramakrishna, K.N. Vishwottam, S. Puran, M. Koteshwara, S. Manoj, M. Santosh, Selective and rapid liquid chromatography–tandem mass spectrometry assay of dutasteride in human plasma, Journal of Chromatography B 809(1) (2004) 117-124. https://doi.org/https://doi.org/10.1016/j.jchromb.2004.06.010.
[7] N. Gomes, A. Pudage, S. Joshi, V. Vaidya, S. Parekh, A. Tamhankar, Rapid and Sensitive LC–MS–MS Method for the Simultaneous Estimation of Alfuzosin and Dutasteride in Human Plasma, Chromatographia 69 (2009) 9-18. https://doi.org/10.1365/s10337-008-0821-1.
[8] B. Prasaja, Y. Harahap, W. Lusthom, L. Yumi, A. Sofiana, M. Sandra, F. Safira, U. Chilmi, Impact of Truncated Area on Point Estimate and Intra-Subject Variability in Bioequivalence of Dutasteride with Long Half-Life, Drug Res (Stuttg) 68(4) (2018) 238-240. https://doi.org/10.1055/s-0043-121695.
[9] I.-H. Beak, M.-S. Kim, Improved Supersaturation and Oral Absorption of Dutasteride by Amorphous Solid Dispersions, Chemical and Pharmaceutical Bulletin 60(11) (2012) 1468-1473. https://doi.org/10.1248/cpb.c12-00563.
[10] M.J. Kang, H. Cho, D. Yeom, Y. Choi, Y. Choi, A Method to Monitor Dutasteride in Rat Plasma Using Liquid-Liquid Extraction and Multiple Reaction Monitoring: Comparisons and Validation, Mass Spectrometry Letters 5 (2014) 79-83. https://doi.org/10.5478/MSL.2014.5.3.79.
[11] S.-W. Seo, J.W. Park, D.-G. Han, J.-M. Kim, S. Kim, T. Park, K.-H. Kang, M.H. Yang, I.-S. Yoon, In Vitro and In Vivo Assessment of Metabolic Drug Interaction Potential of Dutasteride with Ketoconazole, Pharmaceutics 11(12) (2019) 673.

Publikacja 3 (oryginalna)

Gniazdowska E., Goch W., Giebułtowicz J., Rudzki P.J. Replicates number for drug stability testing during bioanalytical method validation – experimental and retrospective approach. Molecules 2022; 27: 457. IF= 4,600; MEiN = 140.

molecules

MDPI

Article

Replicates Number for Drug Stability Testing during Bioanalytical Method Validation—An Experimental and Retrospective Approach

Elżbieta Gniazdowska [1,2], Wojciech Goch [3], Joanna Giebułtowicz [4] and Piotr J. Rudzki [5,*]

[1] Łukasiewicz Research Network, Industrial Chemistry Institute, 8 Rydygiera, 01-793 Warsaw, Poland; elzbieta.gniazdowska@ichp.lukasiewicz.gov.pl or elzbieta.gniazdowska@wum.edu.pl
[2] Department of Bioanalysis and Drugs Analysis, Doctoral School, Medical University of Warsaw, 61 Żwirki i Wigury, 02-091 Warsaw, Poland
[3] Department of Physical Chemistry, Faculty of Pharmacy, Medical University of Warsaw, 1 Banacha, 02-097 Warsaw, Poland; wojciech.goch@wum.edu.pl
[4] Department of Bioanalysis and Drugs Analysis, Faculty of Pharmacy, Medical University of Warsaw, 1 Banacha, 02-097 Warsaw, Poland; joanna.giebultowicz@wum.edu.pl
[5] Celon Pharma S.A., Bioanalytical Laboratory, 15 Marymoncka, 05-152 Kazuń Nowy, Poland
* Correspondence: pj.rudzki@wp.pl

Abstract: Background: The stability of a drug or metabolites in biological matrices is an essential part of bioanalytical method validation, but the justification of its sample size (replicates number) is insufficient. The international guidelines differ in recommended sample size to study stability from no recommendation to at least three quality control samples. Testing of three samples may lead to results biased by a single outlier. We aimed to evaluate the optimal sample size for stability testing based on 90% confidence intervals. Methods: We conducted the experimental, retrospective (264 confidence intervals for the stability of nine drugs during regulatory bioanalytical method validation), and theoretical (mathematical) studies. We generated experimental stability data (40 confidence intervals) for two analytes—tramadol and its major metabolite (O-desmethyl-tramadol)—in two concentrations, two storage conditions, and in five sample sizes (n = 3, 4, 5, 6, or 8). Results: The 90% confidence intervals were wider for low than for high concentrations in 18 out of 20 cases. For n = 5 each stability test passed, and the width of the confidence intervals was below 20%. The results of the retrospective study and the theoretical analysis supported the experimental observations that five or six repetitions ensure that confidence intervals fall within 85–115% acceptance criteria. Conclusions: Five repetitions are optimal for the assessment of analyte stability. We hope to initiate discussion and stimulate further research on the sample size for stability testing.

Keywords: confidence interval; stability; retrospective analysis; sample size; regulatory bioanalysis; bioanalytical method validation

Citation: Gniazdowska, E.; Goch, W.; Giebułtowicz, J.; Rudzki, P.J. Replicates Number for Drug Stability Testing during Bioanalytical Method Validation—An Experimental and Retrospective Approach. *Molecules* **2022**, *27*, 457. https://doi.org/10.3390/molecules27020457

Academic Editor: C. Michael Greenlief

Received: 17 December 2021
Accepted: 7 January 2022
Published: 11 January 2022

1. Introduction

Evaluation of drug or metabolite stability in biological samples in conditions reflecting sample handling and analysis during bioanalytical method validation is recommended by international regulatory guidelines [1,2] and ICH M10 draft guidelines [3]. This evaluation includes stability in the biological matrix (short-term, long-term, and freeze-thaw), in processed samples and solutions (stock and working solutions). Kaza et al. (2019) [4] discussed the differences and similarities in bioanalytical method validation guidelines [1,2], but the authors omitted to mention differences in the recommended sample size (number of samples) for stability testing. The European Medicines Agency (EMA) [1] does not recommend any specific sample size whereas the U.S. Food and Drug Administration (FDA) [2] and ICH [3] recommend a minimum of three quality control samples (QC) per level of concentration of low QC and high QC to assess the stability of an analyte in a

biological matrix. A note from Health Canada does not recommend examining stability using only one repetition of a QC sample [5].

The analyte stability testing refers to other characteristics of the bioanalytical method. The calibration range helps to select studied concentrations (low- and high-quality control samples). However, method precision is important to compare reference samples (e.g., prepared ex tempore) and test samples (i.e., stored for a specified time in specified conditions). Before any regulatory bioanalytical method validation guideline was published, Timm et al. proposed a stability assessment incorporating the precision in the calculation of 95% confidence intervals [6]. However, its application was limited by the assumed equality of variances for the reference and test samples. Rudzki and Leś extended this method for datasets with unequal variances [7]. They also proposed the use of 90% confidence intervals instead of 95% [6] to make the probability equal to the bioequivalence recommendations [8]. Confidence intervals are a good tool for testing stability. Since their introduction by Jerzy Spława Neyman in 1936 [9] they became widely used, including clinical research—for example as bioequivalence criterium [8]. Briefly, the idea of confidence intervals is to define a range of values describing parameters of interest in the population, based on parameter estimates observed in the sample. This estimation has a defined probability—usually 90%, 95%, or 99%. For example, a 90% confidence interval of 85.1–105.2% for mean stability means that there is a 90% probability that the mean stability is between 85.1% and 105.2%. In the case of stability testing, the confidence interval combines central tendency (mean difference between stored and reference samples) and data dispersion (method precision) with a selected probability. This approach is not yet frequently used because it is more restrictive and labor intensive than the guidelines' recommendations. Nevertheless, the confirmation of analyte stability in a biological matrix using this method is associated with a low and pre-defined probability of true instability.

The stability assessment proposed in the draft of the ICH M10 bioanalytical method validation guideline [3] recommends analyzing stored and reference samples but does not include a description of any comparison between them. The lack thereof creates the risk of accepting the method regardless of the 29.8% instability of an analyte [4]. Moreover, there is an insufficient justification of sample size (number of samples) in the stability evaluation. Limiting testing to three samples in each dataset may lead to stability results biased by a single outlier. However, how much do additional analyses increase confidence in the stability results? Is this increase relevant? How to balance it with the cost of extra analyses? Although there may be no universal answer to these questions, further research on sample size for stability assessment is needed.

In this paper, we aim to evaluate the optimal sample size for drug stability testing in human plasma based on confidence intervals [6,7] by conducting an experimental study for tramadol and its major metabolite (O-desmethyl-tramadol), as well as a retrospective data analysis for nine drugs of different structure.

2. Materials and Methods

2.1. Materials

O-desmethyl-tramadol hydrochloride (≤99%) was purchased from LoGiCal (Luckenwalde, Germany) and tramadol hydrochloride (≤99%) was purchased from Saneca Pharmaceuticals (Hlohovec, Slovakia). O-desmethyl-tramadol-d6 (≤98%) and tramadol-d6 hydrochloride (≤99%) were purchased from TLC Pharmaceutical Standards (Newmarket, Ontario, Canada). All other reagents were of analytical grade. Methanol and formic acid were purchased from Merck KGaA (Darmstadt, Germany). Sodium hydroxide was obtained from Chempur (Piekary Śląskie, Poland). Human blank plasma with CPD (citrate, phosphate, dextrose) as an anticoagulant was obtained from the Regional Blood Donation and Blood Therapy Centre (Warsaw, Poland).

2.2. Mass Spectrometric and Chromatographic Conditions

The bioanalytical method was adapted from the previous study [10] with a different chromatographic column and the use of formic acid in the mobile phase instead of acetic acid. The adapted method was validated according to the EMA [1] guidelines, except for long-term stability which was confirmed previously. Instrumental analysis was performed on an Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA), equipped with an autosampler, a degasser, and a binary pump coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer QTRAP 4000 (ABSciex, Framingham, MA, USA). The Turbo Ion Spray source was operated in positive mode with voltage and source temperatures of 5500 V and 550 °C, respectively. The curtain gas, ion source gas 1, ion source gas 2, and collision gas (all high purity nitrogen) were set at 206.84 kPa, 275.79 kPa, 379 kPa, and "high" instrument units, respectively. The target compounds were analyzed in the Multiple Reaction Monitoring (MRM) mode (Table 1).

Table 1. Parameters of MS method.

	Retention Time (min)	MRM [*m*/*z*]	DP [V]	CE [V]	CXP [V]
tramadol	3.4	264.2 > 42.3	51	125	10
tramadol-d6	3.4	270.3 > 252.2	66	17	16
O-desmethyl-tramadol	2.6	250.2 > 232.2	71	17	18
O-desmethyl-tramadol-d6	2.6	256.0 > 238.3	61	17	14

MRM—multiple reaction monitoring; DP—declustering potential; CE—collision energy; CXP—cell exit potential.

Chromatographic separation was achieved with a Kinetex C18 column (100 mm × 4.6 mm, 2.6 µm, Phenomenex, Torrance, CA, USA) using isocratic elution with methanol and 0.1% formic acid in a ratio of 40:60 at a flow rate of 0.3 mL/min. The column and the autosampler temperature was 50 ± 1 °C and 20 ± 1 °C, respectively. The injection volume was 5 µL.

2.3. Stock Solution, Calibration Standards, and Quality Control Samples

The separate standard stock solutions of tramadol, O-desmethyl-tramadol, tramadol-d6, and O-desmethyl-tramadol-d6 were prepared in 50% methanol (*v*/*v*) and were stored at −20 °C. The standard working solution was prepared by mixing stock solutions with an appropriate volume of water. The internal standard working solution (250 ng/mL for tramadol-d6 and 75 ng/mL for O-desmethyl-tramadol-d6) was diluted with water and prepared by mixing both internal standards stock solutions.

All calibration standards and the quality control samples were prepared by spiking blank human plasma with a working solution containing both analytes. The calibration standards contained both tramadol and O-desmethyl-tramadol at eight concentrations ranging from 5.0 to 750 ng/mL and from 2.5 to 150 ng/mL. The quality control samples were prepared at concentrations of 15, 350, and 600 ng/mL for tramadol, and 7.5, 70, and 120 ng/mL for O-desmethyl-tramadol.

2.4. Sample Preparation

The liquid-liquid extraction with *tert*-butyl methyl ether and 1M sodium hydroxide was used for the sample preparation [10]. Internal standards were added in one solution. The ether phase was evaporated in nitrogen gas and the dry residue was reconstituted with 150 µL of the mobile phase.

2.5. Stability Evaluation and Statistical Methods

The short-term stability was evaluated with sets containing an equal number of test and reference-quality control samples (QC): 3, 4, 5, 6, and 8 for low QC (15/7.5 ng/mL tramadol and O-desmethyl-tramadol) and high QC (600/120 ng/mL tramadol and O-desmethyl-tramadol). The reference and test QC samples (plasma fortified with tramadol and O-desmethyl tramadol solution) were prepared. The test QC samples were stored at

room temperature for 24 and 72 h before extraction and LC-MS analysis. Autosampler stability test during the validation method, confirmed that samples are stable for a minimum of 68 h at room temperature [10]. Reference samples were analyzed immediately after preparation, after 24 and 72 h storage in an autosampler at 20 ± 1 °C in the same sequence as test samples. Acceptance criteria were met when the whole confidence interval was within the acceptance range of 85–115%.

The statistical analysis of stability was based on the application of 90% confidence intervals [6,7]. The F-Snedecor test (significance level α = 0.01) was applied to test the hypothesis on variance equality. The influence of the number of repetitions and analyte concentration on the position and width of the confidence interval was analyzed using an analysis of variance (ANOVA, p = 0.05) test with repeated measurements. Normal distribution of the stability was assumed in the estimation of the probability that the confidence interval width is below 30%. The probability $P(CI \subset [85;\ 115])$ was calculated using the equation:

$$P(CI \subset [85;\ 115]) = \chi_{n-1}\left(\frac{225\ n\ (n-1)}{k^2\ {\sigma_S}^2}\right)$$

where:

χ_{n-1}—cumulative distribution function of the chi-square distribution for degrees of freedom (df) = $n - 1$;
n—number of repetitions;
k—the value of the Student t-distribution quantile at a 0.1 significance level for $n - 1$ degrees of freedom (df);
σ_S—standard deviation in stability.

More details on mathematical calculations can be found in the Appendix A.

2.6. Retrospective Analysis

Stability results for nine drugs were recorded during method validations conducted under Good Laboratory Practice conditions at the former Pharmaceutical Research Institute in Warsaw, Poland ([11–15], and unpublished data). The following types of stability were studied: short-term stability, freeze and thaw stability, long-term stability at temperatures of −14 °C and −65 °C. Nine drugs with LC-MS and HPLC-UV methods of determination of varying precision were selected to create the data sets. For each drug and each stability test, n = 6 samples were recorded at each low and high QC concentration. To analyze the worst-case scenario, for each dataset a result lying nearest to the mean of n = 6 results was discarded to obtain n = 5 dataset. The same procedure was used to obtain datasets of n = 4 and n = 3. The final number of calculated confidence intervals was 264. Comparison of the width of the confidence intervals between low and high QC was made using a Wilcoxon signed-rank test (significance level $p < 0.05$). To analyze how differences in one variable (percentage of confidence intervals within acceptance criteria set at 85–115%) can be explained by a difference in a second variable (confidence width or the number of samples), the coefficient of determination was used.

3. Results

3.1. Experimental and Mathematical Studies

Thanks to the design of the experimental study (five sample sizes, two storage durations, two analytes in two concentrations each) we were able to calculate 40 confidence intervals (Figure 1). For 20 pairs of low and high QC concentrations, we recorded 18 cases (90%) where the 90% confidence interval was wider for low than for high concentration. Moreover, the variability of the confidence interval width—presented as relative standard deviation (RSD) in Table 2—was larger for low concentration. It shows the influence of method precision on stability evaluation, as lower concentrations were measured with worse precision.

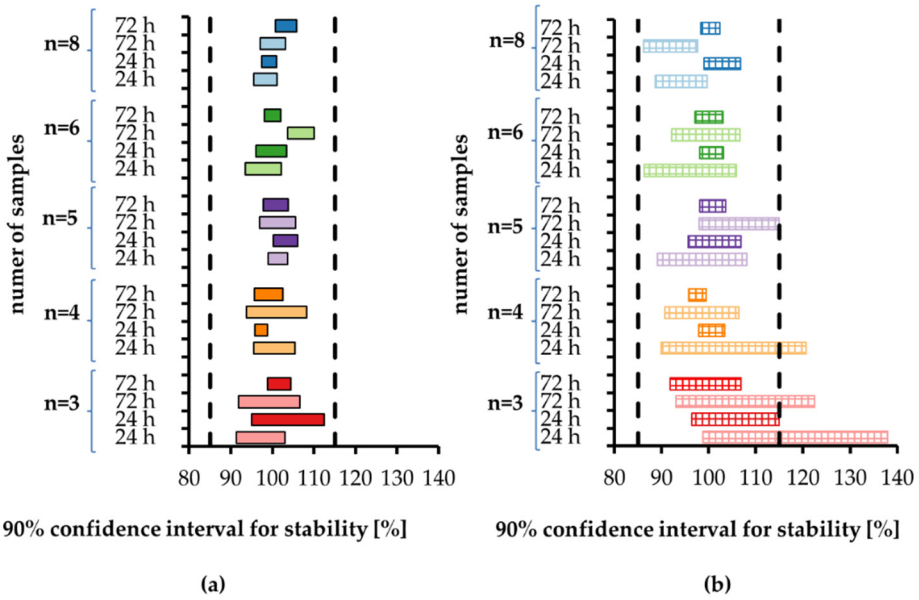


Figure 1. The 90% confidence intervals for the stability calculated according to [7] for (**a**) tramadol and (**b**) O-desmethyl-tramadol in human plasma stored at room temperature for 24 h and 72 h. Each sample size is associated with a different color, with light color indicating low concentration and dark color indicating high concentration. Vertical dashed lines indicate stability limits of 85–115%.

Table 2. Descriptive statistics for the width [%] of a 90% confidence interval. The number of pairs is the equal number of reference and study samples.

	Low QC					High QC				
Number of Pairs	**3**	**4**	**5**	**6**	**8**	**3**	**4**	**5**	**6**	**8**
Experimental Data for Tramadol and O-desmethyl-tramadol (*n* = 4 of results at each column)										
Mean	23.8	17.7	12.4	10.2	8.5	14.2	4.8	7.2	5.5	5.1
Geometric mean	21.1	16.2	10.7	9.7	8.1	12.9	4.6	6.9	5.4	4.9
Median	22.1	15.1	12.8	9.9	8.6	16.3	4.7	6.0	5.5	4.6
Min	11.7	9.9	4.7	6.4	5.5	5.7	3.1	5.5	3.9	3.6
Max	39.1	30.8	19.5	14.5	11.4	18.5	6.8	11.4	7.3	7.7
SD	12.9	9.1	6.9	3.5	3.1	5.8	1.7	2.8	1.4	1.8
RSD [%]	54	51	56	34	37	41	35	39	26	36
Retrospective Analysis (*n* = 33 of results at each column)										
Mean	21.5	14.9	11.4	9.1	-	11.9	8.4	6.4	5.1	-
Geometric mean	18.0	12.9	9.9	8.1	-	10.8	7.9	6.0	4.8	-
Median	18.5	12.8	10.1	7.7	-	10.9	7.6	5.8	4.6	-
Min	2.7	3.9	3.1	2.9	-	3.3	3.0	2.3	1.8	-
Max	54.3	37.6	28.2	23.2	-	28.8	19.5	14.6	12.9	-
SD	13.0	8.4	6.3	5.0	-	5.2	3.2	2.5	2.1	-
RSD [%]	57	54	52	53	-	43	38	38	40	-

Moreover, wider confidence intervals for low concentrations of O-desmethyltramadol than for low concentrations of O-tramadol indicate the importance of method precision. The precision of O-desmethyltramadol determination in quality control samples was 7.38% for low QC (7.5 ng/mL) and 2.90% for high QC (120 ng/mL). The precision of tramadol determination was 6.43% for low QC (15 ng/mL) and 3.07% for high QC (600 ng/mL). For each studied QC level, the mean extraction recovery was consistent for both analytes and their ISs—86.08–87.99% for tramadol, 85.55–86.99% for tramadol-d6, 74.45–78.75% for O-desmethyltramadol, and 74.61–79.07% for O-desmethyltramadol-d6. Thus, we do not expect that extraction recovery influenced stability results.

Visual assessment of low concentration data (Figure 2a) indicates that three and four repetitions are not appropriate due to the width of some confidence intervals over 30%. For five and six repetitions, width is below 20%, while for eight repetitions, width is below 12%. Visual assessment of high concentration data (Figure 2b) is a bit different. For three repetitions the confidence intervals width in 3/4 cases is over 15%, while for all other repetitions it is below 8%, with one exception of 11% (n = 5).

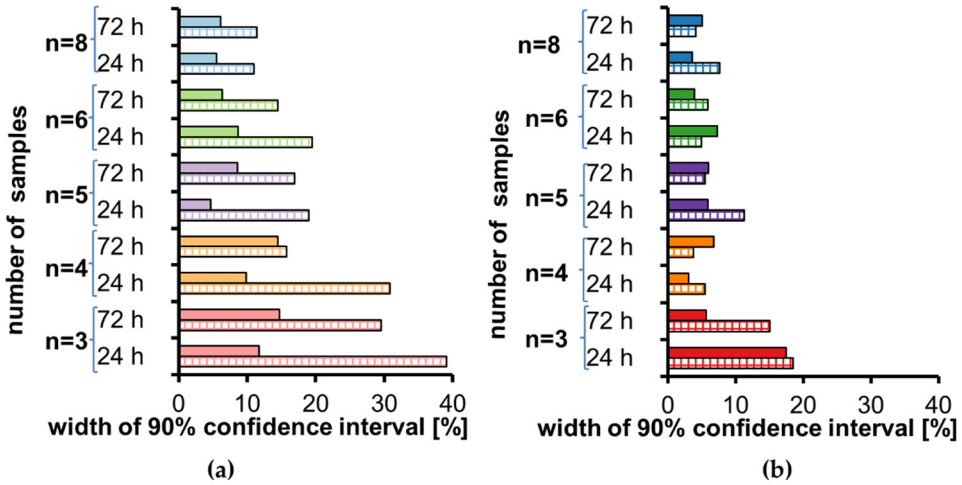


Figure 2. Width of a 90% confidence interval for stability calculated according to [7] for tramadol (full color) and O-desmethyl-tramadol (striped color) in human plasma stored at room temperature for 24 h and 72 h for each sample size: (**a**) low concentration, (**b**) high concentration.

ANOVA showed no dependence of the width of the confidence interval on the analyte concentration (Figure 3) ($p > 0.1187$). Results of the post-hoc least significant difference test (Fisher's LSD test) for sample size showed that the width of the confidence interval for n = 3 statistically significantly differs from more repetitions (n = 4, 5, 6, 8) (p from <0.0001 to 0.0249). The width of the confidence interval for n = 4 differs only from eight repetitions ($p < 0.05$).

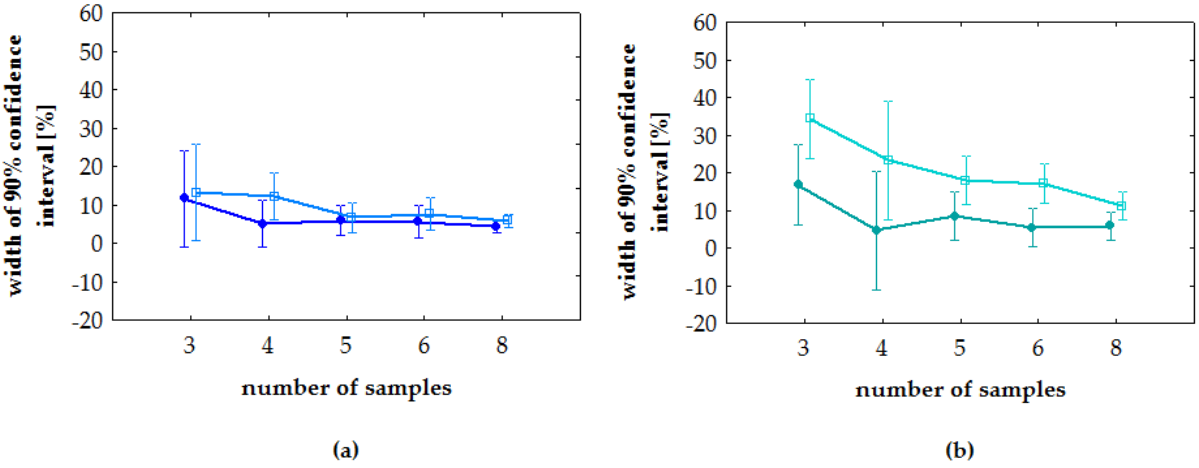


Figure 3. Post-hoc least significant difference test (Fisher's LSD test). Vertical bars means a 90% confidence interval: (**a**) tramadol; (**b**) O-desmethyl-tramadol. On each plot, light color indicates low concentration and dark color indicates high concentration.

Additionally, we have investigated the relation between precision, confidence interval, and the number of repetitions. The length of the confidence interval depends on the sample variance—the greater the n, the shorter the length of the interval (as it is inversely proportional to the square root of n), and the higher the chance the sample variance is

assessed correctly. We calculated the probability that for a given precision, the confidence interval derived from n repetitions falls within a 30% range. As expected, the relation between precision and the number of repetitions is sharp (Figure 4). As an example, for 10% precision, the considered probability is 33% for n = 3, 51% for n = 4, 71% for n = 5, 86% for n = 6, and 98% for n = 8. In general, for a smaller number of repetitions, there is a significant probability that the measurements with even high precision may overestimate the sample variance and consequently the length of the confidence interval. The choice of five or six repetitions proves to be enough to ensure that the confidence intervals will fall within the 85–115% interval.

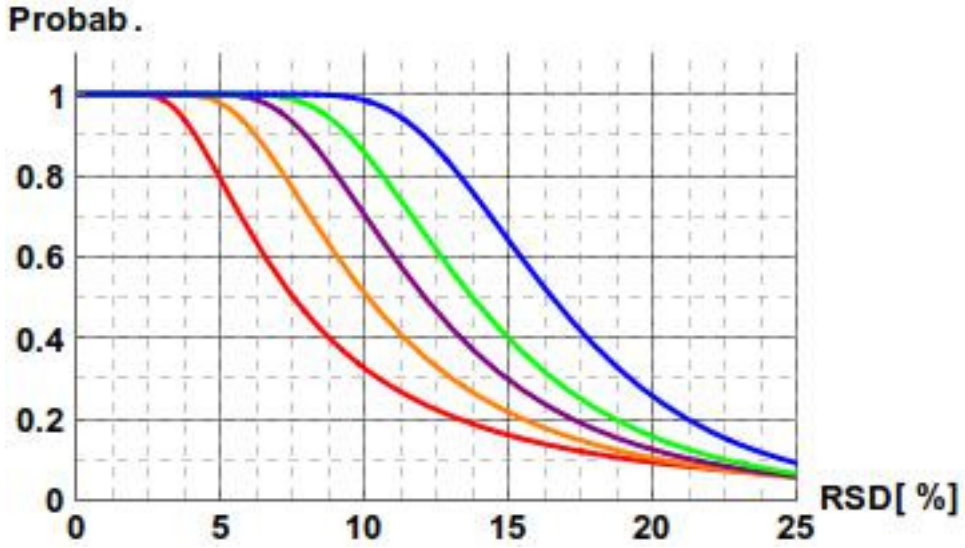


Figure 4. Dependence of the probability that the confidence interval width is below 30% on the precision in measurements. Equal precision for the reference and the studied measurements is assumed. Curves are defined for n = 3 (red), n = 4 (orange), n = 5 (purple), n = 6 (green), and n = 8 (blue).

We postulate that five repetitions of quality control samples at low and high concentration levels are optimal for stability tests during bioanalytical method validation. For each case with n = 5, the stability tests passed and the width of all confidence intervals was below 20%. For n < 5 some of the stability tests failed (part of the confidence interval outside of the acceptance criteria of 85–115%) due to the width of confidence intervals exceeding 30%. Moreover, for n > 5 all stability tests passed and the mean width of the confidence intervals decreased gradually (Table 2).

3.2. Retrospective Study

To verify observations from the experimental and the theoretical studies, we have analyzed human plasma stability data for nine validated bioanalytical methods (Figures 5 and A1). For all data, the percentage of confidence intervals lying within acceptance criteria was acceptable for n = 5 (88% for low and 93% for high concentration, respectively) and reached 100% for n = 6 (Figure 6a). For n = 5, only 5 of 66 results (including four for low QC) were outside of the acceptance limits. The greatest difference between the confidence interval limits and the acceptance criteria was 1.8%.

As expected, a strong positive correlation ($r^2 > 0.96$) was observed between the number of samples and the percentage of confidence intervals within the acceptance criteria (Figure 6a). Consequently, a strong negative correlation ($r^2 > 0.98$) was observed between the confidence interval width and the percentage of confidence intervals within the acceptance criteria (Figure 6b). Among confidence intervals for n = 3, 4, and 5, more than a 2-fold higher percentage of confidence intervals outside of acceptance criteria was observed for the low QC (Figure A2b) than for the high QC (Figure A2c) concentration ($p < 0.00001$). This observation

is consistent with higher values of both width of the confidence interval and its variability expressed as RSD (Table 2, Figures 7 and A4).

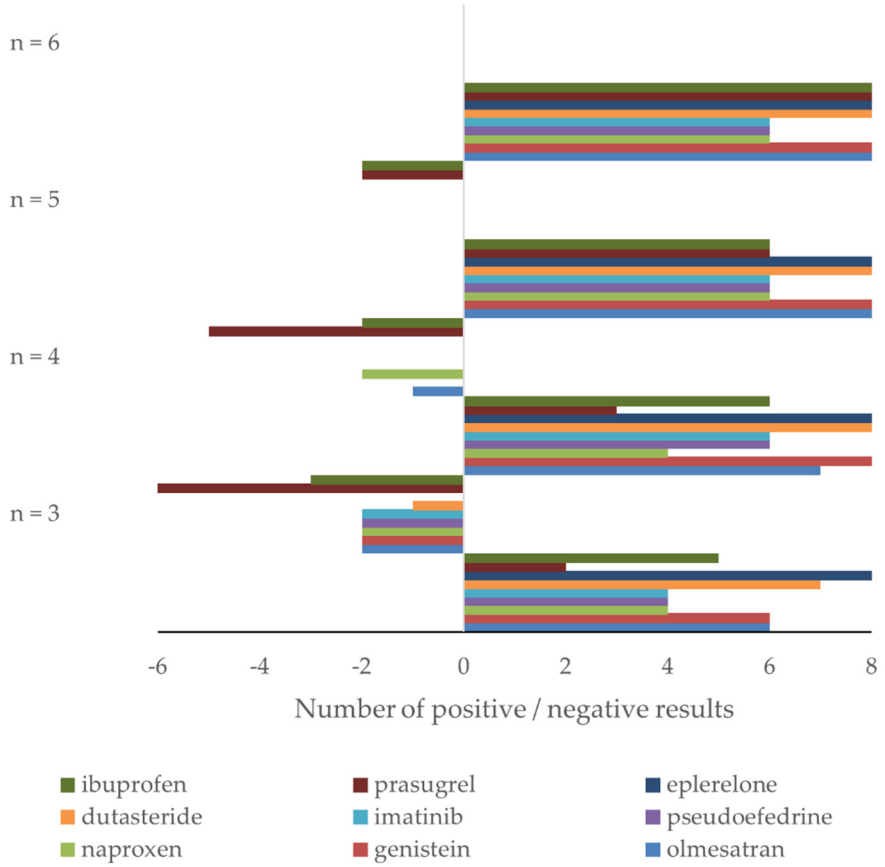
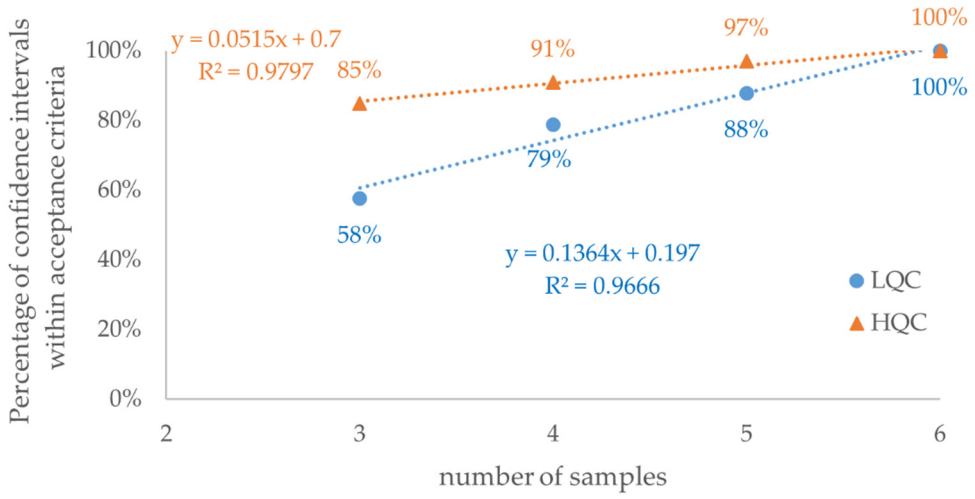
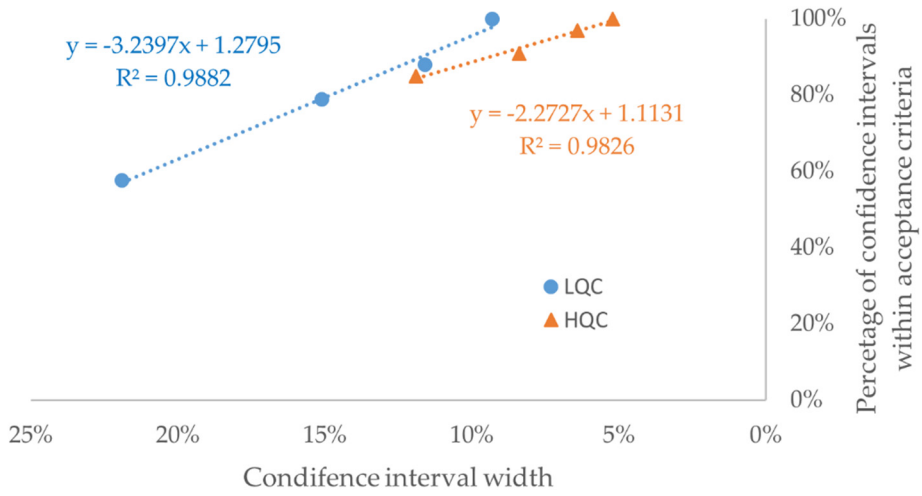


Figure 5. Retrospective study of nine drugs' stability in human plasma: number of confidence intervals within (positive results) and outside (negative results) acceptance criteria for nine drugs using n = 3, 4, 5, and 6 samples for stability testing. High and low concentration data are combined.



(**a**)

Figure 6. *Cont.*



(**b**)

Figure 6. Retrospective study of nine drugs' stability in human plasma: percentage of confidence intervals within acceptance criteria in the function of (**a**) number of samples and (**b**) mean width of the confidence interval for each number of samples (see Table 2). The dataset consisted of 33 confidence intervals for each concentration level: LQC (circle)—low-quality control sample; HQC (triangle)—high-quality control sample.

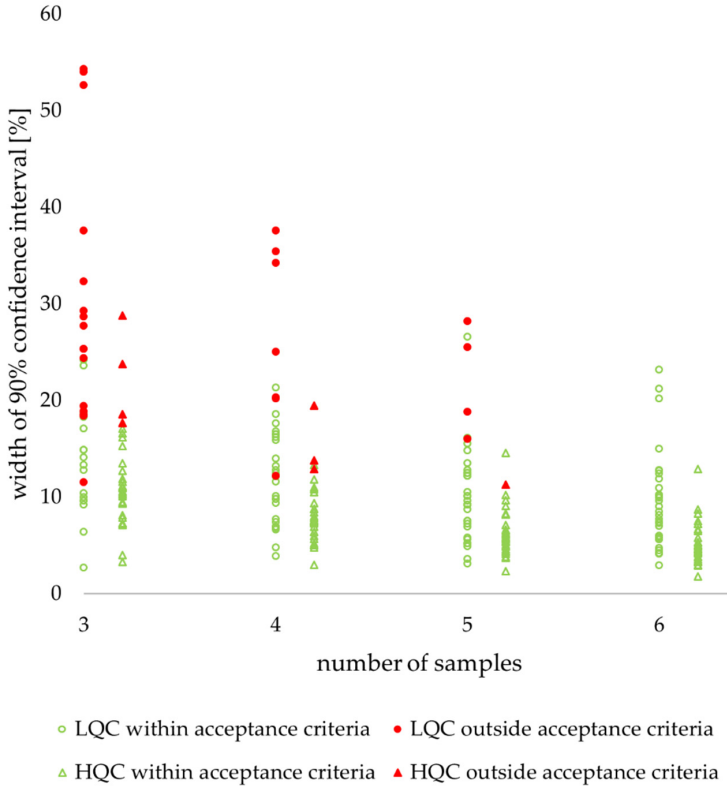


Figure 7. Retrospective study of nine drugs' stability in human plasma: individual values of confidence interval width. LQC (circle)—low-quality control sample, HQC (triangle)—high-quality control sample. Filled red figures indicate values outside acceptance criteria, unfilled green figures indicate values within acceptance criteria.

There were no relevant differences in confidence interval width between stability tests (Figure A3). The highest values for all sample numbers were recorded for the freeze and thaw test, but all other values for each sample number were only 1–2% lower.

4. Discussion

The results of the experimental, theoretical, and retrospective studies are in good agreement indicating that using 90% confidence intervals requires testing of at least five repetitions of quality controls as references and as stability samples. A retrospective study revealed that the percentage of the confidence intervals within acceptance criteria is strongly correlated with the number of samples used for stability testing (positively) and the mean of the width of confidence intervals (negatively). The statistically significant difference between low QC and high QC was observed between the percentage of confidence intervals within the acceptance criteria for a given sample number. The type of stability test did not influence confidence interval width. It seems that the excess work between $n = 5$ and $n = 8$ is not balanced with the benefit of a narrower width of the confidence interval. On the other hand, there would be 72 more analyses during full validation for one analyte, and this number does not include stability testing in solutions. The amount of excess work and resources for additional analyses may not be assessed in general, because it depends on particular method characteristics.

Our experimental study used a single bioanalytical method for the determination of two analytes in a single laboratory. To increase confidence in conclusions, we have reused previously generated stability data for nine drugs. Retrospective analyses are very popular in medicine [16,17], and slightly less popular in pharmacy [18,19]. On the contrary, in analytical chemistry retrospective analyses are used very rarely [20]. Over 20 years ago the concept of green analytical chemistry to protect the environment was established. Recently, its extension was proposed: white analytical chemistry in addition to green aspects also takes into account analytical and practical attributes [21]. Nevertheless, retrospective analysis has even greater ecological aspects since no chemical analysis is required and no waste is generated. Considering the high amount of analytical data produced each year in laboratories, it would be beneficial to explore them all deeply to draw some general conclusions, answer the emerging questions, and contribute to international guidelines development. The retrospective study enabled comparison of data generated using LC-MS and HPLC-UV methods (Table A3). It may be observed that narrower stability confidence intervals were recorded for HPLC-UV determined imatinib than for LC-MS/MS determined prasugrel (Figure A1). On the other hand, narrower stability confidence intervals were recorded for LC-MS determined eplerenone than for HPLC-UV determined ibuprofen (Figure A1). This indicates that the detector type and concentration range are not the appropriate indicator of confidence interval width, which is dependent on method precision.

We limited our study to plasma samples. For neat solutions, due to the lower probability of interferences and lack of variability introduced by sample preparation, the precision should be better and the optimal number of repetitions could be lower. We avoided the exclusion of outlying results. An alternative approach is to use a smaller number of replicates and remove outliers using statistical tests such as the Q-Dixon or Grubbs test. However, this approach—especially for a small number of replicates—may provoke questions from regulatory agencies. Additionally, it does not take into account the precision of the method. Therefore, we do not recommend this approach. The limitation of the retrospective study is that all confidence intervals for $n = 6$ were within the acceptance criteria as we used validated methods. The calculation of a 90% confidence interval may be considered as complicated compared to current bioanalytical method validation guidelines [1,2]. However, an extra effort in data analysis increases the reliability of stability evaluation.

We assumed a normal distribution of concentration data for stability and reference samples. However, stability is a ratio of stability samples over reference samples and the ratio of two normally distributed samples is never normally distributed itself. This

statistical issue is taken into account for bioequivalence testing where the acceptance criteria of 80–125% does not center symmetrically around 100% but does so in log space. Thus, acceptance limits of 85–115% may not be appropriate for stability testing. An approach similar to bioequivalence suggests a criterion of 85.00–117.65%. We have opted to use 85–115% acceptance limits, which are well-established in regulatory guidelines [1,2], but their inconsistency with stability distribution needs further consideration.

Our results are important because the current recommendation of at least three samples for stability testing [2,3] is not sufficient. The proposed $n = 5$ is in line with reports from other laboratories [22–24] where five or six results were used to calculate the 90% confidence intervals for stability. Extending stability acceptance criteria from deviation from nominal concentration by adding a test-to-reference ratio may be considered as an increase of regulatory burden. On the other hand, the reliability of bioanalytical data is crucial for pharmacokinetic calculations and decisions on dosing schemes. The latter impacts drug efficiency and patient safety. Thus, the proper balance between too extensive testing and poor data quality requires further discussion. A possible answer may be a hybrid approach: hard criteria for deviation of the mean from nominal concentration combined with soft criteria for the 90% confidence interval for test-to-reference ratio.

Both experimental and retrospective studies suggest that an optimal number of repetitions is five, as also recommended by the European Bioanalysis Forum [25]. The proper assumption on the relationship between method precision and sample size may be a key factor for successful future simulations. We hope that this paper will initiate discussion and stimulate further research on optimal sample size for stability testing. We expect that further simulations and retrospective studies from other laboratories will support the need for bioanalytical guidelines update.

5. Conclusions

Five sample repetitions are optimal for the assessment of analyte stability during bioanalytical method validation. Experimental, theoretical, and retrospective study results led to similar conclusions. The number of three or four replicates, in spite of being acceptable in some guidelines, is insufficient (in some cases, the width of the confidence intervals for stability exceeded 30%, which precluded meeting the acceptance criteria). In contrast, the excess work between $n = 5$ and $n = 8$ was not balanced with any benefit of narrower confidence interval widths. We hope to initiate a discussion on sample size for stability studies. Such a discussion may result in updated bioanalytical method validation guidelines.

Author Contributions: Conceptualization, P.J.R. and J.G.; methodology, P.J.R. and W.G.; validation, E.G.; formal analysis, E.G. and W.G.; investigation, E.G. and W.G.; resources, E.G.; data curation, W.G., E.G. and P.J.R.; writing—original draft preparation, E.G., J.G., W.G., and P.J.R.; writing—review and editing, E.G., J.G. and P.J.R.; visualization, E.G., W.G. and P.J.R.; supervision, P.J.R.; project administration, J.G.; funding acquisition, E.G. and J.G. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Polish Ministry of Science and Higher Education [DWD/3/6/2019, 21.11.2019]. Recording of data used in retrospective analyses was supported by the European Union (European Regional Development Fund) under the Polish Innovative Economy Operational Programme 2007-2013 [grants UDA-POIG.01.03.01-14-062/09, POIG.01.03.01-14-032/12]; and by the Polish National Centre for Research and Development [grants PBS1/B7/7/2012; INNOTECH-K1/IN1/14/159003/NCBR/12; INNOTECH-K2/IN2/65/182982/NCBR/13].

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data presented in Appendix A.

Acknowledgments: The authors are grateful to Ryszard Marszałek for technical assistance in LC-MS/MS analyses, Edyta Pesta and Krzysztof Abramski for the critical reviewing of the manuscript. The authors gratefully acknowledge the contributions of research teams from the former Pharmaceutical Research Institute (led by Katarzyna Buś-Kwaśnik, Monika Filist, Michał Kaza, and Jacek Musijowski) to generate datasets used in the retrospective analysis. The authors acknowledge the anonymous reviewer for pointing out the inconsistency between acceptance limits and the distribution of variables. The views expressed in this paper are solely those of the authors and do not necessarily reflect their institutions' position on the subject.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Not applicable.

Appendix A

Table A1. Concentrations of tramadol in human plasma during stability testing after storage for 24 h and 72 h at room temperature—low QC (nominal concentration of 15.0 ng/mL) and high QC (nominal concentration of 600 ng/mL).

Number of Samples (n)	Low QC (ng/mL)				High QC (ng/mL)			
	Reference for 24 h	Tested 24 h	Reference for 72 h	Tested 72 h	Reference for 24 h	Tested 24 h	Reference for 72 h	Tested 72 h
8	14.9	14.5	14.2	14.6	573	567	590	640
	15.2	14.7	14.6	14.6	582	569	594	627
	15.4	14.8	14.7	14.6	584	574	572	611
	15.5	15.4	14.9	14.9	586	575	589	578
	15.6	15.4	15.1	14.9	588	579	580	617
	15.8	15.5	15.3	14.9	590	587	609	609
	15.9	15.6	15.5	15.4	594	591	586	584
	16.0	16.3	15.7	16.2	597	615	582	589
6	14.7	14.0	13.9	14.4	568	559	574	582
	14.8	14.5	13.9	15.1	573	564	578	585
	15.1	14.9	14.4	15.3	577	567	583	587
	15.2	15.1	14.5	15.6	587	583	596	591
	15.7	15.3	14.5	15.7	598	591	603	594
	16.3	16.0	14.7	15.7	600	630	606	602
5	14.7	14.8	14.9	14.5	555	572	553	569
	14.9	14.9	15.0	14.8	559	579	585	581
	14.9	15.2	15.1	15.2	566	584	589	588
	15.0	15.4	15.2	15.8	578	601	590	596
	15.4	15.6	15.2	16.1	591	601	594	599
4	14.3	14.3	13.8	14.3	594	571	579	559
	14.9	14.3	14.9	14.7	594	582	579	567
	14.9	15.1	15.5	15.5	595	585	582	592
	15.0	15.7	15.7	15.8	606	588	597	597
3	15.0	14.9	14.6	14.7	570	565	575	584
	15.6	15.2	15.4	15.4	574	578	589	598
	16.4	15.5	16.2	15.6	580	642	592	603

Table A2. Concentrations of O-desmethyl tramadol in human plasma during stability testing after storage for 24 h and 72 h at room temperature—low QC (nominal concentration of 7.50 ng/mL) and high QC (nominal concentration of 120 ng/mL).

Number of Samples (n)	Low QC (ng/mL)				High QC (ng/mL)			
	Reference for 24 h	Tested 24 h	Reference for 72 h	Tested 72 h	Reference for 24 h	Tested 24 h	Reference for 72 h	Tested 72 h
8	7.59	6.87	6.74	6.40	112	132	117	118
	7.21	6.88	7.07	6.47	111	115	118	119
	7.65	6.90	7.55	6.66	112	117	118	121
	7.89	7.22	7.64	6.70	114	117	122	122
	7.93	7.41	7.79	6.95	117	117	122	122
	8.16	7.67	7.92	7.34	117	118	123	123
	8.36	8.02	7.94	7.68	119	119	123	124
	8.37	8.52	8.28	7.69	126	119	127	124
6	7.89	6.37	7.16	6.61	114	117	117	125
	7.35	6.44	7.21	6.81	116	118	118	116
	7.77	8.08	7.23	7.11	116	118	119	119
	8.11	8.19	7.46	7.45	120	120	124	122
	8.22	8.31	7.73	8.04	120	120	124	122
	8.25	8.36	7.82	8.32	124	121	125	123
5	7.02	7.85	6.64	6.87	119	124	117	119
	7.48	6.24	6.71	7.16	113	111	120	120
	7.52	7.70	6.72	7.33	113	114	121	121
	7.87	7.84	7.37	7.69	117	115	122	122
	8.20	7.84	7.68	8.22	122	127	124	127
4	6.98	7.66	6.94	6.40	117	116	122	118
	6.42	7.52	7.02	6.83	117	118	122	119
	8.39	7.55	7.09	6.97	119	119	124	120
	8.46	8.61	7.33	7.74	120	123	124	123
3	7.05	7.64	6.70	6.41	123	117	115	120
	6.39	8.54	6.84	7.54	116	125	127	122
	8.01	8.80	6.88	7.93	117	133	128	124

Table A3. Characteristics of the bioanalytical methods for the determination of the nine drugs used for retrospective analysis.

Drug	Method	Internal Standard	Low/High QC (ng/mL)	Type of Extraction	Source
Dutasteride	HPLC, ESI +	[$^{13}C_6$]-dutasteride	0.3/2.8	LLE	[24]
Eplerenon	HPLC-MS, ESI +	[$^{2}H_3$]-eplerenone	50/1500	LLE	[23]
Genistein	HPLC-MS, ESI −	[$^{2}H_4$]-genistein	50/2000	LLE	N/A
Ibuprofen	HPLC-UV, λ = 220 nm	naproxen	900/24,000	LLE	N/A
Imatinib	HPLC-UV, λ = 265 nm	propranolol hydrochloride	120/3200	LLE	[22]
Naproxen	HPLC-UV, λ = 265 nm	ibuprofen	1500/60,000	LLE	[20]
Olmesartan	HPLC-MS, ESI +	[$^{2}H_6$]-olmesarta	15/2000	LLE	[21]
Prasugrel	HPLC-MS/MS, ESI +	[$^{13}C_6$] R-138727	1.5/200	LLE	N/A
Pseudoephedrine	HPLC-MS/MS, ESI +	[$^{2}H_3$][$^{13}C_6$]-pseudoephedrine	4.5/240	LLE	N/A

LLE—liquid-liquid extraction; ESI—electrospray ionization; N/A—unpublished data.

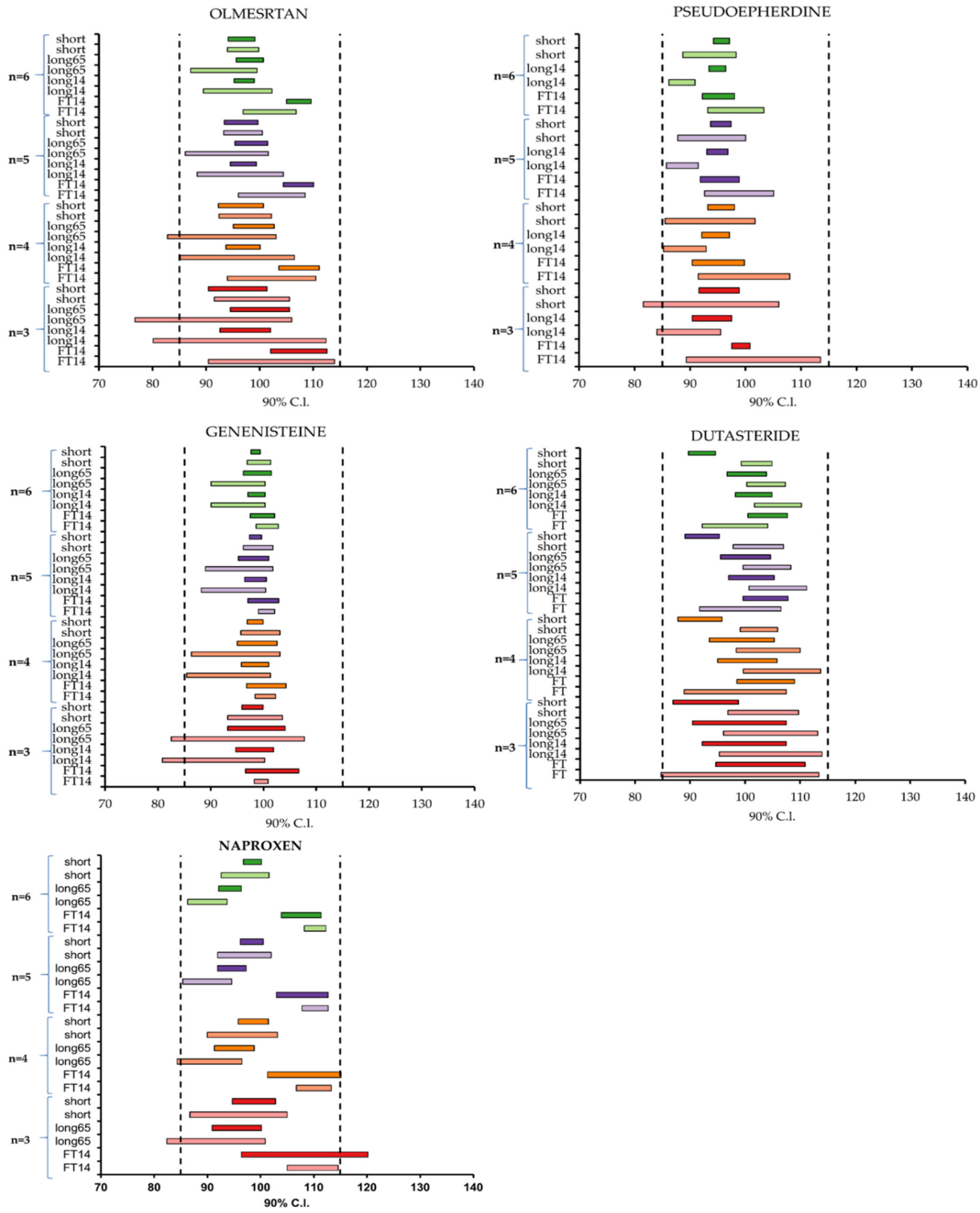


Figure A1. *Cont.*

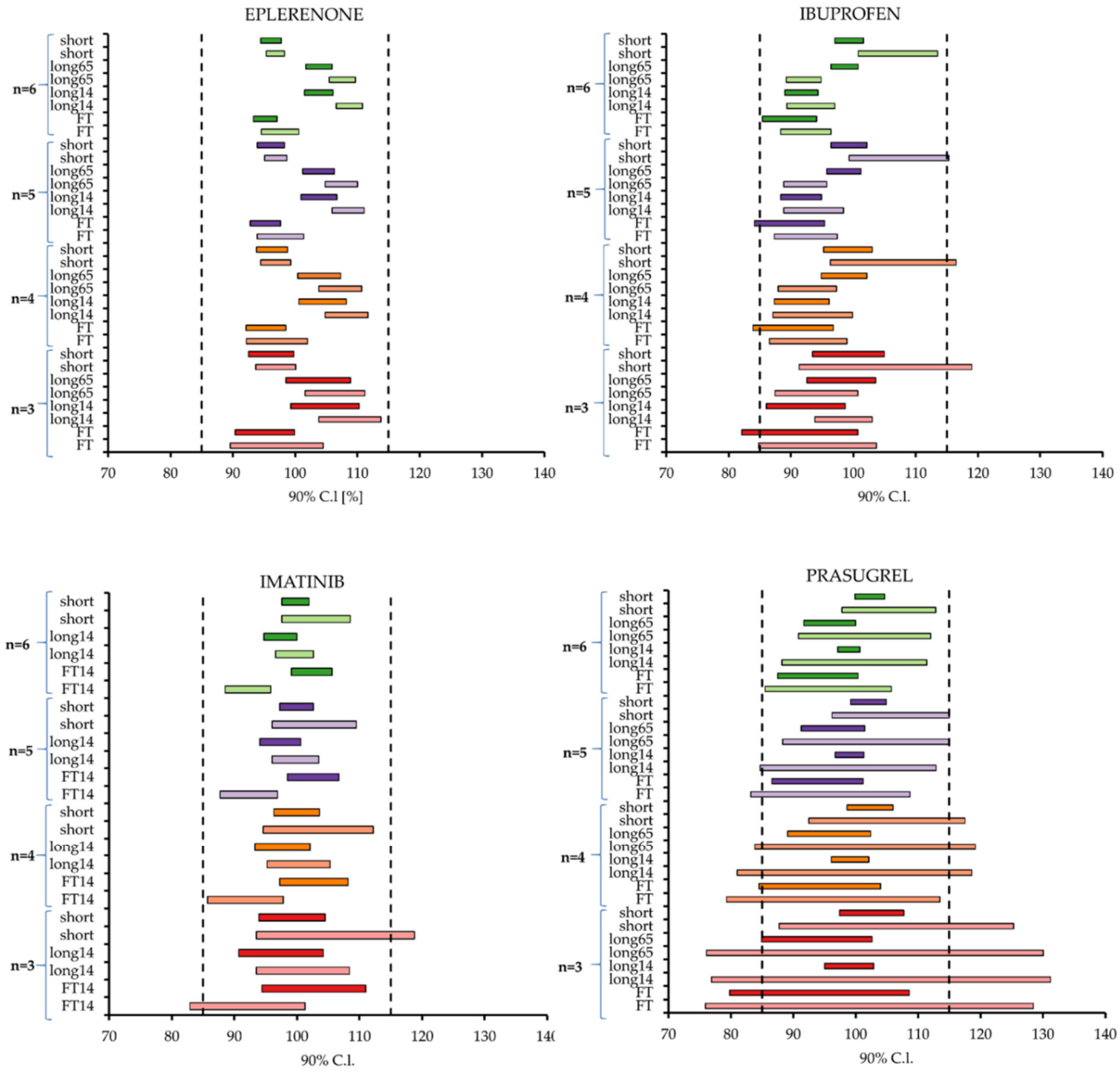


Figure A1. Retrospective study: 90% confidence intervals (90% C.I) for the stability of five drugs using n = 3, 4, 5, and 6. Abbreviations: Short—short-term stability, FT—freeze and thaw stability, long—long-term stability. Numbers 14 and 65 indicate the storage temperature of −14 °C and −65 °C, respectively. Low QC—light color, high QC—intensive color.

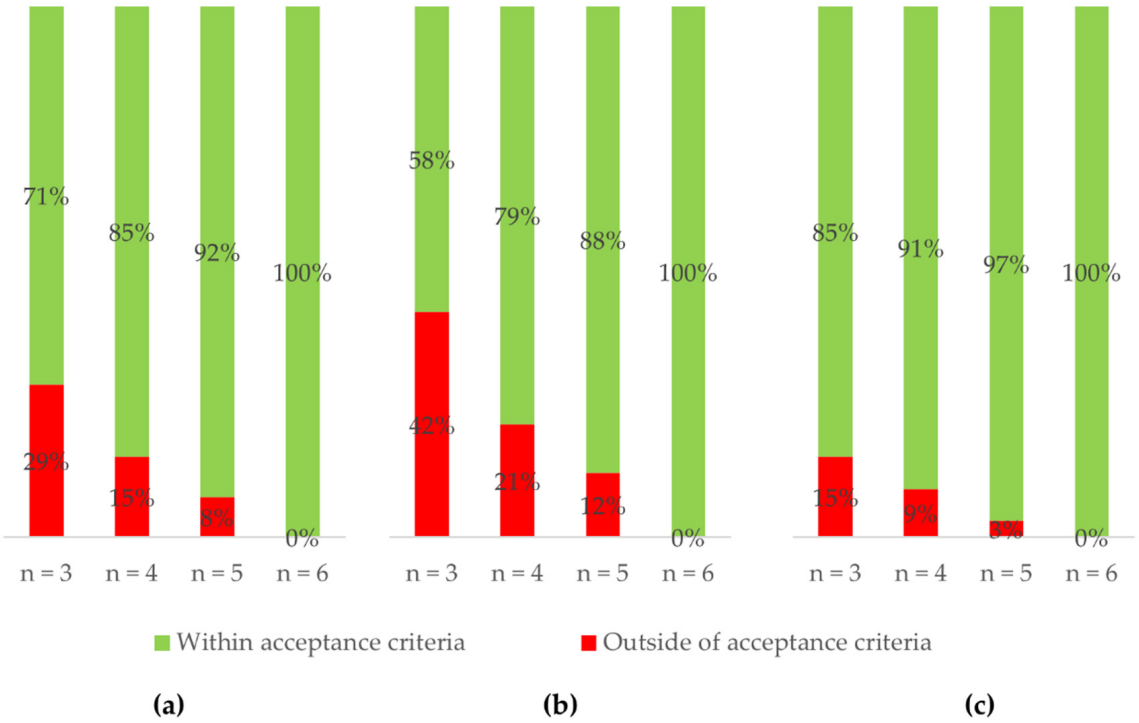


Figure A2. Retrospective study: Percentage of confidence intervals outside (red) and within (green) stability acceptance criteria for (**a**) all data, (**b**) low QC concertation, and (**c**) high QC concertation. Combined data from a retrospective study of nine drugs using n = 3, 4, 5, and 6.

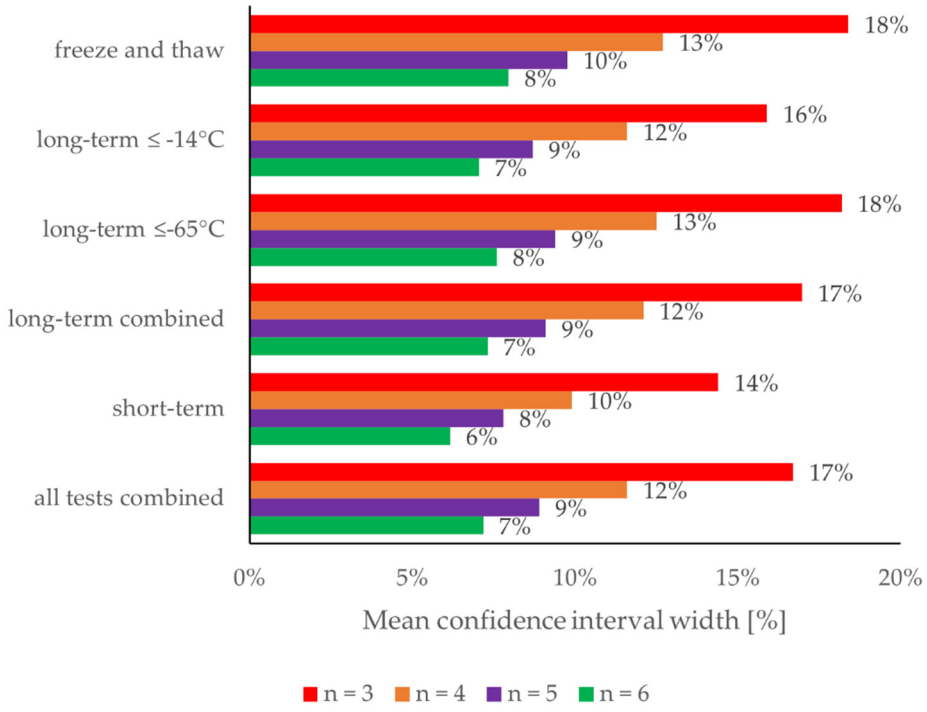


Figure A3. Retrospective study: Relation between mean confidence interval width and type of stability test.

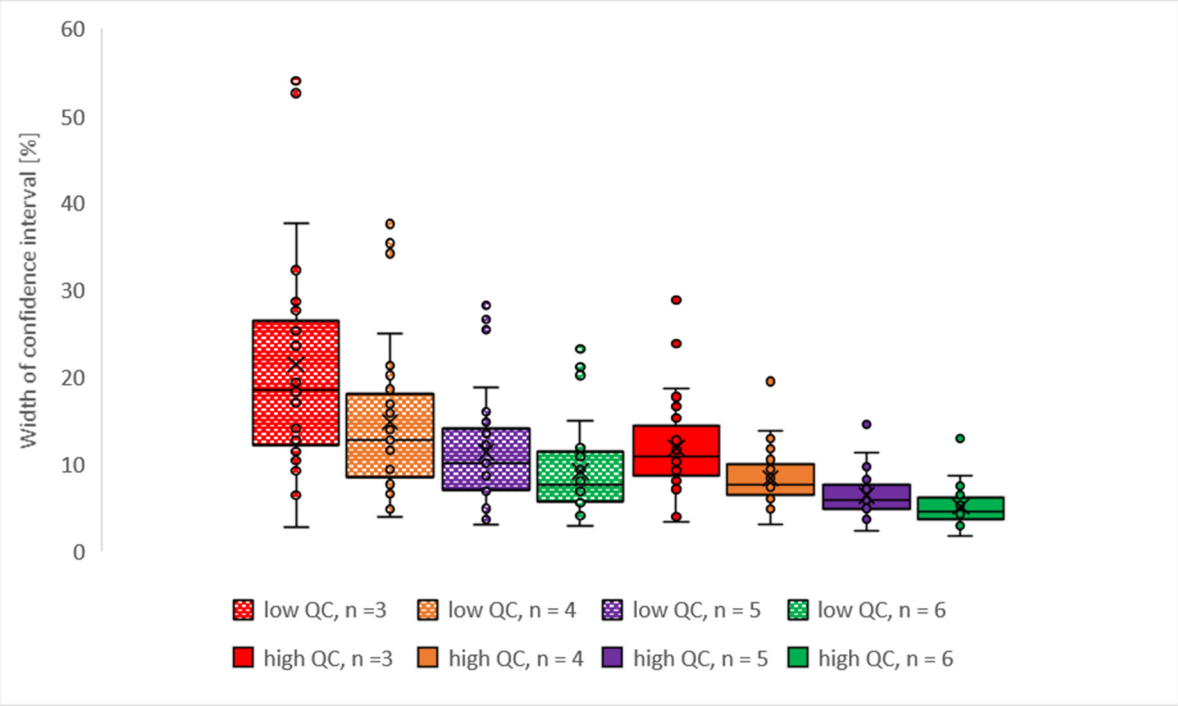


Figure A4. Retrospective study: box & whiskers plot; box indicates 2nd and 3rd quartile, whiskers indicate 1st and 4th quartile, points outside of whiskers indicate outliers.

Relation between precision in measurements and stability:

Stability S is determined as a ratio of two uncorrelated random variables X (tested samples) and Z (reference samples):

$$S = \frac{X}{Z}$$

Our goal is to derive the relation between the standard deviations in X and Z and the standard deviation in S. We start with linearization, which allows us to reformulate the Z variable as follows:

$$Z = \mu Z + \sigma_Z * \widetilde{Z}$$

where $\widetilde{Z}$ is the centralized Z variable (mean = 0, standard deviation = 1). Using linear approximation, we may obtain:

$$\frac{X}{\mu Z + \sigma_Z * \widetilde{Z}} \approx \frac{X}{\mu Z} - \frac{1}{\mu Z^2}\sigma_Z * \widetilde{Z} * X$$

Now:

$$\sigma_S{}^2 = E\left(\left(\frac{X}{Z}\right)^2\right) - \left(E\left(\frac{X}{Z}\right)\right)^2$$

$$\sigma_S{}^2 \approx E\left(\left(\frac{X}{\mu Z} - \frac{1}{\mu Z^2}\sigma_Z * \widetilde{Z} * X\right)^2\right) - \left(E\left(\frac{X}{Z}\right)\right)^2$$

$$E\left(\left(\frac{X}{\mu Z} - \frac{1}{\mu Z^2}\sigma_Z * \widetilde{Z} * X\right)^2\right) = \frac{1}{\mu Z^2}E(X)^2 - 2\frac{dZ}{\mu Z^2}E\left(X^2\widetilde{Z}\right) + \frac{\sigma_Z{}^2}{\mu Z^4}E\left(X^2\widetilde{Z}^2\right) = I + II + III$$

$$I.\quad E(X)^2 = \left(\sigma_X{}^2 + \mu X^2\right)$$

Variables are uncorrelated and expected value of $\widetilde{Z}$ is equal to 0:

$$II.\ E\left(X^2\widetilde{Z}\right) = 0$$

Again, variables are uncorrelated and the standard deviation of $\widetilde{Z}$ is equal to 1:

$$III.\ E\left(X^2\widetilde{Z}^2\right) \approx E\left(X^2\right)E\left(\widetilde{Z}^2\right) = \left({\sigma_X}^2 + \mu X^2\right)$$

Using linearization, we can approximate:

$$E\left(\frac{X}{Z}\right) \approx E\left(\frac{X}{\mu Z} - \frac{1}{\mu Z^2}\sigma_Z * \widetilde{Z} * X\right) = \frac{\mu X}{\mu Z}$$

Finally:

$${\sigma_S}^2 \approx \left({\sigma_X}^2 + \mu X^2\right)\left(\frac{{\sigma_Z}^2}{\mu Z^4} + \frac{1}{\mu Z^2}\right) - \left(\frac{\mu X^2}{\mu Z^2}\right)$$

Probability for the confidence interval:

As demonstrated by Rudzki and Leś, measurements may follow a log-normal distribution [7]. In such a case, the confidence interval can be calculated using logarithmic transformation, which yields a normal distribution of the stability. In order to keep the model simple, from now on we will assume the normal distribution of the stability.

Let us denote the standard deviation in stability as s_S. Under the assumption o the normal distribution, the 90% confidence interval of stability has the following form:

$$CI = \mu_S \pm \frac{s_S\, k}{\sqrt{n}}$$

where:

μ_S is the mean value of stability and k is the value of the Student t-distribution quantile at a 0.1 significance level for $n-1$ degrees of freedom (*df*). In the presented work, we consider only stable analytes, i.e., μ_S =100. The probability that the confidence interval is in the 85–115% interval:

$$P(CI\ \subset [85;\ 115])$$

is equivalent to:

$$P\left(\frac{s_S\, k}{\sqrt{n}} < 15\right) = P\left({s_S}^2 < \left(\frac{15\sqrt{n}}{k}\right)^2\right)$$

Assuming that the true standard deviation in stability is σ_S:

$$P\left({s_S}^2 < \left(\frac{15\sqrt{n}}{k}\right)^2\right) = P\left(\frac{{s_S}^2(n-1)}{{\sigma_S}^2}\ < \frac{225\ n\ (n-1)}{{\sigma_S}^2 k^2}\right)$$

where:

$$\frac{s^2(n-1)}{{\sigma_S}^2}\ \sim\ chi^2(n-1)$$

As a result:

$$P(CI\ \subset [85;\ 115]) = \chi_{n-1}\left(\frac{225\ n\ (n-1)}{k^2\ {\sigma_S}^2}\right)$$

where χ_{n-1} is the cumulative distribution function of the chi-square distribution for df = $n\ -\ 1$.

References

1. *Guideline on Bioanalytical Method Validation*; EMEA/CHMP/EWP/192217/2009; Committee for Medicinal Products for Human Use (CHMP), European Medicines Agency: London, UK, 2011.
2. *Guidance for Industry: Bioanalytical Method Validation*; Food and Drug Administration; Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM): Rockville, MD, USA, 2018.
3. *Draft ICH Guideline M10 on Bioanalytical Method Validation*; EMA/CHMP/ICH/172948/2019; Committee for Human Medicinal Products, European Medicines Agency: London, UK, 2019.
4. Kaza, M.; Karaźniewicz-Łada, M.; Kosicka, K.; Siemiątkowska, A.; Rudzki, P.J. Bioanalytical method validation: New FDA guidance vs. EMA guideline. Better or worse? *J. Pharm. Biomed. Anal.* **2019**, *165*, 381–385. [CrossRef] [PubMed]
5. Health Canada's: Notice Clarification of Bioanalytical Method Validation Procedures. 2015. Available online: https://www.canada.ca/en/health-canada/services/drugs-health-products/drug-products/announcements/notice-clarification-bioanalytical-method-validation-procedures.html (accessed on 6 December 2021).
6. Timm, U.; Wall, M.; Dell, D. A New Approach for Dealing with the Stability of Drugs in Biological Fluids. *J. Pharm. Sci.* **1985**, *74*, 972–977. [CrossRef] [PubMed]
7. Rudzki, P.J.; Leś, A. Application of confidence intervals to bioanalytical method validation-drug stability in biological matrix testing. *Acta Pol. Pharm.* **2008**, *65*, 743–747. [PubMed]
8. *Guideline on the Investigation of Bioequivalence*; CPMP/EWP/QWP/1401/98/Rev. 1/Corr**; Committee for Human Medicinal Products, European Medicines Agency: London, UK, 2010.
9. Neyman, J. Outline of a theory of statistical estimation based on the classical theory of probability. *Philos. Trans. R. Soc. Lond. Ser. A Math. Phys. Sci.* **1937**, *236*, 333–380.
10. Rudzki, P.J.; Jarus-Dziedzic, K.; Filist, M.; Gilant, E.; Buś-Kwaśnik, K.; Leś, A.; Sasinowska-Motyl, M.; Nagraba, Ł.; Bujalska-Zadrożny, M. Evaluation of tramadol human pharmacokinetics and safety after co-administration of magnesium ions in randomized, single- and multiple-dose studies. *Pharmacol. Rep.* **2021**, *73*, 604–614. [CrossRef] [PubMed]
11. Filist, M.; Szlaska, I.; Kaza, M.; Pawiński, T. Validated HPLC-UV method for determination of naproxen in human plasma with proven selectivity against ibuprofen and paracetamol. *Biomed. Chromatogr.* **2016**, *30*, 953–961. [CrossRef] [PubMed]
12. Piórkowska, E.; Musijowski, J.; Buś-Kwaśnik, K.; Rudzki, P.J. Is a deuterated internal standard appropriate for the reliable determination of olmesartan in human plasma? *J. Chrom. B* **2017**, *1040*, 53–59. [CrossRef] [PubMed]
13. Kaza, M.; Piorkowska, E.; Filist, M.; Rudzki, P.J. HPLC-UV assay of imatinib in human plasma optimized for bioequivalence studies. *Acta Pol. Pharm.* **2016**, *73*, 1495–1503. [PubMed]
14. Buś-Kwaśnik, K.; Filist, M.; Rudzki, P.J. Environmentally friendly LC/MS determination of eplerenone in human plasma. *Acta Pol. Pharm.* **2016**, *73*, 1487–1493. [PubMed]
15. Gniazdowska, E.; Kaza, M.; Buś-Kwaśnik, K.; Giebułtowicz, J. LC-MS/MS determination of dutasteride and its major metabolites in human plasma. *J. Pharm. Biomed. Anal.* **2021**, *206*, 114362. [CrossRef] [PubMed]
16. Quaranta, L.; Micheletti, E.; Carassa, R.; Bruttini, C.; Fausto, R.; Katsanos, A.; Riva, I. Efficacy and Safety of PreserFlo® MicroShunt After a Failed Trabeculectomy in Eyes with Primary Open-Angle Glaucoma: A Retrospective Study. *Adv. Ther.* **2021**, *38*, 4403–4412. [CrossRef] [PubMed]
17. Wilde, H.; Dennis, J.M.; McGovern, A.P.; Vollmer, S.J.; Mateen, B.A. A national retrospective study of the association between serious operational problems and COVID-19 specific intensive care mortality risk. *PLoS ONE* **2021**, *16*, e0255377. [CrossRef] [PubMed]
18. Monakhova, Y.B.; Diehl, B.W.K. Retrospective multivariate analysis of pharmaceutical preparations using (1)H nuclear magnetic resonance (NMR) spectroscopy: Example of 990 heparin samples. *J. Pharm. Biomed. Anal.* **2019**, *173*, 18–23. [CrossRef] [PubMed]
19. Ko, Y.; Jeon, W.; Choi, Y.J.; Yang, H.; Lee, J. Impact of drug formulation on outcomes of pharmaceutical poisoning in children aged 7 years or younger: A retrospective observational study in South Korea. *Medicine* **2021**, *100*, e27485. [CrossRef] [PubMed]
20. Yoneyama, T.; Kudo, T.; Jinno, F.; Schmidt, E.R.; Kondo, T. Retrospective Data Analysis and Proposal of a Practical Acceptance Criterion for Inter-laboratory Cross-validation of Bioanalytical Methods Using Liquid Chromatography/Tandem Mass Spectrometry. *AAPS J.* **2014**, *16*, 1226–1236. [CrossRef] [PubMed]
21. Nowak, P.M.; Wietecha-Posłuszny, R.; Pawliszyn, J. White Analytical Chemistry: An approach to reconcile the principles of Green Analytical Chemistry and functionality. *TrAC Trends Anal. Chem.* **2021**, *138*, 116223. [CrossRef]
22. Watanabe, K.; Varesio, E.; Hopfgartner, G. Parallel ultra high pressure liquid chromatography–mass spectrometry for the quantification of HIV protease inhibitors using dried spot sample collection format. *J. Chrom. B* **2014**, *965*, 244–253. [CrossRef] [PubMed]
23. Pihl, S.; Huusom, A.K.T.; Rohde, M.; Poulsen, M.N.; Jørgensen, M.; Kall, M.A. Evaluation of an isochronic study design for long-term frozen stability investigation of drugs in biological matrices. *Bioanalysis* **2010**, *2*, 1041–1049. [CrossRef] [PubMed]
24. Bourgogne, E.; Mathy, F.X.; Boucaut, D.; Boekens, H.; Laprevote, O. Simultaneous quantitation of histamine and its major metabolite 1-methylhistamine in brain dialysates by using precolumn derivatization prior to HILIC-MS/MS analysis. *Anal. Bioanal. Chem.* **2012**, *402*, 449–459. [CrossRef] [PubMed]
25. Wilson, A.; Barker, S.; Freisleben, A.; Laakso, S.; Staelens, L.; White, S.; Timmerman, P. European Bioanalysis Forum recommendation on the best practices to demonstrate processed sample stability. *Bioanalysis* **2019**, *11*, 7–11. [CrossRef] [PubMed]

Publikacja 4 (oryginalna)

Gniazdowska E.M., Giebułtowicz J. and Rudzki P.J. How does the order of sample analysis influence the matrix effect during LC-MS bioanalysis? Journal of Chromatography B 2023; 1227:123800. IF= 3,000; MEiN = 100

Journal of Chromatography B 1227 (2023) 123800

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

How does the order of sample analysis influence the matrix effect during LC-MS bioanalysis?

Elżbieta Gniazdowska [a,b,*], Joanna Giebułtowicz [c], Piotr J. Rudzki [d]

[a] Łukasiewicz Research Network - Industrial Chemistry Institute, Pharmaceutical Analysis Laboratory, 8 Rydygiera, 01-793 Warsaw, Poland
[b] Department of Drug Chemistry, Doctoral School, Medical University of Warsaw, 61 Żwirki i Wigury, 02-091 Warsaw, Poland
[c] Department of Drug Chemistry, Faculty of Pharmacy, Medical University of Warsaw, 1 Banacha, 02-097 Warsaw, Poland
[d] Celon Pharma S.A., Bioanalytical Laboratory, 15 Marymoncka, 05-152 Kazuń Nowy, Poland

ARTICLE INFO

Keywords:
LC-MS
Matrix effect
Matrix factor
Bioanalytical method validation
Chemometrics
Lipemic plasma
Phospholipids

ABSTRACT

Mass spectrometry coupled with liquid chromatography is a valuable tool for drug development and personalised drug therapy. The matrix effect is caused by enhancing or suppressing the analyte signal intensity by the interfering compounds of biological fluids. The matrix effect may influence the reliability of the quantitative results. Thus, its evaluation is a critical part of bioanalytical method validation. Identified factors affecting the matrix effect are the physicochemical properties of the analyte, type of biological material, analytical conditions, the ion source construction and calculation method. The order of analysis of test samples (pure solutions and post-extraction spiked samples) is another factor possibly affecting quantifying the matrix effect variability between sources. Our primary goal was to find which experimental design – interleaved or set of blocks – is more sensitive to detect matrix effect variability. Additionally, to better understand the reason of variability, we evaluated the influence of chromatographic elution and the type of plasma (normal, lipemic or hemolyzed), co-elution, and carry-over of phospholipids. We used chemometric methods: Principal Component Analysis and Partial Least-Squares Discriminant Analysis. Although a comparable (but statistically different) matrix effect ($\%RSD_{MF}$) is observed using the interleaved and block schemes, for some compounds, the order of the samples strongly influences the results. The interleaved scheme was generally more sensitive in detecting the matrix effect than the block scheme. Thus, reporting the order of samples is needed to ensure the repeatability of experiments. Chemometrics suggests that lipemic samples analyzed in isocratic conditions are most prone to the matrix effect. Different compositions of matrix lots of the same type – especially lipemic – may influence method reliability. Thus, evaluating more than one source of lipemic and hemolyzed plasma is recommended.

1. Introduction

Mass spectrometry coupled to liquid chromatography is a useful tool for drug development and personalized drug therapy. It allows studying of pharmacokinetics and toxicokinetics due to its high throughput, high sensitivity, and specificity [1]. However, the endogenous or exogenous compounds of biological fluids (matrix) may influence the quantitative results' reliability by enhancing or suppressing the analyte signal intensity. This phenomenon is called the matrix effect [2–4], and its evaluation is a critical part of bioanalytical method validation. The matrix effect was also listed as one of the essential factors for plasma sample preparation before metabolomics [5].

Matrix effect can be calculated as absolute matrix effect, as the variation of the calibration curve slopes [6], as matrix factor (MF) [7], and as combined qualitative and quantitative assessment [8]. The matrix effect can be also calculated as an instrumental and global matrix effect [9]. The regulatory guidelines for bioanalytical method validation do not set criteria regarding absolute matrix effect, but its variability

Abbreviations: CI, confidence interval; EMA, European Medicines Agency; FDA, U.S. Food and Drug Administration; ICH, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; MF, matrix factor; *m/z*, mass to charge ratio; n, number of observations; ROC, receiver operating characteristic; SST, system suitability test; $\%RSD_{MF}$, percent relative standard deviation of matrix factor.

* Corresponding author at: Pharmaceutical Analysis Laboratory, Łukasiewicz Research Network - Industrial Chemistry Institute, 8 Rydygiera, 01-793 Warsaw, Poland.

E-mail addresses: elzbieta.gniazdowska@ichp.lukasiewicz.gov.pl (E. Gniazdowska), joanna.giebultowicz@wum.edu.pl (J. Giebułtowicz), piotr.rudzki@celonpharma.com (P.J. Rudzki).

https://doi.org/10.1016/j.jchromb.2023.123800
Received 20 January 2023; Received in revised form 4 June 2023; Accepted 14 June 2023
Available online 23 June 2023

between matrix sources (%RSD$_{MF}$) is limited to 15% for small molecules [7,10]. The matrix effect should be evaluated using different matrix sources, including hemolyzed and lipemic sources in the case of plasma [3,6,7,10–16]. However, the influence of an experimental design on this test results seems to remain an unaddressed topic as regulatory guidelines do not recommend the specific order of samples [7,10]. Furthermore, each of the 8 random publications from the 2022 and 2023 years we found in PubMed using the keywords "bioanalytical method validation" and "matrix effect plasma LC-MS'' of the matrix effect determination – like the order of samples (interleaved pairs or sets of blocks) [17–24]. The novelty of the study is the examination if the order of samples (neat solution of standards and post-extraction spiked plasma samples) in the analytical run influences on results of %RSD$_{MF}$.

Despite numerous studies on the matrix effect, the background of the phenomenon is not fully understood, and the occurrence of the matrix effect is difficult to predict. Among factors suggested affecting the matrix effect are physicochemical properties of the analyte, type of biological material (the more complex the highest probability of matrix effect), sample preparation technique, chromatographic conditions, ionisation type and the ion source construction [2,13] as well as calculation method [2,6,11,25–27]. The easiest way to reduce the probability of a matrix effect occurrence is to prepare the sample for analysis, but the most straightforward technique – protein precipitation – is the most prone to matrix effect [28]. The interference with phospholipids richly present in lipemic plasma mainly causes a matrix effect [11,12]. Research on specific factors influencing the matrix effect focuses on the absolute matrix effect, not its between-source variability, which is more important [2,4,12,13,28].

Our study draws attention to the order of analysis of test samples (neat solution of standards and post-extraction spiked plasma samples) as another factor that may affect the quantification of matrix effect between-source variability. The primary goal was to find which experimental design – interleaved or set of blocks – is more sensitive to detect %RSD$_{MF}$. We intentionally avoided using the internal standard and chose the protein precipitation as a sample preparation technique to observe the significant matrix effect. Additionally, in order to interpret the recorded data, we assessed the influence of three chromatographic elution conditions and the three types of plasma (normal, lipemic or hemolyzed) on %RSD$_{MF}$ using chemometric methods. We also evaluated co-elution and carry-over of phospholipids better to understand the reasons of the high %RSD$_{MF}$.

2. Materials and methods

The design and workflow of research is presented in Fig. 1.

2.1. Chemicals and reagents

Reference standards ($n = 20$, purity $\geq 98\%$): amitriptyline, bezafibrate, clomipramine, darunavir, desipramine, fexofenadine, hydroxyzine, imipramine, levopromazine, maprotiline, orphenadrine, promazine, propafenone, protriptyline, quinapril, ramipril, rifampicin, roxithromycin, trimipramine, and verapamil were purchased from Sigma-Aldrich (Darmstadt, Germany). Acetonitrile MS grade and formic acid (anhydrous for analysis, EMSURE®) were purchased from Merck KGaA (Darmstadt, Germany). Purified water from Milli-Q system, Millipore (Molsheim, France), was used throughout the study. Blank human plasma with anticoagulant sodium citrate was purchased from the Regional Centre of Blood Donation and Blood Therapy in Warsaw, Poland. Hemolyzed plasma was prepared by addition of whole blood to plasma in final concentration 2% (v/v). Plasma with visible lipemia was obtained from the Regional Centre of Blood Donation and Blood Therapy in Warsaw, Poland.

2.2. Chromatographic and mass spectrometric conditions

Instrumental analysis was performed on Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, US), equipped with an autosampler, degasser, and binary pump coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer QTRAP 4000 (ABSciex, Framingham, MA, US). The data was processed by Analyst software 1.6.2 and MultiQuant software version 3.03 (ABSciex, Framingham, MA, US). The Turbo Ion Spray source was operated in positive mode. The ion spray voltage and source temperature were 5000 V and 600 °C, respectively. The curtain gas, ion source gas 1, ion source gas 2, and collision gas were set at 35 psi, 60 psi, 40 psi, and "medium" instrument units, respectively. The target

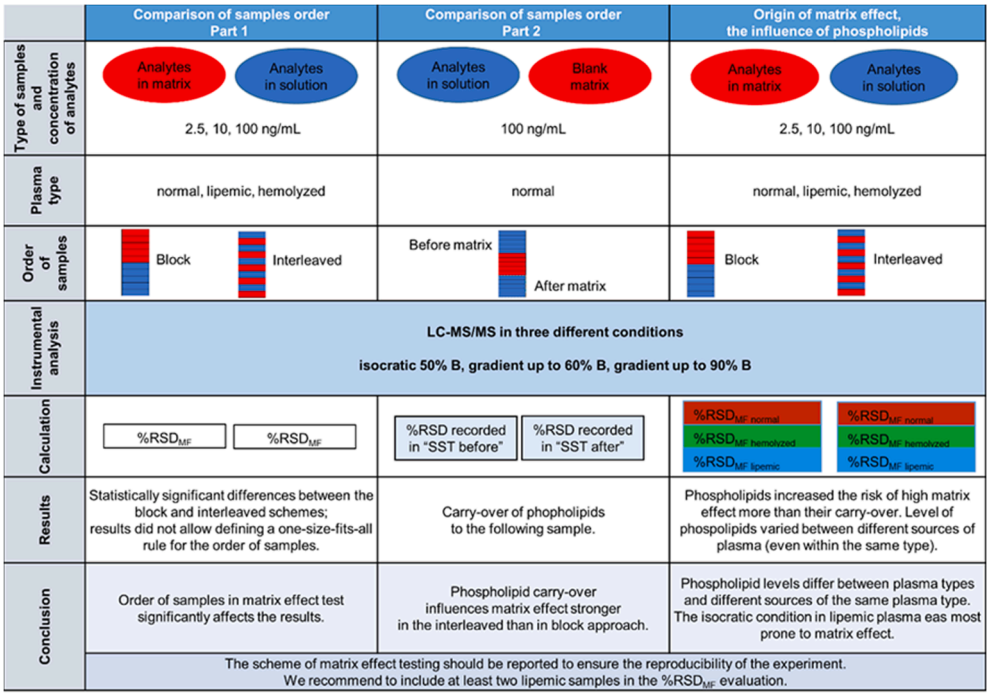


Fig. 1. The design and workflow of research.

compounds were optimised and analyzed in the Selected Reaction Monitoring (SRM) mode (Table S1, Supplementary data). The phospholipids were monitored as well (Table S1, Supplementary data).

Mobile phase A consisted of 0.2% formic acid *(v/v)*, and mobile phase B consisted of 0.2% formic acid in acetonitrile *(v/v)*. The experiments were conducted on column Bionacom Bionacore C18 (4.6 × 100 mm; 2.7 μm). The injection volume was 10 μL, and the flow rate was 0.5 mL/min. The samples were analyzed in the three elution conditions:

1. isocratic: 5-minute isocratic elution 50% B;
2. gradient 60: 12-minute gradient of 20–60% B (0–1 min −20% B, 7 min – 60% B, 9 min – 60% B, 9.2 min – 20% B, 12 min – 20% B);
3. gradient 90: 12-minute gradient of 10–90% B (0–1 min −10% B, 7 min – 90% B, 9 min – 90% B, 9.2 min – 10% B, 12 min – 10% B).

The cleaning gradient (2 injections of 25 min) (0–15 min −80% B, 15 min – 90% B, 22 min – 90% B, 22.2 min – 10% B, 25 min – 10% B) was applied before changing elution's condition.

2.3. Sample preparation (standard solutions, neat solution of standards and plasma samples)

We prepared stock solutions for each of the 20 compounds at 1 mg/mL in methanol. Spiking solutions were prepared by mixing 20 standard stock solutions and diluting them with water to a concentration of 30 μg/mL, then diluting with water again to 300, 30, and 7.5 ng/mL. System Suitability Test (SST) is performed to assess the system's ability to generate a reproducible peak area and analyte retention time. For this purpose, SST samples are repeatedly injected. SST sample was a mixture of analytes at the concentration of 100 ng/mL in water: methanol (4:1, v/v). Part 1: Neat solution of standards was a mixture of analytes at the same concentrations as the post-extraction spiked plasma samples (Fig. 1, "analytes in solution") i.e. 2.5, 10 or 100 ng/mL. The solution was prepared in water: acetonitrile and was used with the post-extraction spiked plasma sample to calculate the matrix factor. Both samples contained the same amount of acetonitrile. To prepare the neat solution of standards for the matrix effect study, we diluted spiking solutions (300 μL) with acetonitrile (200 μL) and water (400 μL). The final concentration of all analytes in neat solution of standards and plasma samples were further named as low (2.5 ng/mL), medium (10 ng/mL) and high (100 ng/mL), respectively. The final concentration of all analytes in neat solution of standards (described as samples A in [2]) corresponds to post-extraction spiked plasma samples (described as samples B [2]).

Post-extraction spiked plasma samples (Fig. 1, "analytes in matrix") were prepared in each concentration, in six different lots of human plasma, including two hemolyzed, two normal and two lipemic ones. Blank human plasma (300 μL) was mixed with cold acetonitrile in the ratio 1:2 (*v/v*), mixed on vortex for 20 s and centrifuged at 10 000 rpm for 1 min. Then, 300 μL of supernatant was mixed in vial with 300 μL of water to dilute the extract and 300 μL of aqueous spiking solution (with 20 compounds) at an appropriate concentration (7.5 ng/mL, 30 ng/mL and 300 ng/mL).

Part 2: Blank sample (Fig. 1, "blank matrix") is the blank plasma without the analytes (no spiking was performed before or after extraction). Blank human plasma (300 μL) was mixed with cold acetonitrile in the ratio 1:2 (*v/v*), mixed on vortex for 20 s and centrifuged at 10 000 rpm for 1 min. Then, 300 μL of supernatant (the same volume as in Part 1) was mixed with 600 μL of water.

2.4. Experimental design

In the first step of analytes selection, we analyzed 100 various pharmaceutically active substances and selected 20 of them with reproducible SST (%RSD < 4.5%) under all selected chromatographic conditions: isocratic, gradient 60 and gradient 90 (see par. 2.2). The drugs selected for this study belong to different therapeutic classes including antidepressants, cardiovascular drugs and antibiotics.

2.4.1. Part 1 – post-extraction spiked samples and neat solution of standards

Before the start of the analysis, the column equilibration with the mobile phase was performed. Then, ten SST samples were injected to ensure the chromatographic system generates repeatable peak area and analyte retention time. Then neat solution of standards and post-extraction spiked plasma samples were analyzed at low (2.5 ng/mL), medium (10 ng/mL) and high (100 ng/mL) concentrations. For each chromatographic condition, the set of samples described below was analyzed in two orders within one analytical run:

(1) set of blocks: 6 post-extraction spiked plasma samples then 6 neat solution of standards at corresponding concentration;

(2) interleaved six pairs of 1 injection of neat solution of standards and 1 injection of post-extraction spiked plasma sample at the corresponding concentration for each of 6 plasma sources (two normal, two haemolyzed and two lipemic). For each concentration additional neat solution of standards was analyzed at the end (post-extraction spiked plasma samples were bracketed by neat solution of standards). The method was not validated due to the other aim of the study than developing a new method of determination 20 drugs. However, it should be highlighted that we selected the analytes with satisfactory repeatability of the peak area.

2.4.2. Part 2 – SST and blank plasma

For 20 selected drugs, two kinds of samples were prepared: SST solution (20 compounds in water:methanol (4:1, *v/v*) at a concentration of 100 ng/mL each) and blank human plasma samples after protein precipitation with acetonitrile. In each of the three chromatographic conditions (see par. 2.2), after the stabilization of the chromatographic system, the samples were analyzed in the following sequence:

(1) 10 injections of SST solution,
(2) 10 blank plasma samples after protein precipitation with acetonitrile,
(3) 10 injections of SST solution.

The stability of the analytes in solution was confirmed for at least 12 h.

2.5. Calculations, chemometric and statistical methods

For samples analyzed in Part 1 (post-extraction spiked plasma samples and neat solution of standards described in section 2.4.1.) in interleaved pairs and sets of blocks, matrix factor (MF, Equation (1) and $\%RSD_{MF}$ (Equation (2) were calculated according to the EMA guideline [7].

$$MF = \frac{\text{Analyte response in the post-extraction spiked plasma}}{\text{Analyte response in the neat solution of standards}} \quad (1)$$

$$\%RSD_{MF} = \frac{100*S}{\overline{MF}} \quad (2)$$

Where:
S = standard deviation of the matrix factor.
$\overline{MF}$ = mean matrix factor.

Analysis Of Variance (ANOVA) compares variances across the means of different groups based on the assumption of normal distribution and homogeneity of variances. We used ANOVA for paired samples to compare difference in the $\%RSD_{MF}$ between both schemes (interleaved pairs and sets of blocks). In Part 2, using ANOVA, we compared the %RSD (for each drug) calculated for SST samples injected before (n = 6)

and after (n = 6) the blank plasma samples.

Principal Component Analysis (PCA) – a multivariate data analysis and data reduction technique [29] – was used to evaluate the influence of chromatographic conditions as well as the occurrence of lipemia and hemolysis. PCA allows the data visualisation by reducing the number of original variables in the analysed dataset and represents them in a visually understandable space as new latent variables, otherwise known as principal components (PCs). A set of data points is represented in a

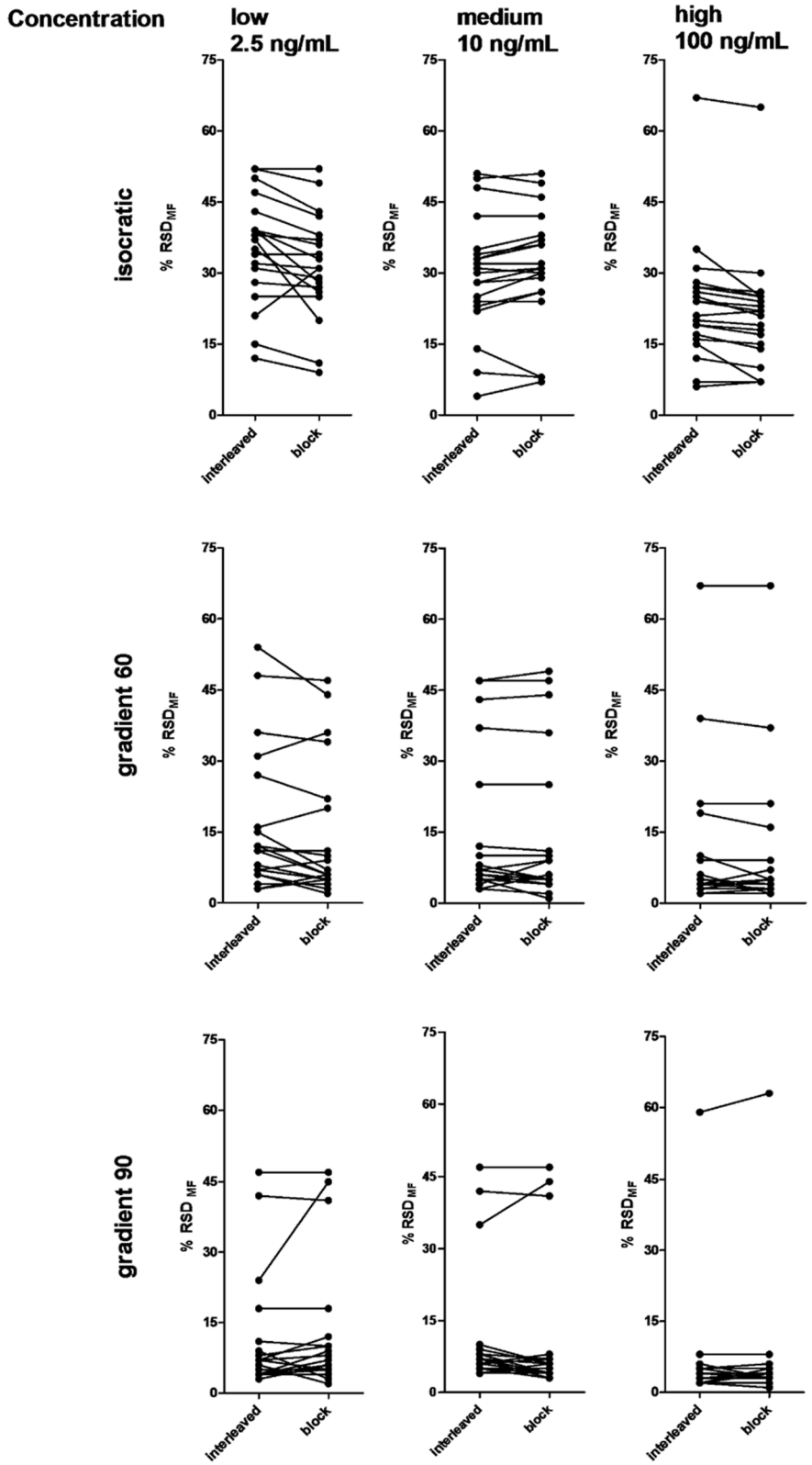


Fig. 2. The %RSD_{MF} calculated for all plasma samples for three elution types and three concentrations (2.5 ng/mL, 10 ng/mL, 100 ng/mL) of n = 20 studied drugs. The linked dots represent %RSD_{MF} of a particular drug in both block and interleaved schemes. The segment slope (from left to right) indicates more sensitive sample order to detect matrix effect: upward – block, downward – interleaved. Data and compound names are presented in Table S2, Supplementary Material.

two-dimensional system of coordinates, PC is a line that passes in the direction in which the maximum approximation to as many points as possible. In this way, most of the data variability is captured and as little information as possible is lost. The remaining variability is explained by the next PC, which is orthogonal to the previous one [30,31].

Partial Least-Squares Discriminant Analysis (PLS-DA) is a parametric and linear method that identifies PCs in the featured spaces, which have maximal covariance within the predictor variables. This method is therefore supervised, in contrast to PCA, which is a unsupervised method [30]. PLS-DA is - a useful feature selector and classifier tool in machine learning – was also used to check the influence of chromatographic conditions on $\%RSD_{MF}$ in hemolyzed and lipemic plasma and also to check the influence of the molecular descriptors of compound (dipole moment, polar surface area, polar volume, total area, total volume, calculated logP, molar mass, amount of acceptor HB and amount of donor HB) (Table S3, Supplementary Data) on susceptibility for the occurrence of matrix effect.

The areas under the curve of the receiver operating characteristic (ROC) curves were used to determine the discriminating ability and diagnostic accuracy of the experimental design – interleaved and block schemes – in detecting the matrix effect. The ROC plot shows sensitivity (true positive fraction) on the horizontal axis against 1-specificity (false positive fraction) on the vertical axis over all possible decision thresholds. The sensitivity and specificity of the experimental designs in the detection of matrix effect were compared [32]. Heatmaps were used to visualise the data. All the analyses were performed using the online tool, Metaboanalyst 5.0, after the log transformation of variables.

3. Results

3.1. Comparison of analysis order Part 1 – post-extraction spiked matrix samples and neat solution of standards

To determine which order of analysis (interleaved or set of blocks) has a better ability to detect matrix effect ($\%RSD_{MF} > 15\%$) in at least one of the chromatographic conditions, we performed ROC analysis. Sensitivity to detect matrix effect was calculated to be 0.973 (95% CI 0.933–1.000) and 1.000 (95% CI 1.000–1.000), specificity was estimated to be 1.000 (95% CI 1.000–1.000) and 0.981 (95% CI 0.947–1.000) for block and interleaved, respectively. Both experimental designs have similar ability to detect matrix effect. The interleaved analytical run was more sensitive in detecting the matrix effect in general, but there were exceptions (Fig. 2).

We observed differences in MF and $\%RSD_{MF}$ between the order of analysis of test samples and between different elution conditions. Regardless of the analysis order used in the analytical run, only quinapril passed the 15% acceptance criterion of $\%RSD_{MF}$ in all elution conditions (Fig. 3). A $\%RSD_{MF} > 15\%$ was observed for:

- 90% of the drugs under isocratic conditions (all except ramipril and quinapril)
- 30% of drugs (orphenadrine, levopromazine, promazine, ramipril, darunavir, rifampicin) in gradient 60
- and 20% of drugs (hydroxyzine, promazine, darunavir and rifampicin) in gradient 90.

ANOVA for paired samples for complete dataset indicates that $\%RSD_{MF}$ was statistically higher in the interleaved than in the block scheme ($p = 0.0130$). The same statistical test was performed in subgroups corresponding to different elution conditions. The differences $\%RSD_{MF}$ between interleaved and block schemes were only statistically significant for isocratic elution ($p = 0.0012$), but not for gradients ($p = 0.0811$ for gradient 60 and $p = 0.4972$ for gradient 90). In general, $\%RSD_{MF}$ for two schemes of matrix effect analyses were lower in gradient than in isocratic elution; thus, the percentage of random errors is higher in the first one. It can be a reason for the lack of statistically significant differences between $\%RSD_{MF}$ in interleaved and block schemes in gradient elution. To sum up, the order of analysis affects $\%RSD_{MF}$.

3.2. Comparison of analysis order Part 2 – SST and blank plasma

To better understand the source of the differences between the two orders of analyses from Part 1, we studied the effect of blank plasma constituents on the peak area of drugs in SST solution (Part 2). The %RSD of injection of STT solutions before (SST before) and after (SST after) plasma injections are presented in Fig. 4. For some analytes, the peak area in the first "SST after" was significantly lower than in "SST before", and gradually increased with each subsequent injection. This resulted in a higher %RSD recorded in "SST after" (Fig. 4, Figure S1, Supplementary data). The PLS-DA analysis showed a higher polar surface area and more hydrogen donors for analytes with high differences in %RSD (>5 pp), than for these with lower differences in %RSD. The influence of elution types was also noted. For 14 out of 20 drugs a larger difference between "SST after" and "SST before" in %RSD was determined for both gradients compared to isocratic elution (Fig. 4, the relevant difference was set as 5%).

Thus, one might expect greater differences between $\%RSD_{MF}$ in the block and interleaved approaches for these 14 drugs. But it is not in line with the results of Part 1. Even when only $\%RSD_{MF}$ of normal plasma

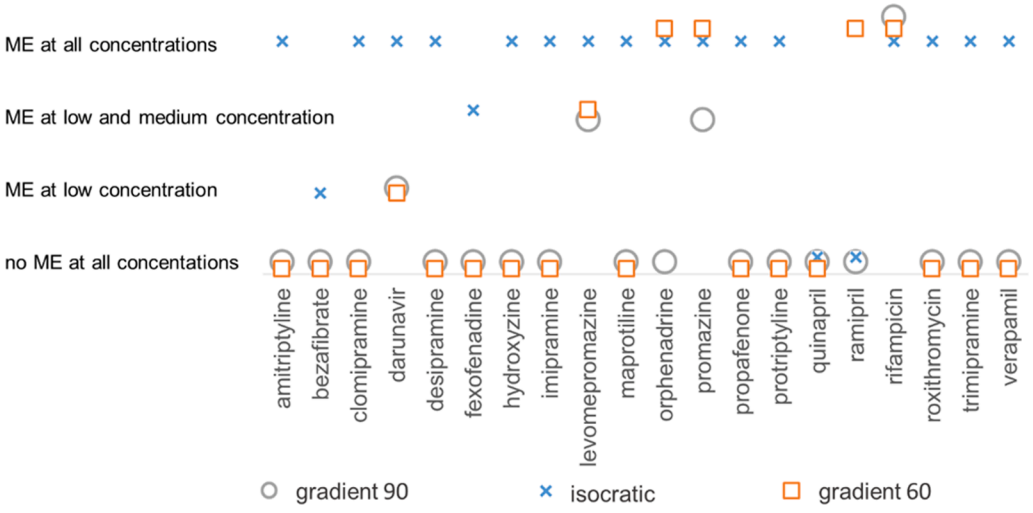


Fig. 3. Matrix effect ($\%RSD_{MF} \leq 15\%$) in different elution conditions for interleaved approach.

Elution Condition's	Isocratic			Gradient 60			Gradient 90		
Drug	SST before	SST after	Differences	SST before	SST after	Differences	SST before	SST after	Differences
Amitriptyline	1.4%	2.3%	1.0%	2.0%	16.0%	**14.0%**	3.3%	7.3%	4.0%
Bezafibrate	2.9%	2.7%	-0.2%	1.8%	1.3%	-0.5%	2.7%	1.0%	-1.7%
Clomipramine	3.4%	3.5%	0.1%	3.1%	3.6%	0.4%	3.0%	3.3%	0.3%
Darunavir	1.7%	12.8%	**11.1%**	2.2%	2.0%	-0.1%	3.3%	2.0%	-1.3%
Desipramine	3.0%	1.5%	-1.5%	2.2%	4.3%	2.1%	3.4%	11.1%	**7.6%**
Fexofenadine	2.0%	2.7%	0.6%	3.3%	8.2%	4.9%	3.5%	3.6%	0.1%
Hydroxyzine	3.3%	4.2%	0.9%	2.3%	3.1%	0.8%	2.1%	5.0%	2.9%
Imipramine	2.0%	2.5%	0.5%	2.9%	9.7%	**6.8%**	1.7%	9.2%	**7.6%**
Levomepromazine	1.8%	1.8%	0.0%	3.9%	8.0%	4.1%	3.3%	5.0%	1.7%
Maprotiline	3.1%	3.1%	0.0%	3.9%	13.8%	**9.9%**	1.1%	7.1%	**6.0%**
Orphenadrine	2.7%	3.3%	0.6%	2.3%	2.6%	0.3%	2.2%	11.5%	**9.3%**
Promazine	2.0%	3.1%	1.2%	2.7%	3.2%	0.4%	2.5%	13.3%	**10.8%**
Propafenone	3.2%	3.0%	-0.2%	2.6%	2.8%	0.1%	3.2%	4.3%	1.1%
Protriptyline	1.5%	2.6%	1.1%	2.4%	3.3%	0.9%	0.8%	9.3%	**8.5%**
Quinapril	3.1%	2.1%	-1.0%	2.8%	6.0%	3.2%	3.0%	5.8%	2.8%
Ramipril	3.3%	2.6%	-0.7%	1.4%	2.5%	1.2%	2.0%	9.7%	**7.7%**
Rifampicin	2.2%	1.3%	-0.8%	7.0%	2.8%	-4.2%	3.2%	6.2%	3.0%
Roxithromycin	3.1%	3.3%	0.3%	2.0%	9.8%	**7.8%**	2.2%	10.6%	**8.4%**
Trimipramine	2.8%	2.5%	-0.2%	1.4%	7.4%	**6.0%**	2.6%	7.9%	**5.3%**
Verapamil	4.4%	2.2%	-2.1%	3.0%	9.0%	**6.1%**	1.2%	4.3%	3.1%

Fig. 4. Change from baseline RSD of analyte instrument response [%] calculated for each of 20 drugs mixed at 100 ng/mL (SST) analyzed before (6 last injections) and after (6 first injections) injection of blank human plasma extract ($n = 10$). In bold we marked the differences between "SST before" and "SST after" higher than 5 pp.

was considered, the gradient did not reveal greater differences between block and interleaved approaches compared to isocratic elution (Figure S2, Table S2, Supplementary data). Thus, we hypothesise that different concentrations of the interfering compounds (in various plasma sources) affect $\%RSD_{MF}$ more than the carry-over of interfering compounds to the following sample. Only one source of normal plasma was used in Part 2. Thus, we could not evaluate the influence of different matrix compositions on the method performance. But we showed that performing the SST test directly after the analysis of blank plasma samples may indicate the susceptibility of the analyte to signal suppression or enhancement.

In conclusion, a carry-over of phospholipids from plasma to the following sample was noted. However, this negative effect had less impact on $\%RSD_{MF}$ than the variability of phospholipid levels in different plasma sources.

3.3. Origin of matrix effect – the influence of phospholipids

The phospholipids analysis was performed to understand better the differences between interleaved and block schemes found in Part 1 as well as verify the hypothesis from Part 2. Among all monitored phospholipids, a significantly higher signal in the sample injected after the plasma sample compared to the neat solution of standards was observed for the sum of phosphatidylcholines and sphingomyelins (sum-PC), the sum of 1-mono (2-lyso)glycerophosphocholines (sum-LPC), lysophosphatidylcholine-1 (LPC-1) and lysophosphatidylcholine-2 (LPC-2) (Fig. 5, Figures S3-S8, Supplementary data). We observed the phospholipids carry-over from the plasma sample to the neat solution of standards. The chromatogram of phospholipids in the neat solution of standards injected after plasma differs within the same plasma types. Thus, intensity and type of co-eluting phospholipids' can influence the ionisation of the analytes, presumably depending on the physicochemical properties of the analyte. It can explain a higher $\%RSD_{MF}$ in interleaved than block approach.

Phospholipids are claimed to be the most important cause of the matrix effect. In Part 2 we hypothesised that the variability in the concentration of the interferences affects $\%RSD_{MF}$ more than the carry-over of interferences to the following sample. We have observed that phospholipid levels differ strongly between plasma types and different sources of the same plasma type. In isocratic elution, almost all analytes eluted around 2.2–2.5 min except for darunavir (3.5 min) and bezafibrate (4.5 min) (Figure S9, Supplementary data). All analytes except bezafibrate co-eluted with phospholipids (sum-PC peak from 2.1 to 2.5 min (Fig. 6A), LPC-1: 3.0–3.7 min (Figure S6). Thus, in the case of isocratic elution, the phospholipids elution profile did not indicate analytes more prone to matrix effect. Nor did the molecular descriptors (Table S4, Supplementary data).

In gradient 60, almost all analytes eluted around 6.3–6.9 min except for clomipramine (7.2 min), rifampicin (8.3 min), darunavir (9.1 min) and bezafibrate (9.2 min) (Figure S10, Supplementary data). A high $\%RSD_{MF}$ were observed for orphenadrine (6.4 min), levopromazine (6.8 min), ramipril (6.3 min), darunavir (9.1 min), and rifampicin (6.3 min). However, only darunavir co-eluted with phospholipids (LPC-2: 9–10 min) (Figure S7) together with bezafibrate, which have similar retention time but reveal low $\%RSD_{MF}$.

In gradient 90, almost all analytes eluted around 6.0–6.9 min except for rifampicin (7.0 min), darunavir (7.6 min) and bezafibrate (7.7 min) (Figure S11, Supplementary data). A high $\%RSD_{MF}$ were observed for darunavir (7.6 min), levopromazine (6.3 min), promazine (6.1 min), and rifampicin (7.0 min). However, only darunavir and rifampicin, eluted with phospholipids (sum-LPC and LPC-1 peak from 7.0 to 9.0 min, sum-PC: 5.0–12.0) (Fig. 6C and Figure S8, Supplementary data).

We conclude that phospholipids increase the risk of high matrix effect, but cannot be used as a definitive predictor of high $\%RSD_{MF}$. Their levels vary between plasma types and between different sources of the same plasma type, what increases MF variability. The random selection of lipemic sample can strongly influence the validation test results. Therefore, we recommend using at least two lipemic samples in the matrix effect evaluation to minimize the risk of irrelevant conclusion.

3.4. Origin of matrix effect - chemometric methods

Using PCA, we reduced the dimensions of the dataset to two principal components (PC), accounting for 75.4% of the total variation (PC1 – 59%, PC2 – 16.4%). PC1 best accounts for the plasma type, whereas PC2 for chromatographic conditions. Including both factors into two primary PCs confirms that both contribute considerably to $\%RSD_{MF}$. It can be observed that one variable is significantly separated from the others – isocratic conditions for the lipemic plasma sample (Figure S12A). This suggests that the effect of lipemia on $\%RSD_{MF}$ was the greatest in isocratic elution, which is in line with the experimental results of Part 1.

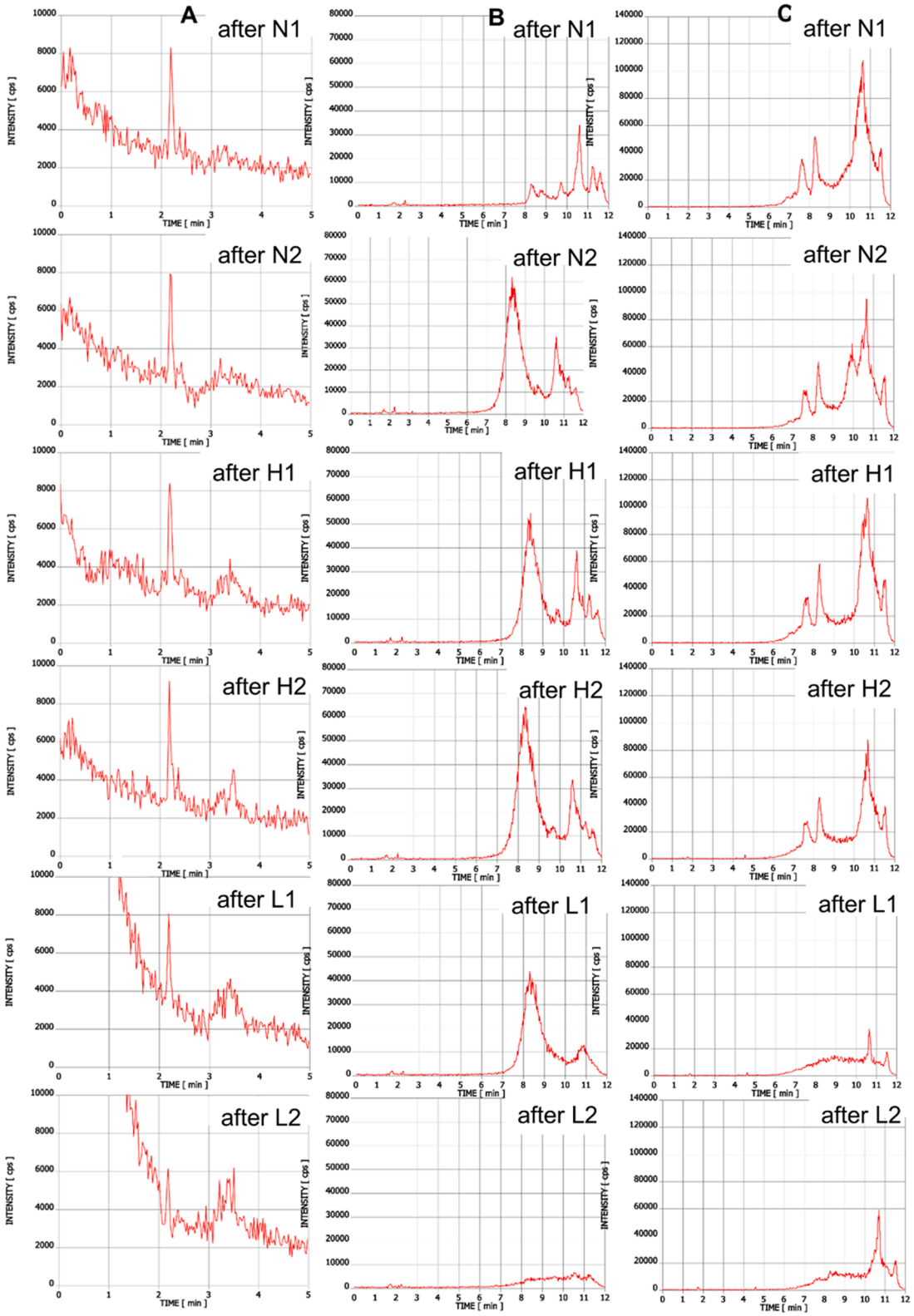


Fig. 5. Extracted ion chromatograms of the sum of phosphatidylcholines and sphingomyelins detected in the neat solution of standards injected after plasma sample (**A**) isocratic elution, (**B**) gradient 20–60%, (**C**) gradient 10–90%; N1, N2– normal plasma; H1, H2– hemolyzed plasma; L1, L2– lipemic plasma.

The results of PLS-DA (goodness of fit $R^2 = 0.80$; cross-validated R^2-$Q^2 = 0.45$; $p < 0.01$), confirmed the strong influence of the isocratic condition on the value of $\%RSD_{MF}$ obtained for analytes in lipemic plasma. We observed that some compounds are more prone to the influence of hemolysis and lipemia than others. These compounds included rifampicin, levomepromazine, orphenadrine, promazine and clomipramine, and to a lesser extent protriptyline, desipramine, roxithromycin and fexofenadine (Figure S12B). No association of the susceptibility with the compound structure – i.e. molecular descriptors – was found (Table S3). This is in agreement with the work by Kojro et al.

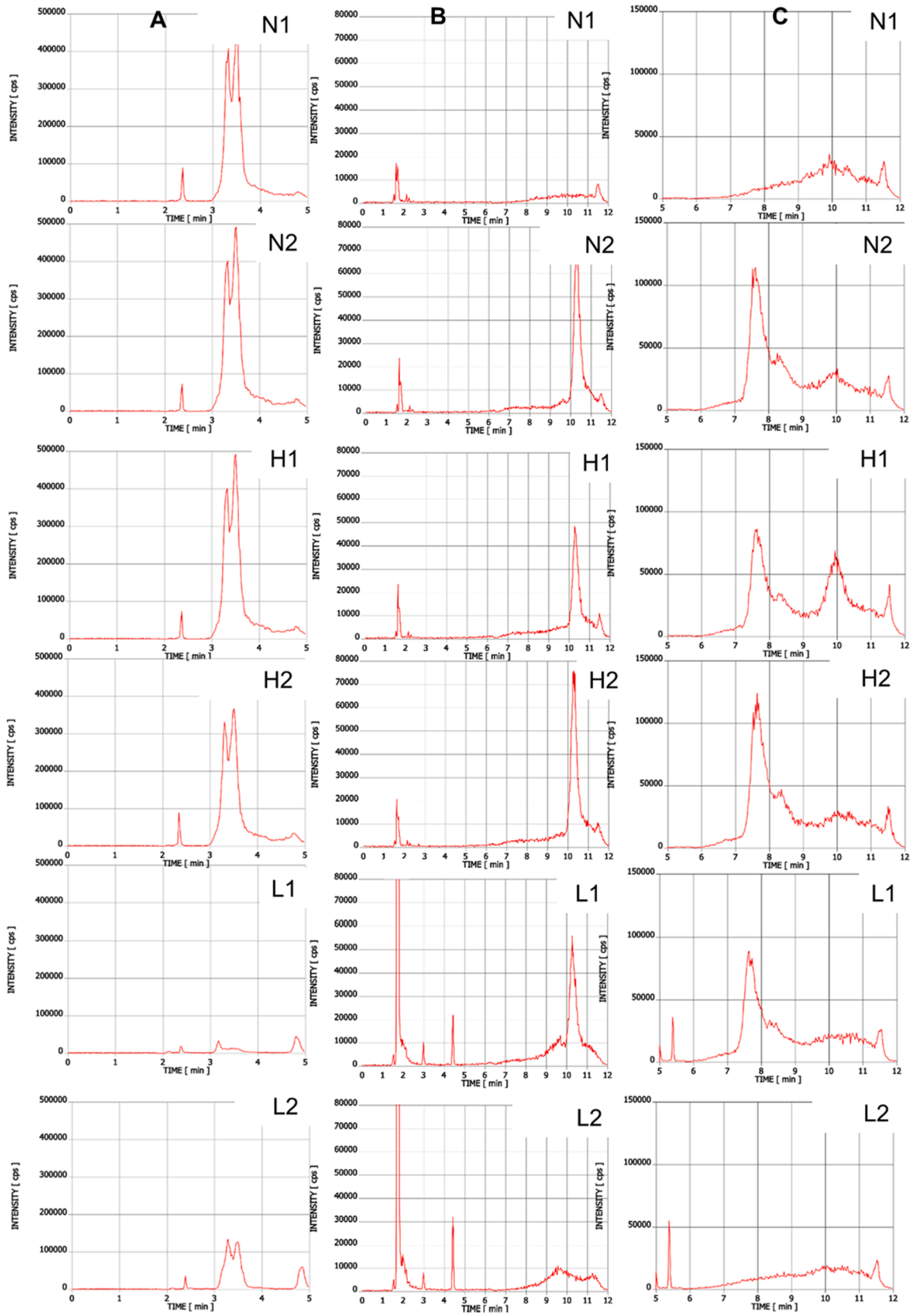


Fig. 6. Extracted ion chromatograms of sum of phosphatidylcholines and sphingomyelins detected in the plasma sample separated using (**A**) isocratic elution, (**B**) gradient 20–60%, (**C**) gradient 10–90%, N1,N2– normal plasma; H1,H2– hemolyzed plasma; L1,L2– lipemic plasma.

[33], where no correlation of molecular descriptors with absolute matrix effect was noted for plasma samples prepared using cloud point extraction. However, the influence of surfactant (Triton X-114) on the ionisation suppression/enhancement was observed for the lipophilic drugs characterised by the low polar volume, PSA and dipole moment.

The strong effect of lipemia on $\%RSD_{MF}$ during isocratic separation was confirmed using heat maps with clustering (Figure S12C). We observed that $\%RSD_{MF}$ was the highest for almost all compounds. Among all chromatographic conditions and plasma types, clusters were formed more for chromatographic conditions. In isocratic elution, compounds were more prone to matrix effect, with an average $\%RSD_{MF}$ of 29% for isocratic elution, 14% for gradient 60 and 10% for gradient 90. For 13 out of 20 compounds, $\%RSD_{MF}$ (in at least one concentration) was observed only under isocratic conditions, but only 1 of 20 under gradient 60. In the case of 4 out of 20 drugs (rifampicin, levopromazine, promazine, darunavir), high $\%RSD_{MF}$ was observed regardless of the gradient used. To sum up, the influence of lipemia an hemolysis on $\%RSD_{MF}$ depends on the chromatographic method and compound, but cannot be simply predicted by molecular desriptors.

4. Discussion

The interleaved scheme was more sensitive in detecting the matrix effect and produced a higher $\%RSD_{MF}$ than the block scheme. The latter statement was statistically significant for all chromatographic conditions combined and for isocratic, but not for gradients. $\%RSD_{MF}$ for two schemes of matrix effect analyses were lower in gradient than in isocratic elution. But for some compounds, relevant differences were observed between interleaved and block schemes, even for gradient elution. However, a higher $\%RSD_{MF}$ in the interleaved scheme may be due to the carry-over of some compounds from plasma (like phospholipids) to the neat solution of standards injected next. These results do not allow defining a one-size-fits-all rule for the order of samples, which increases the probability of observing the matrix effect. But our study strongly highlights that the order of analysis of samples may influence the test results. To ensure reproducibility of experiments, it is necessary to describe in detail the methodology for evaluating the effect of the matrix, including the order of analysis of samples.

The influence of haemolysis [14–16], lipemia/phospholipids [12,28,34–36] on matrix effect in bioanalysis is widely described. Our study enabled us to find the most susceptible conditions in 2D space of plasma type and chromatographic conditions. Chemometric methods draw the primary attention to lipemic samples analyzed in isocratic conditions as the most prone to variable matrix effect (Figure S12C, Supplementary data). We also confirmed that not all the phospholipids are eluted off the column during a single analysis (Fig. 3, Figure S3-Figure S5, Supplementary data), as reported by Xia and Jemal [12]]. To remove lipids from the reverse-phase column, a relatively long wash with acetonitrile/isopropanol for most phospholipid classes (phosphatidylcholines and sphingomyelins) and with methanol for the other (glycerophosphocholines) is required [11,12] (at least 15-fold column volumes for 95% ACN) [12]. Such a long process is not used in routine analytical methods and thus may affect the results of matrix effect assessment in the interleaved scheme. If ME is unacceptable in lipemic plasma, it is recommended that other sample preparation methods are used to remove components that may cause a matrix effect. A number of techniques can be chosen, including liquid–liquid extraction or solid-phase extraction, particularly using molecularly imprinted polymers as highly specific sorbents [37]. Careful optimisation of these techniques is required to obtain acceptable ME [28]. The influence of hemolysis is less complex than in the case of lipemic one. The factors that may cause matrix effect in hemolyzed plasma are the degree of hemolysis (concentration of free plasma hemolysis) [14] and compound-dependent factors like the red blood cell: plasma drug's concentration ratio. Tan et al. reported that the impact of hemolysis is more significant (in the same degree of hemolysis) in increasing the drug level of affinity to red blood cells. The results of the concentration of the drug in hemolyzed plasma samples for a drug with a high affinity to red blood cells may be unreliable [38].

After the experimental part of our study was finished, ICH M10 guideline was issued [39]. The ICH M10 guideline [39] recommends in section 3.2.3. Matrix effect: "An additional evaluation of the matrix effect is recommended using haemolysed or lipaemic matrix samples during method validation on a case-by-case basis". Thus, there is no indication of the number of lipemic sources to be used. However, in section 3.2.1. it is recommended "For the investigation of selectivity in lipemic matrices at least one source of matrix should be used". Triplicate evaluation of each source and calculation procedures are novelties which were not implemented in our work. Our results suggest that the differences in the composition of various matrix lots of the same type – especially lipemic – may influence method reliability. Thus, we recommend evaluating more than one lipemic and hemolyzed plasma source. Previously, it has been emphasised for normal plasma that acceptable results in one plasma source may not be sufficient to ensure the method's validity in multiple sources [34]. Here, we proved that more significant differences could be observed for lipemic one. The main limitations of our study are the number of analytes (n = 20), the number of chromatographic conditions (3 variants) and the number of plasma sources studied. The number of combinations of studied drugs and possible sample preparation methods, chromatographic and MS conditions is aiming at infinity. However, 20 analytes represent different chemical structures, chromatographic conditions include isocratic and two different gradients, while 6 plasma sources included two matrices of each kind: normal, hemolyzed and lipemic. The studied combinations of analytes and experimental conditions allowed us to form the conclusion that sample order for matrix effect testing may influence test results and it should be reported. We suppose that this conclusion would be similar if different combinations were studied. We conducted our study without internal standards, while in regulatory bioanalysis, stable isotope labelled internal standards are recommended. We avoided internal standards intentionally not to minimalize matrix effect and to increase matrix effect variability. The way of matter allows better evaluate "susceptible" conditions and compares experimental design – the interleaved and block schemes order in the analytical run of test samples (neat solution of standards and post-extraction spiked plasma samples). Also, the analyte concentrations did not correspond with the human pharmacokinetic ranges. But unified concentrations for all analytes eliminated one of the possible sources of variability. The analytical methods used, especially the one based on gradient elution, are quite lengthy, as an High Performance Liquid Chromatograph (HPLC) was used. To reduce the run time and the cost of gradient chromatography analyses, it is suggested that Ultra Performance Liquid Chromatography (UPLC) methods be used with shorter columns and smaller particle diameters. We could not find common molecular descriptors for the analytes more prone to variable matrix effect ($\%RSD_{MF}$). But in Part 2 of the study, we observed that higher polar surface area and a larger number of hydrogen donors are associated with high RSD of the analyte's peak areas in a series of SST solutions analyzed after analysis of blank samples. This indicates that the carry-over of plasma interferences impacts more polar analytes to a larger extent.

Although the matrix effect in bioanalysis is known for many years, this topic is still gaining scientific interest [40–43]. Future studies on predicting matrix effect based on molecular descriptors, chromatographic conditions, co-elution or carry-over of phospholipids may reveal associations not observed in our work.

5. Conclusions

Although a comparable (but even statistically different) matrix effect ($\%RSD_{MF}$) is observed using the interleaved and block schemes, for some pharmaceuticals the order of the sample analysis strongly influences the results. The interleaved scheme was generally more sensitive in

detecting the matrix effect than the block scheme. Thus, the scheme of matrix effect testing should be reported to ensure the reproducibility of the experiments. In addition, as different composition of various sources of the matrix of the same type, especially lipemic, affect the method's reliability, evaluating more than one source of lipemic and hemolyzed plasma is advised.

CRediT authorship contribution statement

Elżbieta Gniazdowska: Investigation, Formal analysis, Writing – original draft. **Joanna Giebułtowicz:** Methodology, Resources, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Piotr J. Rudzki:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The Ministry of Education and Science (Poland) grant DWD/3/6/2019 and Subsidy for Łukasiewicz Research Network – Industrial Chemistry Institute No. 841345A financially supported the experimental studies. The funding sources was not involved in the research.

The authors gratefully acknowledge the contributions of Ryszard Marszałek for technical assistance in LC-MS/MS analyses. Thanks are also due to Dr. Katarzyna Buś-Kwaśnik from the Pharmacokinetics Section of Pharmaceutical Analysis Laboratory, Łukasiewicz Research Network – Industrial Chemistry Institute (Warsaw, Poland) for her critical reviewing of the manuscript. The views expressed in this paper are solely those of the authors and do not necessarily reflect their institutions' position on the subject.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2023.123800.

References

[1] J.-J. Cui, L.-Y. Wang, Z.-R. Tan, H.-H. Zhou, X. Zhan, J.-Y. Yin, Mass spectrometry-based personalized drug therapy, Mass Spectrom. Rev. 39 (5–6) (2020) 523–552, https://doi.org/10.1002/mas.21620.

[2] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC−MS/MS, Anal. Chem. 75 (13) (2003) 3019–3030, https://doi.org/10.1021/ac020361s.

[3] A. Nasiri, R. Jahani, S. Mokhtari, H. Yazdanpanah, B. Daraei, M. Faizi, F. Kobarfard, Overview, consequences, and strategies for overcoming matrix effects in LC-MS analysis: a critical review, Analyst 146 (20) (2021) 6049–6063, https://doi.org/10.1039/D1AN01047F.

[4] P.J. Taylor, Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry, Clin. Biochem. 38 (4) (2005) 328–334, https://doi.org/10.1016/j.clinbiochem.2004.11.007.

[5] H.-W. Liao, Y.-W. Cheng, S.-C. Tang, C.-H. Kuo, Bias caused by incomplete metabolite extraction and matrix effect: evaluation of critical factors for plasma sample preparation prior to metabolomics, J. Pharm. Biomed. Anal. 219 (2022), 114930, https://doi.org/10.1016/j.jpba.2022.114930.

[6] B.K. Matuszewski, Standard line slopes as a measure of a relative matrix effect in quantitative HPLC–MS bioanalysis, J. Chromatogr. B 830 (2) (2006) 293–300, https://doi.org/10.1016/j.jchromb.2005.11.009.

[7] Guideline on bioanalytical method validation, EMEA/CHMP/EWP/QWP/1401/98/ Rev. 1 Corr .2**, European Medicines Agency (2011).

[8] P. Adamowicz, W. Wrzesień, Simple approach for evaluation of matrix effect in the mass spectrometry of synthetic cannabinoids, J. Anal. Chem. 71 (8) (2016) 794–802, https://doi.org/10.1134/S1061934816080025.

[9] J.-F. Bienvenu, G. Provencher, P. Bélanger, R. Bérubé, P. Dumas, S. Gagné, É. Gaudreau, N. Fleury, Standardized Procedure for the Simultaneous Determination of the Matrix Effect, Recovery, Process Efficiency, and Internal Standard Association, Anal Chem 89 (14) (2017) 7560–7568, https://doi.org/10.1021/acs.analchem.7b01383.

[10] Guidance for Industry: Bioanalytical Method Validation, Food and Drug Administration. Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine (CVM) (2018).

[11] J.L. Little, M.F. Wempe, C.M. Buchanan, Liquid chromatography–mass spectrometry/mass spectrometry method development for drug metabolism studies: Examining lipid matrix ionization effects in plasma, J Chromatogr B Biomed Appl 833 (2) (2006) 219–230, https://doi.org/10.1016/j.jchromb.2006.02.011.

[12] Y.Q. Xia, M. Jemal, Phospholipids in liquid chromatography/mass spectrometry bioanalysis: comparison of three tandem mass spectrometric techniques for monitoring plasma phospholipids, the effect of mobile phase composition on phospholipids elution and the association of phospholipids with matrix effects, Rapid Commun Mass Spectrom 23 (14) (2009) 2125–2138, https://doi.org/10.1002/rcm.4121.

[13] V. Trivedi, V. Upadhyay, M. Yadav, P.S. Shrivastav, M. Sanyal, Impact of electrospray ion source platforms on matrix effect due to plasma phospholipids in the determination of rivastigmine by LC-MS/MS, Bioanalysis 6 (17) (2014) 2301–2316, https://doi.org/10.4155/bio.14.85.

[14] E.N. Fung, A.-F. Aubry, A. Allentoff, Q.C. Ji, Utilizing Internal Standard Responses to Assess Risk on Reporting Bioanalytical Results from Hemolyzed Samples, AAPS J. 17 (5) (2015) 1168–1176, https://doi.org/10.1208/s12248-015-9783-z.

[15] D. Tang, E. Thomas, Strategies for dealing with hemolyzed samples in regulated LC–MS/MS bioanalysis, Bioanalysis 4 (22) (2012) 2715–2724, https://doi.org/10.4155/bio.12.229.

[16] N.C. Hughes, N. Bajaj, J. Fan, E.Y. Wong, Assessing the matrix effects of hemolyzed samples in bioanalysis, Bioanalysis 1 (6) (2009) 1057–1066, https://doi.org/10.4155/bio.09.91.

[17] Y. Cheng, M. Chen, B. Zhang, H. Lin, X. Li, Y. Cai, H. Zhang, W. Que, M. Liu, H. Qiu, Rapid, simple, and economical LC–MS/MS method for simultaneous determination of ceftazidime and avibactam in human plasma and its application in therapeutic drug monitoring, J. Clin. Pharm. Ther. 47 (9) (2022) 1426–1437, https://doi.org/10.1111/jcpt.13693.

[18] V. Desfontaine, S. Guinchard, S. Marques, A. Vocat, F. Moulfi, F. Versace, J. Huser-Pitteloud, A. Ivanyuk, C. Bardinet, V. Makarov, O. Ryabova, P. André, S. Prod'Hom, H. Chtioui, T. Buclin, S.T. Cole, L. Decosterd, Optimized LC-MS/MS quantification of tuberculosis drug candidate macozinone (PBTZ169), its dearomatized Meisenheimer Complex and other metabolites, in human plasma and urine, J. Chromatogr. B 1215 (2023), https://doi.org/10.1016/j.jchromb.2022.123555.

[19] A. Fischle, R. Schwarz, F. Wendt, M. Kordt, R. Ramer, L. Boeckmann, M. Hein, P. Langer, S. Emmert, B. Vollmar, B. Hinz, A Sensitive LC-MS/MS Method for the Simultaneous Determination of Two Thia-Analogous Indirubin N-Glycosides and Indirubin-3′-Monoxime in Plasma and Cell Culture Medium, Molecules 27 (9) (2022) 3031, https://doi.org/10.3390/molecules27093031.

[20] M.M. Islam, E.F. Kub, V. Rajaratnam, S.P. Mirza, The development and validation of sensitive LC-MS/MS method for quantitative bioanalysis of carmofur in mouse plasma and its application to pharmacokinetic study, J. Chromatogr. B 1212 (2022), https://doi.org/10.1016/j.jchromb.2022.123516.

[21] A.P. Lakeev, E.A. Yanovskaya, V.A. Yanovsky, M.O. Andropov, G.A. Frelikh, I. Y. Chukicheva, A.V. Kutchin, LC-MS/MS method for the determination of a semi-synthetic phenolic antioxidant 2,6-diisobornyl-4-methylphenol in rats after different administration routes, J. Chromatogr. B 1213 (2022), https://doi.org/10.1016/j.jchromb.2022.123537.

[22] T.-T.-L. Nguyen, J.W. Kim, H.-I. Choi, H.-J. Maeng, T.-S. Koo, Development of an LC-MS/MS Method for ARV-110, a PROTAC Molecule, and Applications to Pharmacokinetic Studies, Molecules 27 (6) (2022) 1977, https://doi.org/10.3390/molecules27061977.

[23] P. Wong, A. Akrami, B. Houk, I. Vuu, C.A. James, Validation of an LC–MS/MS method for the determination of sotorasib, a KRASG12C inhibitor, in human plasma, Bioanalysis 14 (19) (2022) 1281–1292, https://doi.org/10.4155/bio-2022-0173.

[24] J. Thomann, L. Ley, A. Klaiber, M.E. Liechti, U. Duthaler, Development and validation of an LC-MS/MS method for the quantification of mescaline and major metabolites in human plasma, J Pharm Biomed Anal 220 (2022), 114980, https://doi.org/10.1016/j.jpba.2022.114980.

[25] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds, Rapid Commun Mass Spectrom 13 (12) (1999) 1175–1185, https://doi.org/10.1002/(sici)1097-0231(19990630)13:12<1175::aid-rcm639>3.0.co;2-0.

[26] Y. Huang, R. Shi, W. Gee, R. Bonderud, Matrix effect and recovery terminology issues in regulated drug bioanalysis, Bioanalysis 4 (3) (2012) 271–279, https://doi.org/10.4155/bio.11.315.

[27] P.J. Rudzki, E. Gniazdowska, K. Buś-Kwaśnik, Quantitative evaluation of the matrix effect in bioanalytical methods based on LC-MS: A comparison of two approaches, J Pharm Biomed Anal 155 (2018) 314–319, https://doi.org/10.1016/j.jpba.2018.03.052.

[28] X. Guo, E. Lankmayr, Phospholipid-based matrix effects in LC–MS bioanalysis, Bioanalysis 3 (4) (2011) 349–352, https://doi.org/10.4155/bio.10.213.

[29] R. Bro, A.K. Smilde, Principal component analysis, Anal. Methods 6 (9) (2014) 2812–2831, https://doi.org/10.1039/C3AY41907J.

[30] J. Djuris, S. Ibric, Z. Djuric, 4 - Chemometric methods application in pharmaceutical products and processes analysis and control, in: J. Djuris (Ed.), Computer-Aided Applications in Pharmaceutical Technology, Woodhead Publishing, 2013, pp. 57–90, https://doi.org/10.1533/9781908818324.57.
[31] L.s.F. Sidou, E.M. Borges, Teaching Principal Component Analysis Using a Free and Open Source Software Program and Exercises Applying PCA to Real-World Examples, J. Chem. Educ. 97 (6) (2020) 1666–1676, https://doi.org/10.1021/acs.jchemed.9b00924.
[32] M.H. Zweig, G. Campbell, Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine, Clin. Chem. 39 (4) (1993) 561–577, https://doi.org/10.1093/clinchem/39.4.561.
[33] G. Kojro, P.J. Rudzki, D.M. Pisklak, J. Giebułtowicz, Matrix effect screening for cloud-point extraction combined with liquid chromatography coupled to mass spectrometry: Bioanalysis of pharmaceuticals, J. Chrom. A 1591 (2019) 44–54, https://doi.org/10.1016/j.chroma.2019.01.031.
[34] O.A. Ismaiel, M.S. Halquist, M.Y. Elmamly, A. Shalaby, H. Thomas Karnes, Monitoring phospholipids for assessment of ion enhancement and ion suppression in ESI and APCI LC/MS/MS for chlorpheniramine in human plasma and the importance of multiple source matrix effect evaluations, J Chromatogr B Analyt Technol Biomed Life Sci 875 (2) (2008) 333–343, https://doi.org/10.1016/j.jchromb.2008.08.032.
[35] O. Ismaiel, T. Zhangc, R. Jenkins, H. Karnesa, Investigation of endogenous blood plasma phospholipids, cholesterol and glycerides that contribute to matrix effects in bioanalysis by liquid chromatography/mass spectrometry, J. Chromatogr. B 878 (2010) 3303–3316, https://doi.org/10.1016/j.jchromb.2010.10.012.
[36] G. Kojro, P. Wroczyński, Cloud Point Extraction in the Determination of Drugs in Biological Matrices, J. Chromatogr. Sci. 58 (2) (2020) 151–162, https://doi.org/10.1093/chromsci/bmz064.
[37] M. Sobiech, J. Giebułtowicz, P. Luliński, Computational and experimental studies of magnetic molecularly imprinted sorbent with high specificity towards aceclofenac, Microchem. J. 186 (2023), https://doi.org/10.1016/j.microc.2022.108272.
[38] A. Tan, S. Gagné, I.A. Lévesque, S. Lachance, N. Boudreau, A. Lévesque, Impact of hemolysis during sample collection: How different is drug concentration in hemolyzed plasma from that of normal plasma? J. Chromatogr. B 901 (2012) 79–84, https://doi.org/10.1016/j.jchromb.2012.06.002.
[39] ICH guideline M10 on bioanalytical method validation and study sample analysis, Committee for Medicinal Products for Human Use (CHMP), EMA/CHMP/ICH/172948/2019, European Medicines Agency, 2022.
[40] Z.-M. Li, M. Lakuleswaran, K. Kannan, LC-MS/MS methods for the determination of 30 quaternary ammonium compounds including benzalkonium and paraquat in human serum and urine, J. Chromatogr. B 1214 (2023), https://doi.org/10.1016/j.jchromb.2022.123562.
[41] Y.E. Sim, J.W. Kim, B.J. Ko, J.Y. Kim, J.C. Cheong, J. Pyo, Determination of urinary metabolites of cannabidiol, Δ8-tetrahydrocannabinol, and Δ9-tetrahydrocannabinol by automated online μSPE–LC–MS/MS method, J. Chromatogr. B 1214 (2023), https://doi.org/10.1016/j.jchromb.2022.123568.
[42] K.N. Nguyen, R. Saxena, D.B. Re, B. Yan, Rapid LC-MS/MS quantification of Organophosphate non-specific metabolites in hair using alkaline extraction approach, J. Chromatogr. B 1217 (2023), https://doi.org/10.1016/j.jchromb.2023.123619.
[43] S. Emami, A.Y. Taha, Effects of carotenoid pigmentation in salmon on antibiotic extraction recovery, matrix effects and accuracy of quantification by ultrahigh performance liquid chromatography coupled to tandem mass spectrometry, J. Chromatogr. B 1216 (2023), https://doi.org/10.1016/j.jchromb.2022.123585.

Supplementary material

How does the order of sample analysis influence the matrix effect during LC-MS bioanalysis?

Elżbieta Gniazdowska[a,b], Piotr J. Rudzki[c*], Joanna Giebułtowicz[d]

[a] Pharmaceutical Analysis Laboratory, Łukasiewicz Research Network - Industrial Chemistry Institute, 8 Rydygiera, 01-793 Warsaw, Poland.

[b] Department of Drug Chemistry, Doctoral School, Medical University of Warsaw, 61 Żwirki i Wigury, 02-091 Warsaw, Poland

[c] Celon Pharma S.A. Bioanalytical Laboratory, 15 Marymoncka, 05-152 Kazuń Nowy, Poland

[d] Department of Drug Chemistry, Faculty of Pharmacy. Medical University of Warsaw, 1 Banacha. 02-097 Warsaw, Poland. Electronic address: joanna.giebultowicz@wum.edu.pl

[*] Corresponding author: Elżbieta Gniazdowska, Pharmaceutical Analysis Laboratory, Łukasiewicz Research Network - Industrial Chemistry Institute, 8 Rydygiera, 01-793 Warsaw, Poland. Electronic address: elzbieta.gniazdowska@ichp.lukasiewicz.gov.pl

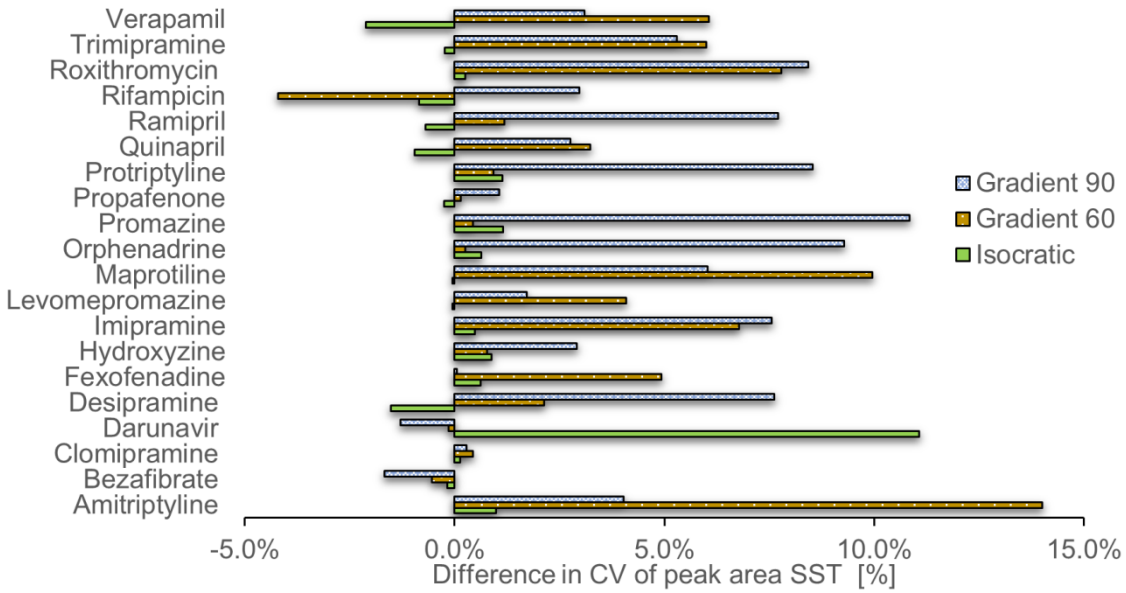


Figure S1. The difference between %RSD of peak area of SST solution injected before and after blank plasma sample was calculated for n=6 samples injected before and after normal blank plasma extracts (n = 10).

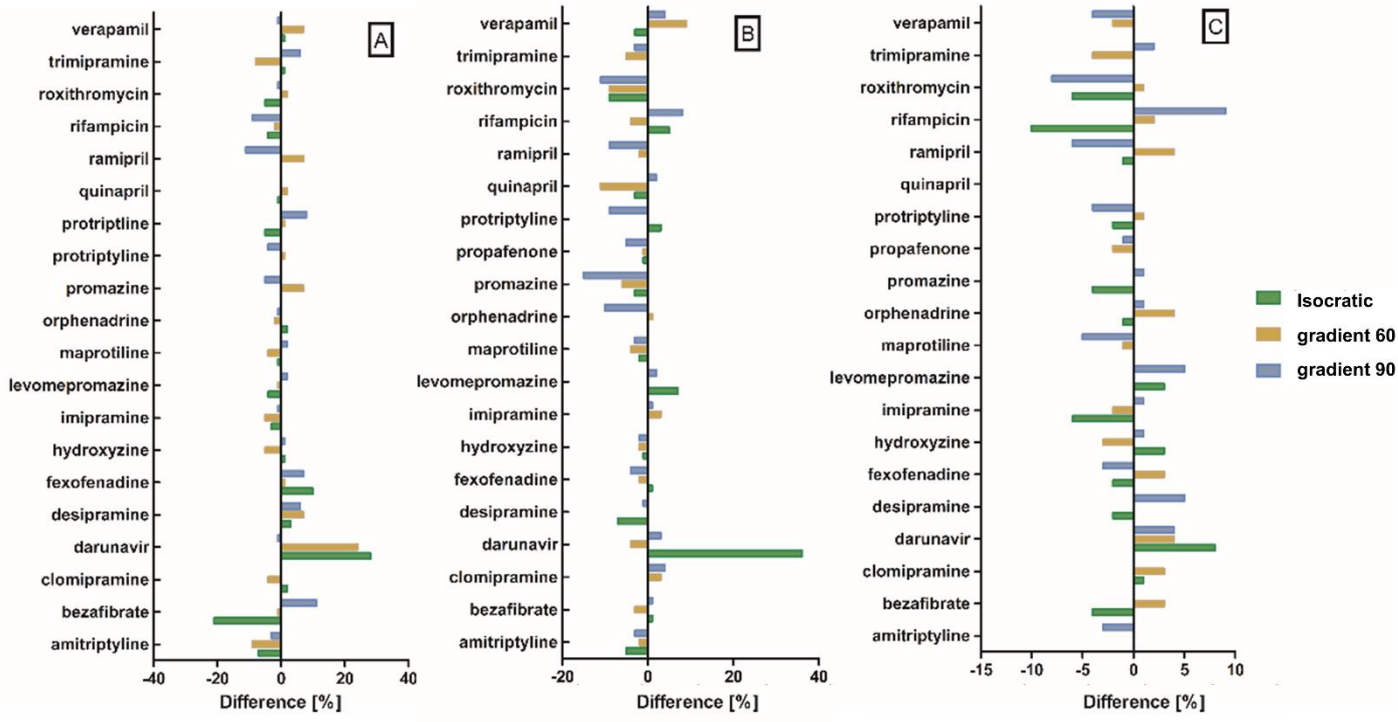


Figure S2. Difference between %RSD $_{MF}$ calculated only for normal plasma samples using block and interleaved approaches for three elution types (A) 2.5 ng/mL, (B) 10 ng/mL, (C) 100 ng/mL

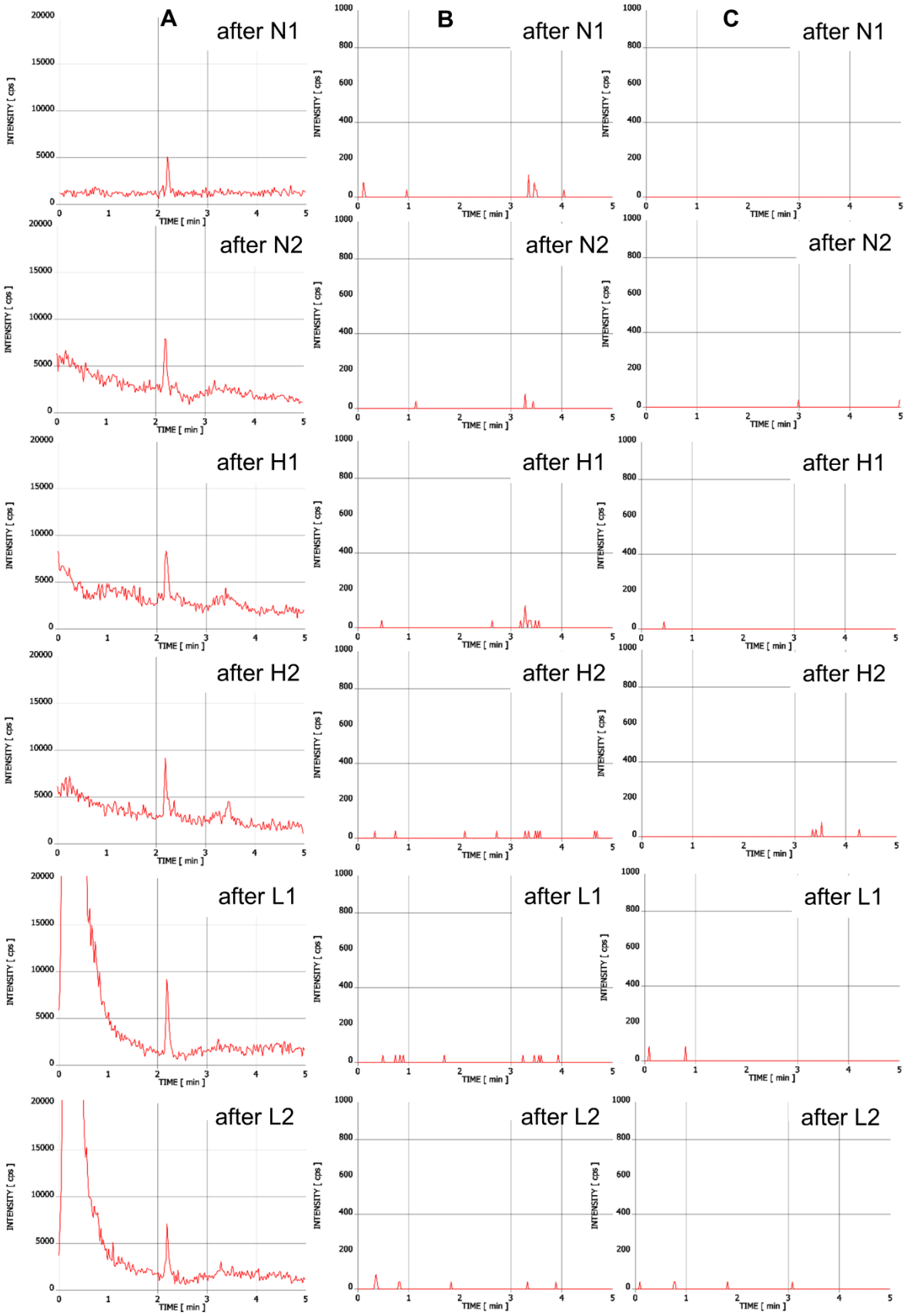


Figure S3. Extracted ion chromatograms of phospholipids detected in the neat solution injected after plasma sample using isocratic elution (**A**) the sum of 1-mono (2-lyso)glycerophosphocholines (sum-LPC), (**B**) lysophosphatidylcholine-1 (LPC-1) and (**C**) lysophosphatidylcholine-2 (LPC-2); N1, N2- normal plasma; H1, H2- hemolyzed plasma; L1, L2- lipemic plasma

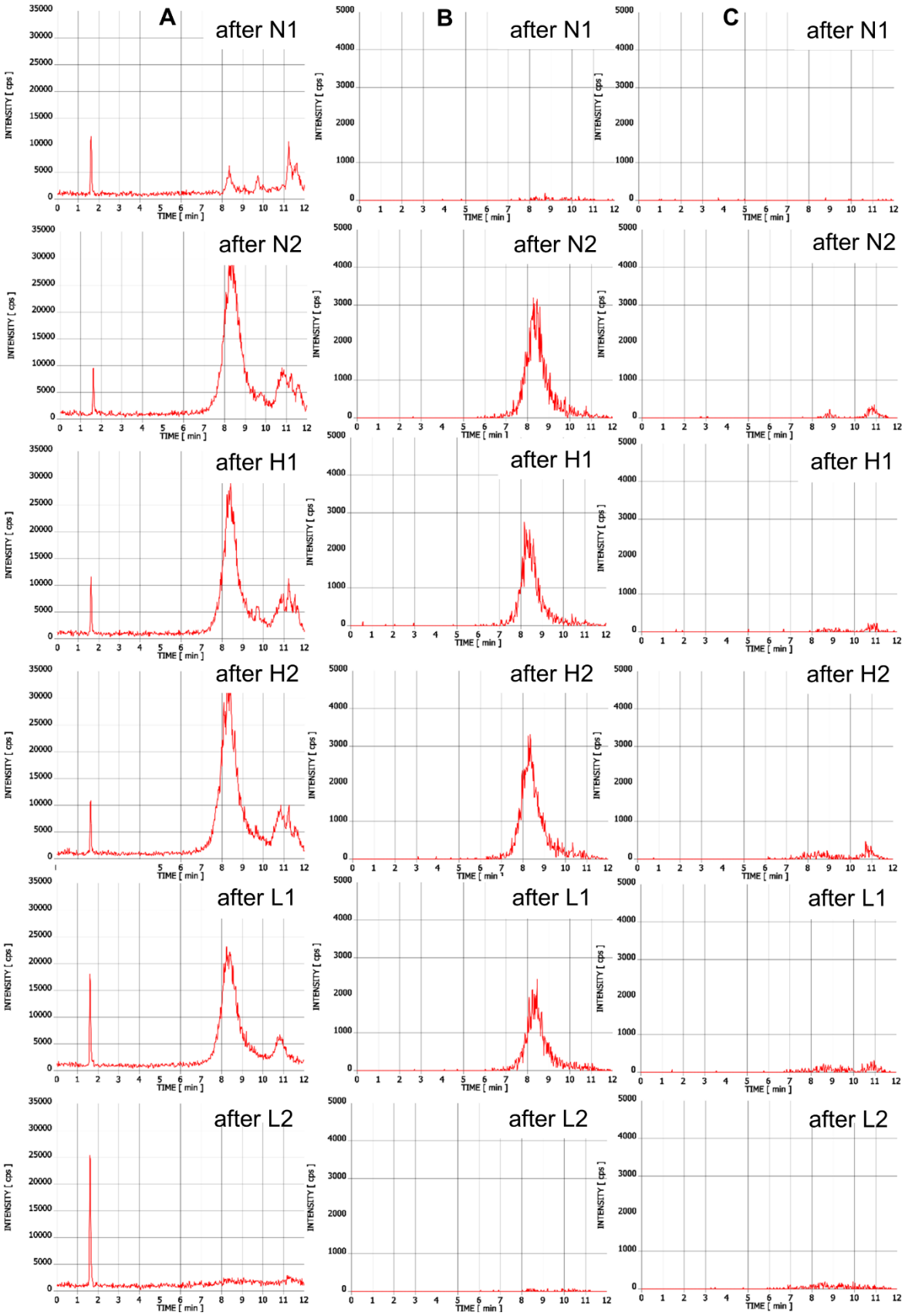


Figure S4. Gradient 60: extracted ion chromatograms of phospholipids detected in the neat solution injected after plasma sample (**B**) the sum of 1-mono (2-lyso)glycerophosphocholines (sum-LPC), (**C**) lysophosphatidylcholine-1 (LPC-1) and (**D**) lysophosphatidylcholine-2 (LPC-2); N1, N2- normal plasma; H1,H2- hemolyzed plasma; L1, L2- lipemic plasma

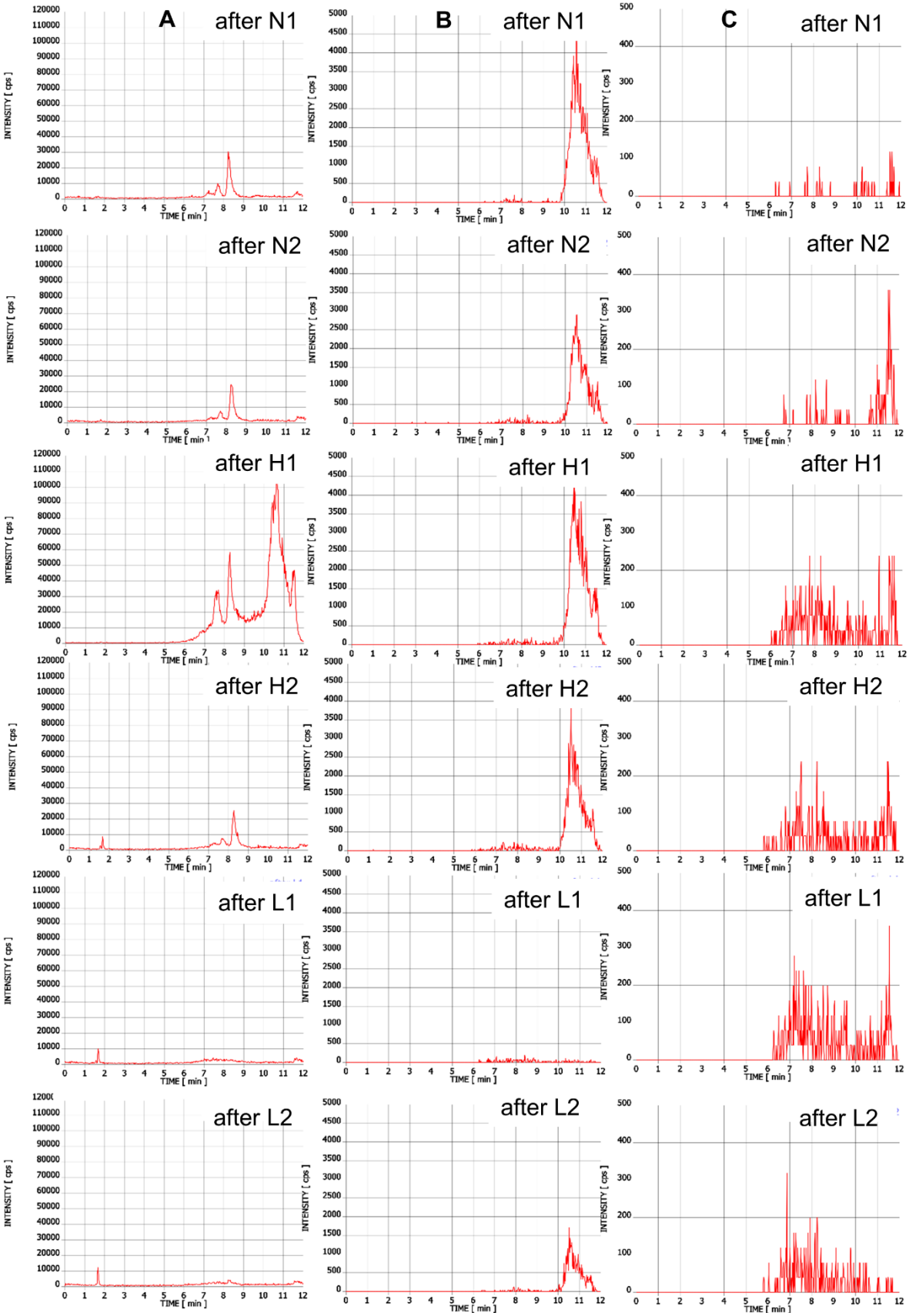


Figure S5: Gradient 90: extracted ion chromatograms of phospholipids detected in the neat solution injected after plasma sample (**A**) the sum of 1-mono(2-lyso)glycerophosphocholines (sum-LPC), (**B**) lysophosphatidylcholine-1 (LPC-1) and (**C**) lysophosphatidylcholine-2 (LPC-2); N1, N2- normal plasma; H1,H2- hemolyzed plasma; L1, L2- lipemic plasma

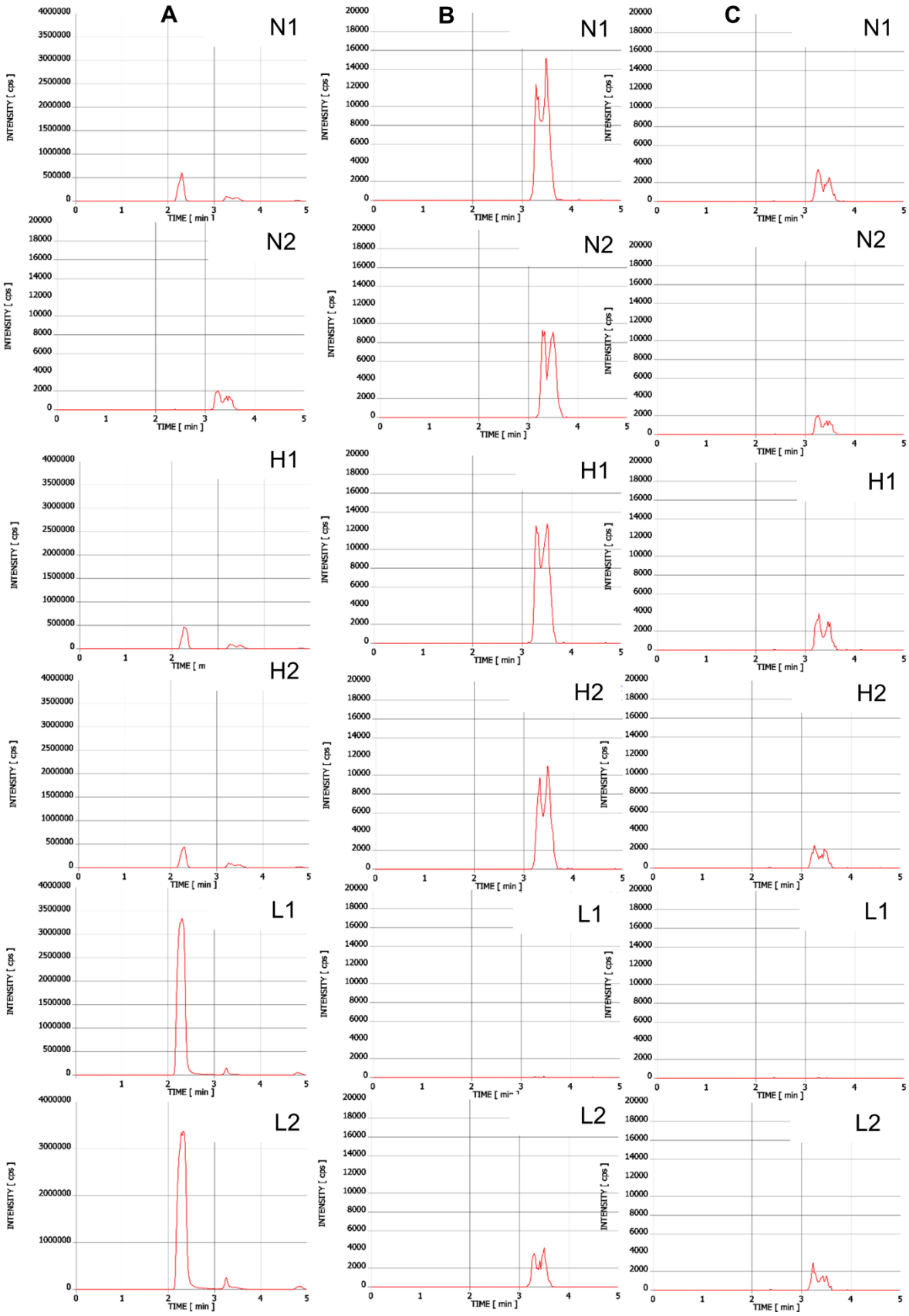


Figure S6. Extracted ion chromatograms of phospholipids detected in the plasma sample and separated using isocratic elution (**A**) the sum of 1-mono (2-lyso)glycerophosphocholines (sum-LPC), (**B**) lysophosphatidylcholine-1 (LPC-1) and (**C**) lysophosphatidylcholine-2 (LPC-2); N1, N2- normal plasma; H1,H2- hemolyzed plasma; L1, L2- lipemic plasma

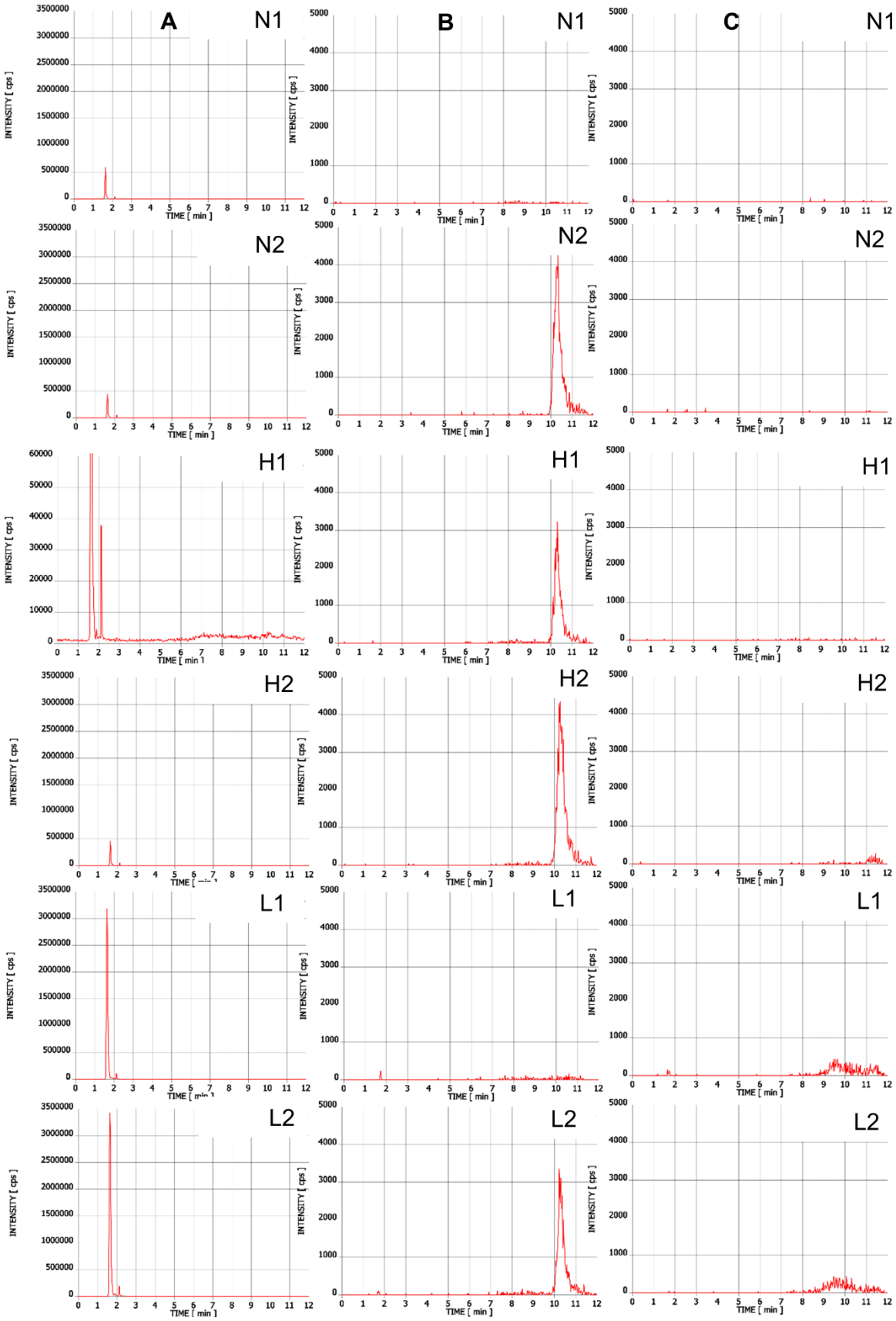


Figure S7. Extracted ion chromatograms of phospholipids detected in the plasma sample and separated using gradient 20-60% B (**A**) sum of phosphatidylcholines and sphingomyelins (sum-PC), (**B**) the sum of 1-mono (2-lyso)glycerophosphocholines (sum-LPC), (**C**) lysophosphatidylcholine-1 (LPC-1) and (**D**) lysophosphatidylcholine-2 (LPC-2); N1, N2- normal plasma; H1, H2- hemolyzed plasma; L1, L2- lipemic plasma

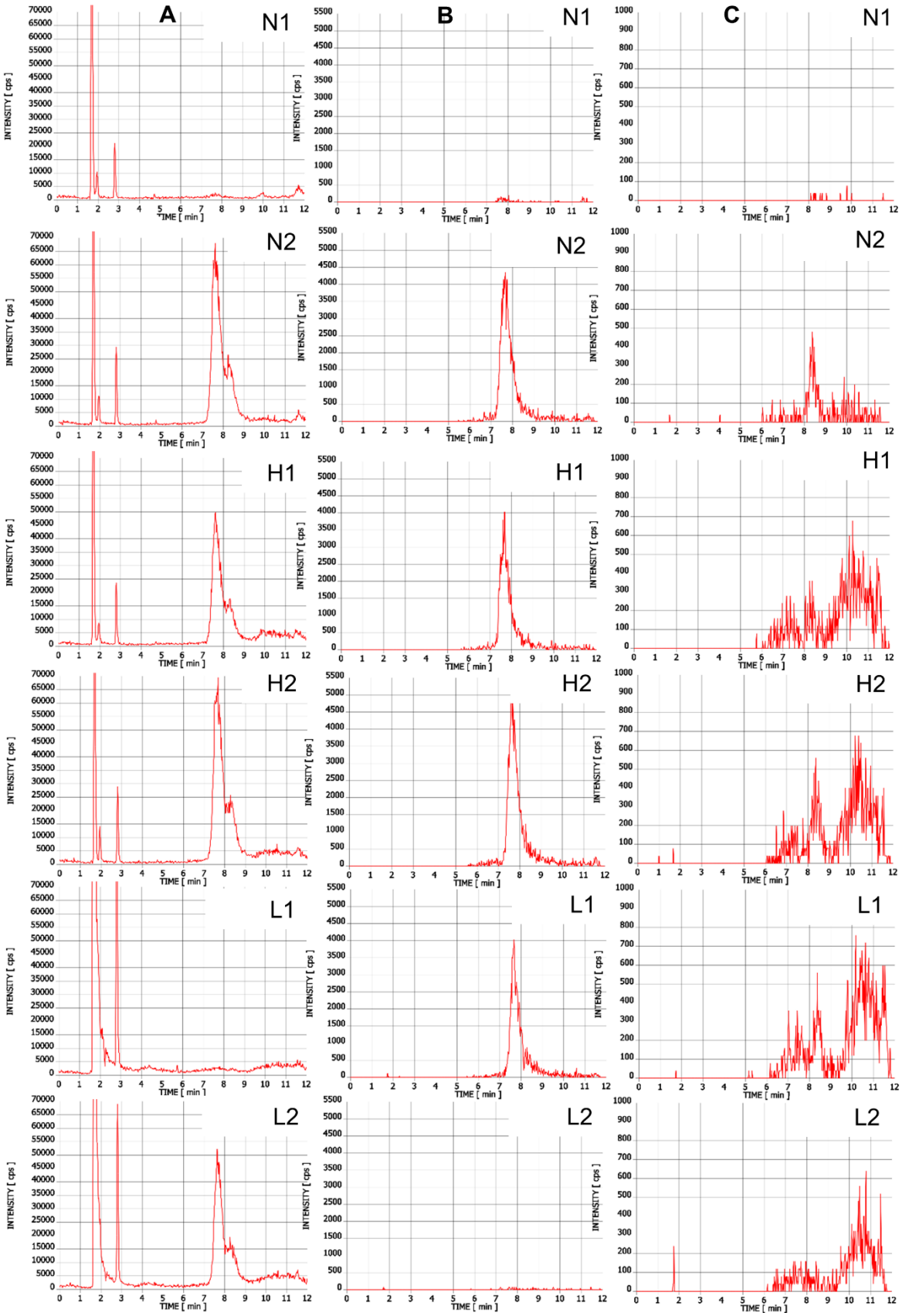


Figure S8. Extracted ion chromatograms of phospholipids detected in the plasma sample and separated gradient 10-90% B, (**A**) the sum of 1-mono (2-lyso)glycerophosphocholines (sum-LPC), (**B**) lysophosphatidylcholine-1 (LPC-1) and (**C**) lysophosphatidylcholine-2 (LPC-2); N1, N2- normal plasma; H1,H2- hemolyzed plasma; L1, L2- lipemic plasma

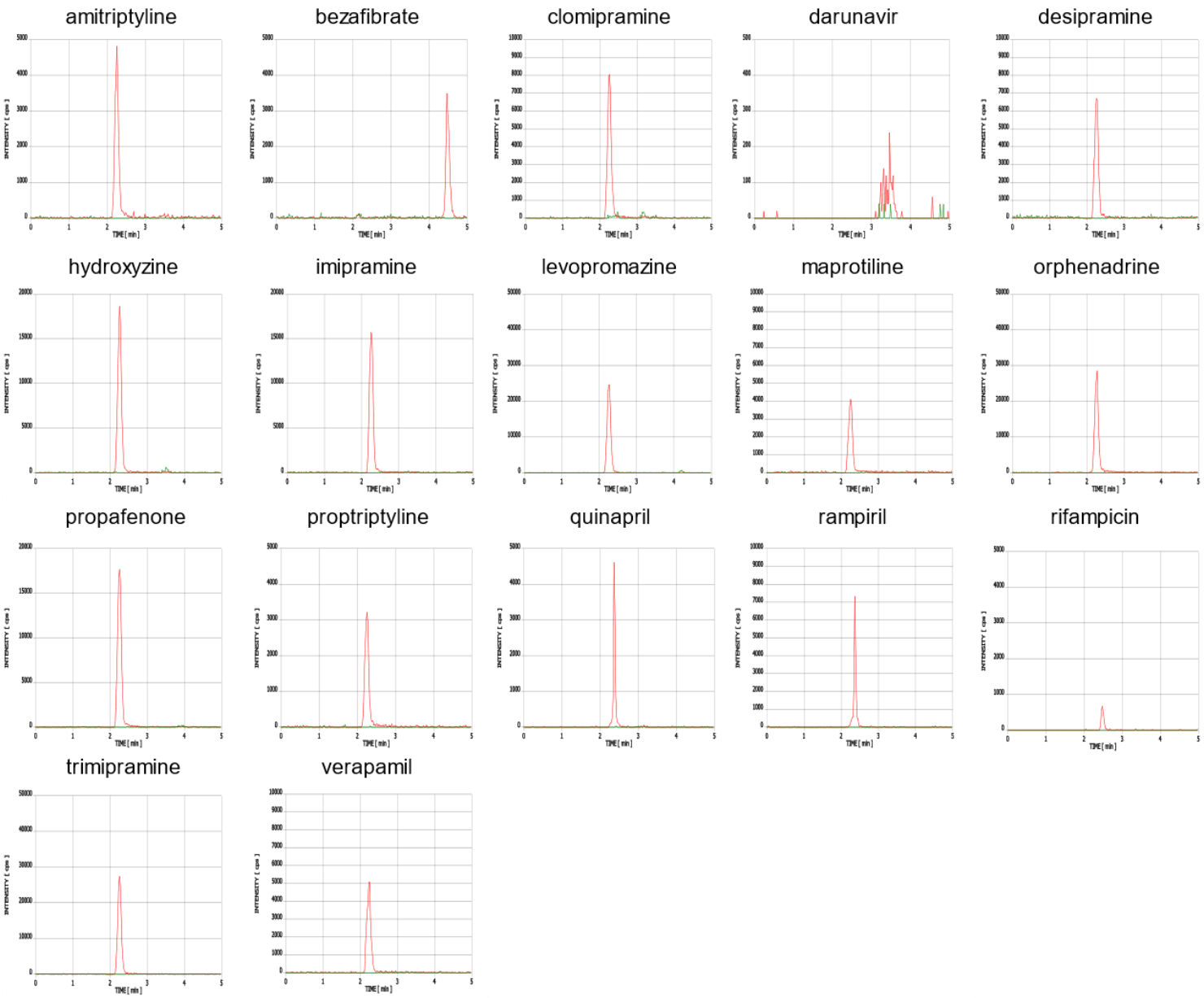


Figure S9. Extracted ion chromatograms of standards in blank plasma (green) and plasma spiked with standards (red) (low concentration - please see the materials and methods section in the main body of the manuscript) separated using isocratic elution.

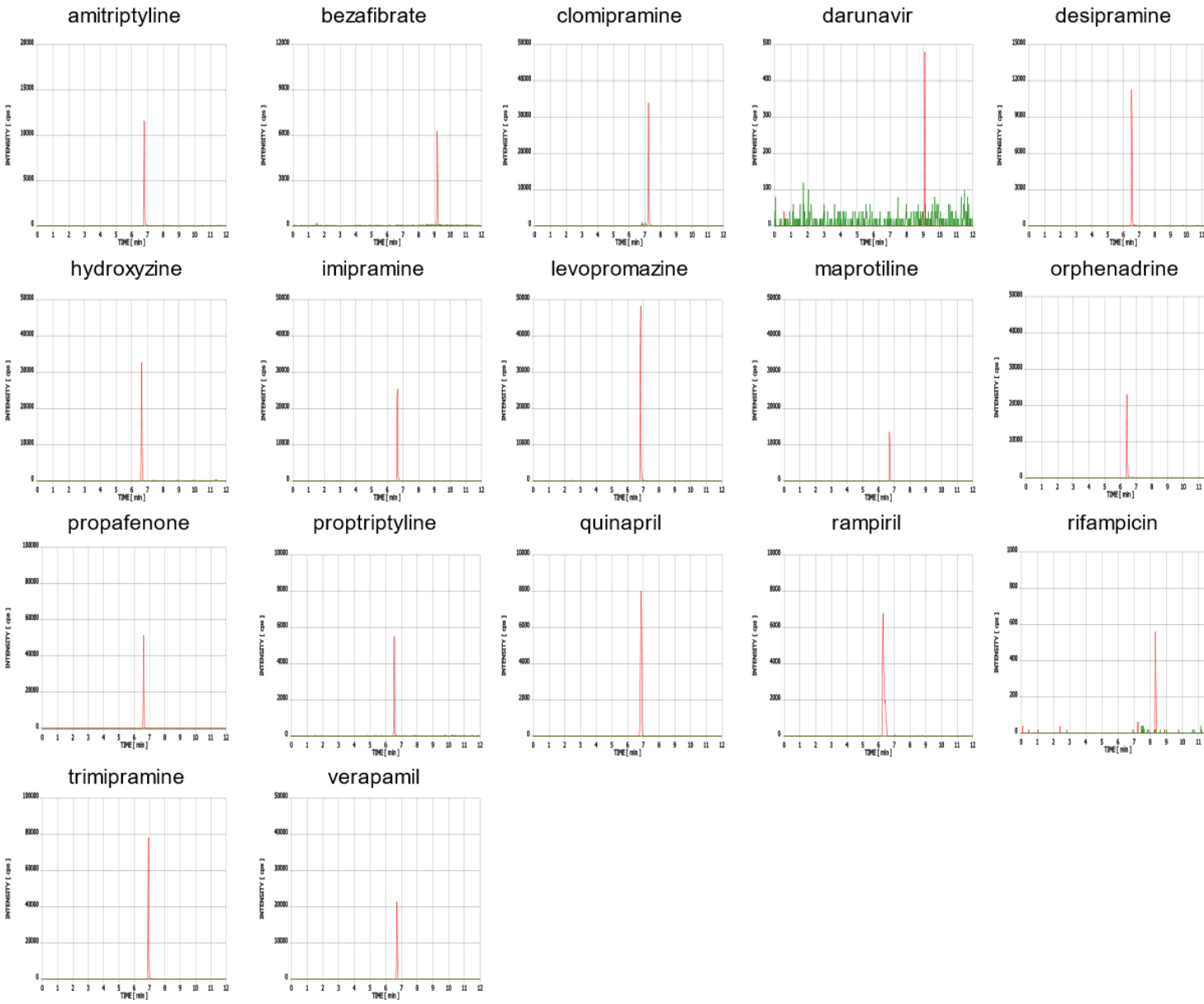


Figure S10. Extracted ion chromatograms of standards in blank plasma (green) and plasma spiked with standards (red) (low concentration - please see the materials and methods section in the main body of the manuscript) separated using gradient 20-60% B.

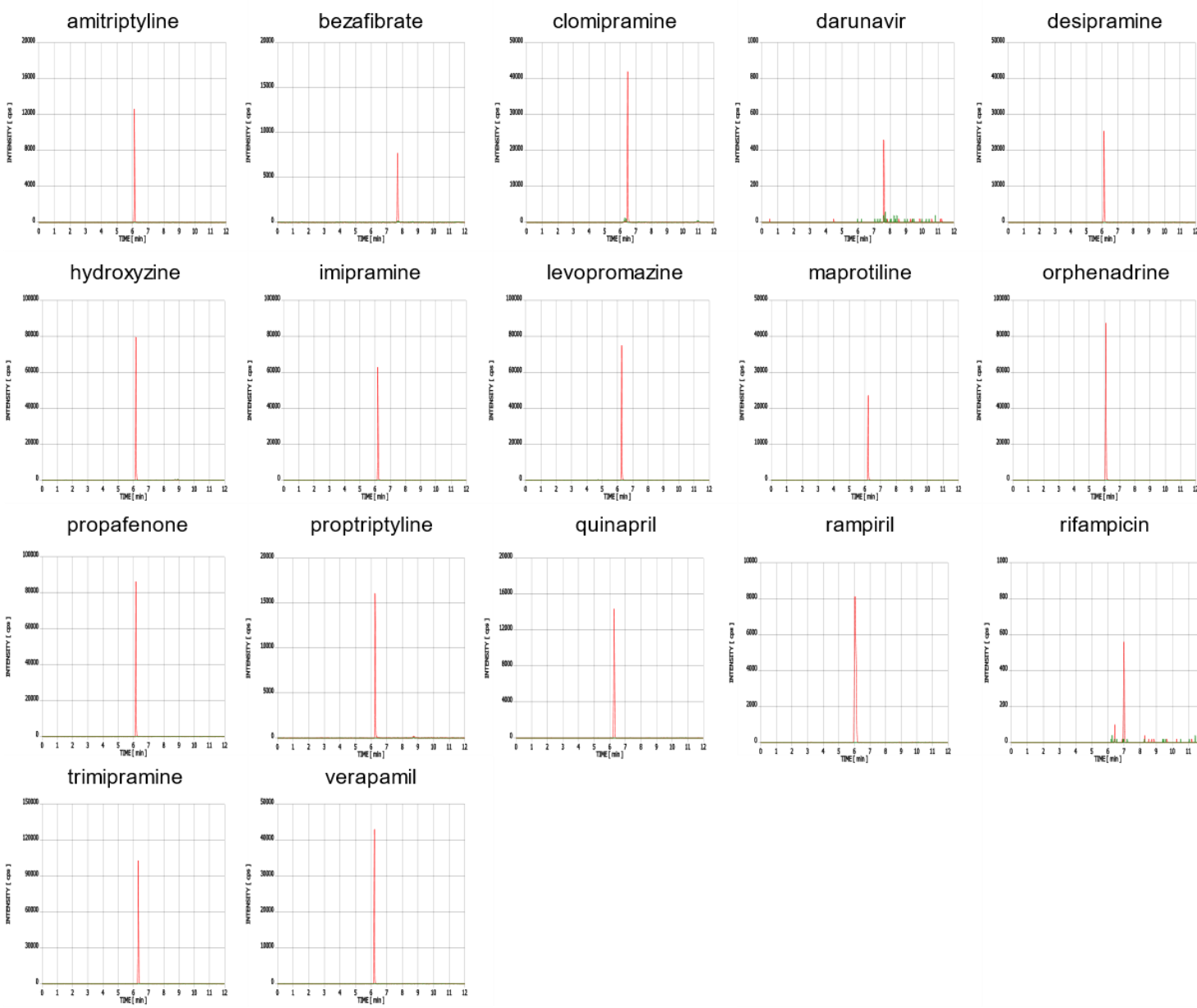


Figure S11. Extracted ion chromatograms of standards in blank plasma (green) and plasma spiked with standards (red) (low concentration - please see the materials and methods section in the main body of the manuscript) separated using gradient 10-90% B.

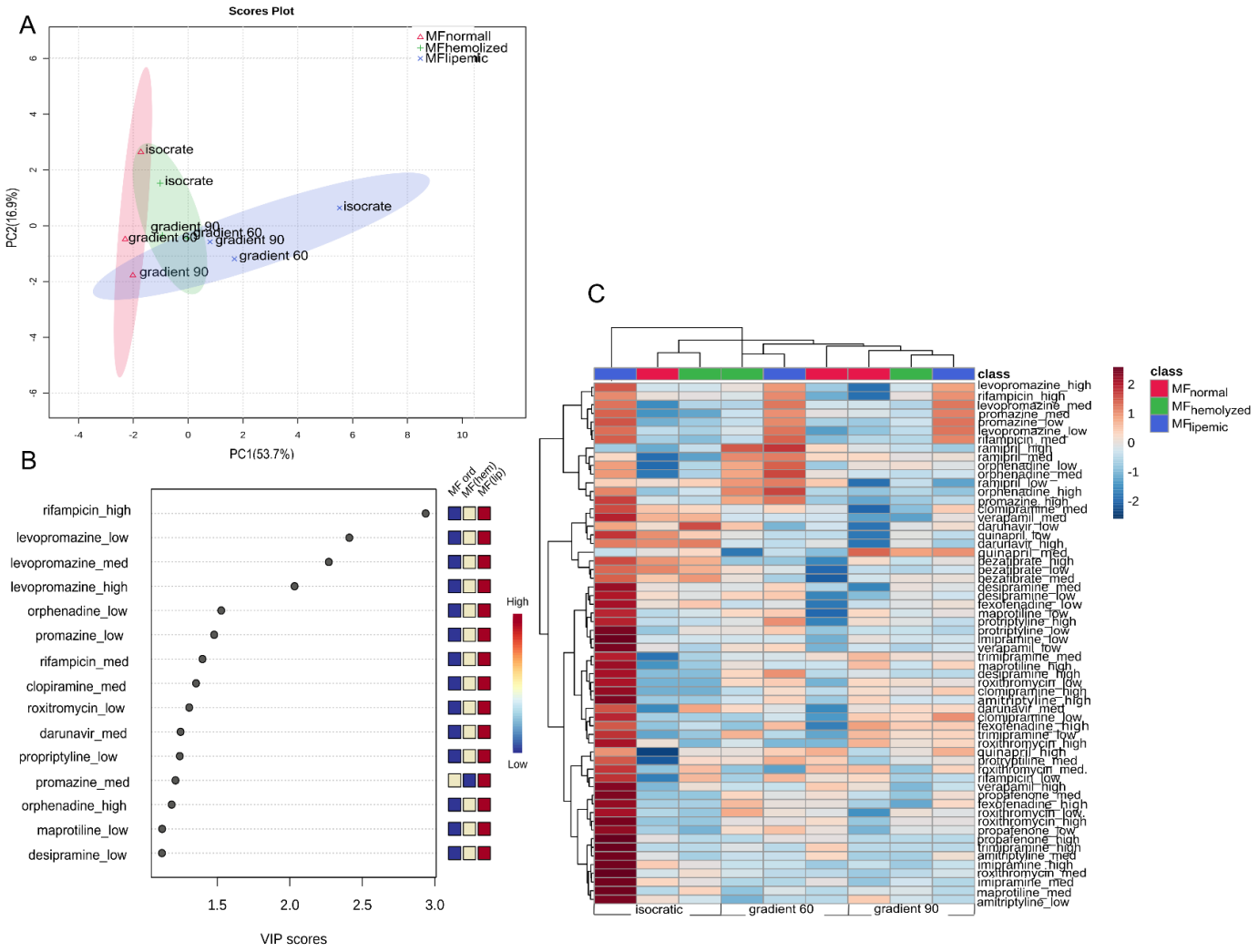


Figure S12. The chemometric analysis on the influence of chromatographic conditions, the occurrence of lipemia and hemolysis (type of plasma) on the $\%RSD_{MF}$ (A) Score plot obtained using Principal Component Analysis (PCA). (B) The variable's importance in the Partial Least-Squares Discriminant Analysis (PLS-DA) model. The coloured boxes on the right indicate the standardised value of the $\%RSD_{MF}$ in each studied group. Abbreviations after the drug name indicate concentration: low - 2.5 ng/mL, med – 10 ng/mL, high – 100 ng/mL. (C) Heatmap illustrating the data. Each coloured cell on the map corresponds to the standardised $\%RSD_{MF}$. The color key indicates the standardised $\%RSD_{MF}$; dark blue: lowest; dark red: highest. Abbreviations after the drug name indicate concentration: low - 2.5 ng/mL, med – 10 ng/mL, high – 100 ng/mL.

Table S1. Selected Reaction Monitoring (SRM) and mass spectrometric conditions

Drug	Q1 [m/z]	Q3 [m/z]	DP [V]	CE [V]	CXP [V]
Amitriptyline	278.1	233.1	91	25	14
Bezafibrate	362.2	316.1	86	21	6
Clomipramine	315.2	86.1	81	29	8
Darunavir	548.3	392.2	96	21	12
Desipramine	267.2	72.1	81	31	10
Fexofenadine	502.4	466.2	116	39	8
Hydroxyzine	375.2	201.0	66	25	18
Imipramine	281.2	86.1	76	25	12
Levomepromazine	329.	100.1	66	29	16
Maprotiline	278.1	250.1	86	27	10
Orphenadrine	270.1	181.1	46	19	6
Promazine	285.0	86.1	66	29	6
Propafenone	342.2	116.1	81	33	10
Protriptyline	264.1	191.1	96	41	10
Quinapril	439.3	234.1	86	29	14
Ramipril	417.2	234.2	86	31	6
Rifampicin	823.7	791.3	101	25	10
Roxithromycin	837.5	158.1	96	49	10
Trimipramine	295.2	100.2	71	25	14
Verapamil	455.3	165.1	111	39	14
Sum of Phosphatidylocholine (PC) and Spningomyeline (SM)	184.0	184.0	121	7	12
(2-lyso) glycerophosphocholines	104.0	104.0	105	6	12
lysophosphatidylcholine-1	496.0	184.0	100	12	12
lysophosphatidylcholine-2	524.0	184.0	100	13	12
Spningomyeline (SM) (34:1)	704.0	184.0	100	14	12
Phosphatidylocholine (16:0/18:2)	758.0	184.0	100	15	12
Phosphatidylocholine (14:0/16:0)	786.0	184.0	100	16	12
Phosphatidylocholine (38:6)	806.0	184.0	100	17	12
Phosphatidylocholine (14:0/16:0)	786.0	184.0	100	16	12

DP - declustering potential; CE - collision energy; CXP - collision cell exit potential

Table S2. The $\%RSD_{MF}$ was calculated for all plasma samples using block and interleaved approaches for three elution types and three concentrations of drugs (2.5 ng/mL, 10 ng/mL, 100 ng/mL). Data used for preparation Figure S2.

Type of elution	**Isocratic**						**gradient 60**						**gradient 90**					
	2.5 ng/mL		**10 ng/mL**		**100 ug/mL**		**2.5 ng/mL**		**10 ng/mL**		**100 ug/mL**		**2.5 ng/mL**		**10 ng/mL**		**100 ug/mL**	
Compound	**I**	**b**	**i**	**b**	**i**	**b**	**i**	**b**	**i**	**b**	**i**	**b**	**I**	**B**	**i**	**b**	**i**	**b**
Amitriptyline	31	29	30	31	24	22	6	2	5	5	4	4	7	5	6	7	2	1
Bezafibrate	37	20	14	8	15	7	12	6	5	1	2	3	7	12	5	3	3	3
Clomipramine	21	31	32	32	28	25	3	5	5	4	3	3	8	10	6	4	4	3
Darunavir	35	26	28	29	35	25	16	20	8	5	3	5	18	18	9	6	5	6
Desipramine	39	36	33	37	25	21	11	11	10	10	9	9	5	5	8	4	2	5
Fexofenadine	39	28	22	26	12	10	4	4	6	5	6	2	4	9	5	3	5	3
Hydroxyzine	47	42	42	42	31	30	7	5	5	4	4	4	4	7	5	4	4	4
Imipramine	34	34	34	36	27	26	3	6	4	9	4	7	4	5	5	5	4	3
Levomepromazine	52	52	50	51	19	18	48	47	47	47	10	5	47	47	47	47	8	8
Maprotiline	15	31	25	30	17	14	6	3	6	4	5	4	4	4	6	8	5	3
Orphenadrine	25	25	24	24	20	19	54	44	43	44	39	37	9	3	7	6	2	4
Promazine	52	49	51	49	27	25	36	34	37	36	19	16	42	41	42	41	5	6
Propafenone	43	38	35	38	26	24	8	5	5	5	2	2	6	2	4	6	2	2
Protriptyline	38	37	33	36	21	22	12	10	12	11	9	9	7	8	7	6	3	3
Quinapril	15	11	4,2	7	7	7	4	4	3	2	4	5	4	5	7	3	5	5
Ramipril	12	9	9	8	6	7	27	22	25	25	21	21	4	6	7	4	4	4
Rifampicin	50	43	48	46	67	65	31	36	47	49	67	67	24	45	35	44	59	63
Roxithromycin	28	27	23	26	19	17	15	7	7	5	5	4	11	10	10	6	6	3
Trimipramine	31	29	31	30	24	23	11	6	7	9	4	3	7	8	8	7	3	4
Verapamil	39	33	28	31	16	15	7	9	3	6	3	3	3	6	4	4	3	4

i- interleaved plasma sample with solution, b – block – plasma samples and solutions

Table S3. Molecular descriptors.

Compound	Dipole moment	PSA	Polar volume	Total area	Total volume	clogP	Molar mass	Amount of acceptor HB	Amount of donors HB
Amitriptyline	2.2908	0.733	100.469	530.286	880.562	4.8510	277.403	1	0
Bezafibrate	3.552	129.67	127.3	581.000	981.06	3.6980	361.81	5	2
Clomipramine	1.7898	2.907	60.735	547.228	928.415	5.9208	314.852	2	0
Darunavir	1.638	167.94	431.04	818.39	1516.69	2.8870	547.66	5	10
Desipramine	1.9038	20.366	130.271	523.616	859.462	4.4680	266.381	2	1
Fexofenadine	4.873	86.99	205.42	751.23	1415.15	1.9570	501.66	5	3
Hydroxyzine	1.947	50.92	118.53	674.41	1136.54	3.9950	374.9	1	4
Imipramine	1.9652	0.627	129.28	536.551	907.152	5.0370	280.407	2	0
Levomepromazine	1.813	73.17	219.34	530.750	903.41	4.1310	314.44	3	1
Maprotiline	4.1271	24.95	119.866	531.122	864.948	4.5240	277.403	1	1
Orphenadrine	2.7183	5.098	146.13	536.933	906.234	3.9012	269.381	2	0
Promazine	0.711	11.91	190.51	493.030	828.83	4.3990	284.42	2	0
Propafenone	4.9865	77.756	164.858	615.987	1050.28	3.6363	341.444	4	2
Protriptyline	1.4995	25.487	118.413	528.457	868.947	4.8650	263.377	1	1
Quinapril	1.8308	137.777	252.292	733.547	1270.38	1.7359	438.516	7	2
Ramipril	7.5804	92.184	277.769	673.719	1197.38	1.5399	416.511	7	2
Rifampicin	5.094	213.28	356.8	1011.23	2033.88	3.7100	822.94	6	16
Roxithromycin	9.342	135.3	506.94	1027.62	2140.54	2.2920	837.05	5	17
Trimipramine	0.9726	0.095	133.364	539.804	931.055	5.4360	294.434	2	0
Verapamil	5.2859	86.399	223.49	776.052	1383.63	4.4660	454.602	6	0

Publikacja 5 (przeglądowa)

Gniazdowska E., Gilant E., Buś-Kwaśnik K. Wytyczna ICH M10 – Jednolite podejście do bioanalizy na całym świecie. Prospects in Pharmaceutical Sciences 2023; 21(3):57-63. IF = 0,100; MEiN: 20

PROSPECTS
IN PHARMACEUTICAL SCIENCES

Prospects in Pharmaceutical Sciences, 21 (3), 57-63
https://prospects.wum.edu.pl/

Review Article

ICH M10 GUIDELINE - A HARMONIZED GLOBAL APPROACH TO BIOANALYSIS

WYTYCZNA ICH M10 – JEDNOLITE PODEJŚCIE DO BIOANALIZY NA CAŁYM ŚWIECIE

Elżbieta Gniazdowska*[1,2], Edyta Gilant[1], Katarzyna Buś-Kwaśnik[1]

[1] *Łukasiewicz Research Network - Industrial Chemistry Institute, Pharmaceutical Analysis Laboratory, 8 Rydygiera, 01-793 Warsaw, Poland*

[2] *Department of Drug Chemistry, Doctoral School, Medical University of Warsaw, 61 Żwirki i Wigury, 02-091 Warsaw, Poland.*

* Correspondence, e-mail: elzbieta.gniazdowska@ichp.lukasiewicz.gov.pl

Received: 19.06.2023 / Accepted: 28.08.2023 / Published: 22.09.2023

ABSTRACT

Bioanalytical methods are used in research on small-molecule and large-molecule drug products to determine analytes and their metabolites in biological matrices such as blood, plasma, serum, urine, feces, saliva, other biological fluids, or tissues. Validation of a bioanalytical method is the essential step before the implementation of the method into routine use in toxicokinetic or pharmacokinetic studies. Harmonization of recommendations for the validation of bioanalytical methods has been advocated for many years. In 2022, The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) finished the work on final version of the ICH M10 guideline, as a combination of four regional guidelines (American, European, Brazilian and Japanise). The document uniforms rules for the performance of the bioanalytical method validation and documentation of sample analysis from clinical and non-clinical studies in most countries around the world, which are submitted to registration authorities.

KEYWORDS: bioanalytical method validation, reliability, harmonization, bioavailability, pharmacokinetics.

STRESZCZENIE

Metody bioanalityczne wykorzystywane są w badaniach produktów leczniczych do oznaczania stężeń analitów i ich metabolitów w matrycach biologicznych (krew, osocze, surowica, mocz, kał, ślina, inne płyny biologiczne lub tkanki). Metody przed zastosowaniem do rutynowych analiz w badaniach farmakokinetycznych i toksykokinetycznych powinny być zwalidowane. Od wielu lat dążono do ogólnoświatowego ujednolicenia zaleceń dotyczących zakresu i sposobu przeprowadzania walidacji metod bioanalitycznych. W 2022 roku Międzynarodowa Rada Harmonizacji Wymagań Technicznych dla Rejestracji Produktów Leczniczych Stosowanych u Ludzi (*ang. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use – ICH*) ukończyła prace nad ostateczną wersją wytycznej ICH M10 będącą połączeniem 4 regionalnych wytycznych (amerykańskiej, europejskiej, brazylijskiej i japońskiej). Dokument scala zasady przeprowadzania walidacji metod bioanalitycznych i dokumentowania wyników z badań klinicznych i nieklinicznych prowadzonych w większości krajów na całym świecie na potrzeby rejestracji produktów leczniczych.

SŁOWA KLUCZOWE: walidacja metod bioanalitycznych, wiarygodność, harmonizacja, biodostępność, farmakokinetyka.

1. Zastosowanie metod bioanalitycznych i znaczenie harmonizacji

Metody bioanalityczne są stosowane w badaniach rozwojowych produktów leczniczych zawierających substancje drobnocząsteczkowe i wielkocząsteczkowe, do oznaczania substancji czynnych i/lub metabolitów w materiale biologicznym zwierzęcym lub ludzkim. Opracowywane są w matrycach biologicznych takich jak krew, osocze, surowica, mocz, kał, ślina, inne płyny biologiczne lub tkanki [1]. Oznaczenia analitu w materiale biologicznym należy przeprowadzić z zastosowaniem zwalidowanej metody bioanalitycznej. Spełnienie kryteriów akceptacji dla

wszystkich testów walidacyjnych potwierdza, że metoda jest adekwatna do zamierzonego celu, a otrzymane wyniki analizy próbek badanych są wiarygodne i powtarzalne [1-3]. Z procesu walidacji metody sporządzany jest raport przygotowany zgodnie z formatem Wspólnego Dokumentu Technicznego *(ang. Common Technical Document – CTD)* dla badań prowadzonych na materiale biologicznym ludzkim (CTD 5.3.1.2) i zwierzęcym (CTD 4.2.2.1)[4], które następnie przez Sponsora badania przedkładane są organom regulacyjnym w procesie dopuszczenia leku do obrotu [1].

Walidacja metod bioanalitycznych była do 23.01.2023 prowadzona zgodnie z wytyczną obowiązującą na danym obszarze i wydaną przez następujące instytucje:

- w USA przez amerykańską Agencję ds. Leków i Żywności (*ang. Food and Drug Administration, US FDA*) z 2018 r. [3],
- w Europie przez Europejską Agencję Leków (*ang. European Medicines Agency, EMA*) z 2011 r. [2],
- w Brazylii przez Narodową Agencję Nadzoru Zdrowia (*ang. The National Health Surveillance Agency, ANVISA*) [5],
- w Japonii przez Ministerstwo Zdrowia i Opieki Społecznej (*ang. Ministry of Health, Labour and Welfare of Japan, MHLW*) z 2013 r. dla małych cząsteczek [6] oraz z 2014 r. dla związków wielkocząsteczkowych [7],
- w Kanadzie przez EMA [2] oraz uzupełnioną przez *Health Canada*.

Od momentu implementacji wytycznej ICH M10 [1] będącej połączeniem [8] wytycznych FDA, EMA i MHLW [2,3,6,7] w większości krajów na świecie obowiązują jednolite zasady przeprowadzania walidacji metod bioanalitycznych i dokumentowania analizy próbek z wszystkich faz badań klinicznych, w tym z porównawczych badań dostępności biologicznej/ równoważności biologicznej (*ang. bioavailability/bioequivalence study – BA/BE study*) oraz nieklinicznych badań z zakresu toksykokinetyki lub farmakokinetyki [1].

Ujednolicenie rekomendacji dotyczących walidacji metod bioanalitycznych było postulowane od wielu lat [9]. Harmonizacja umożliwia stosowanie wspólnych zaleceń przez większość organów dopuszczających produkty lecznicze do obrotu, co niesie korzyści dla firm oraz pacjentów:

- poprawę efektywności procesu oceny dokumentacji rejestracyjnej,
- skrócenie czasu wprowadzenia produktu leczniczego do obrotu,
- zmniejszenie kosztów dla pacjenta wynikających z niepotrzebnego powielania badań klinicznych [10].

Harmonizacja wytycznej wpisuje się także w zasadę 3R (*ang. Reduce, Refine, Replace*) [11] prowadzącą do zmniejszenia liczby niepotrzebnych badań na zwierzętach bez uszczerbku dla badania oceny bezpieczeństwa i skuteczności leczenia.

Wytyczne organów rejestracyjnych, tworzone przez ekspertów z komitetów naukowych i grup roboczych, zapewniają spójne zasady rozwoju produktów leczniczych zgodnie z najwyższymi standardami jakości i spójności danych bioanalitycznych. Wytyczne są zbiorami zaleceń służących zapewnieniu dostępności wysokiej jakości, skutecznych i bezpiecznych produktów leczniczych z korzyścią dla pacjentów. Prace nad zarysem i koncepcją wytycznej ICH M10 rozpoczęto w 2016 roku [8]. Następnie w okresie 14.03-01.09.2019 projekt wytycznej poddano konsultacjom społecznym przeprowadzonym przez 10 organów rejestracyjnych (ICH, EMA, FDA, MHLW/PMDA, Health Canada, Swissmedic, ANVISA, MFDS, NMPA, TFDA) [12]. Wdrożenie ICH M10 w poszczególnych rejonach rejestracyjnych na dzień opracowania niniejszej pracy przedstawiono w Tabeli 1.

Tabela 1. Wdrożenie wytycznej ICH M10 przez niektórych członków ICH [13].

Nazwa członka ICH	Data implementacji/ planowanej implementacji*
Health Canada (Kanada)	20 stycznia 2023
EC – Komisja Europejska / EMA (Europa)	21 stycznia 2023
Swissmedic (Szwajacaria)	25 maja 2022
US FDA (Stany Zjednoczone)	7 listopada 2022
MFDS (Republika Korei)	1 października 2023*
NMPA (Chiny)	29 czerwca 2023

2. ICH M10 – zmiany w prowadzeniu testów walidacyjnych w bioanalizie

Wytyczna ICH M10 [1] wprowadza modyfikacje w testach prowadzonych podczas walidacji metod bioanalitycznych w porównaniu z wytyczną EMA [2] oraz FDA [3]. W Tabeli 2 opisano najważniejsze zmiany w ocenie parametrów walidacyjnych dla leków chemicznych i biologicznych analizowanych metodami chromatograficznymi z pominięciem wymagań dla techniki wiązania ligandów ze względu na specyfikę badań prowadzonych przez autorów pracy. Laboratoria pracujące dotychczas zgodnie z wytyczną FDA [3] powinny zaktualizować lub zmodyfikować Standardowe Procedury Operacyjne w opisie testów: specyficzność, selektywność, wpływ matrycy, powtarzalność ponownego dozowania oraz stabilność. Dla laboratoriów prowowadzących walidację metod bioanalitycznych zgodnie z wytyczną EMA [2] zmianie ulegnie sposób postępowania podczas oceny wpływu matrycy, specyficzności, selektywności, stabilności oraz dodatkowo wprowadzony zostanie opis dotyczący oceny odzysku i powtarzalności dozowania.

2.1. Selektywność, specyficzność

Zalecenia dotyczące oceny selektywności metody bioanalitycznej w obecności matrycy i substancji interferujących, wstecznej konwersji i wpływu matrycy zawarte były w jednym punkcie *Selektywność* w przypadku wytycznej EMA [2] lub *Selektywność i Specyficzność* w przypadku wytycznej FDA [3]. Test selektywności i specyficzności w wytycznej ICH M10 [1] zostały rozdzielone na osobne punkty.

Test selektywności zgodnie z nową wytyczną dotyczy tylko używanej matrycy biologicznej, a mianowicie obecnych w niej substancji zakłócających. Metoda analityczna powinna wykazywać selektywność w danej matrycy wobec oznaczanych analitów i wzorców wewnętrznych [1]. Wytyczna ICH M10 [1] zaleca wykonanie testu na minimum 6 różnych źródłach osocza niehemolizowanego, nielipemicznego oraz dodatkowo na minimum 1 źródle osocza hemolizowanego i lipemicznego. Nowością w wytycznej jest wprowadzenie definicji wyżej wspomnianych osoczy oraz sposobu ich przygotowania. Problem braku jednoznacznej definicji obu rodzajów osocza był poruszany już w 2014 roku przez Europejskie Forum Bioanalityczne (*ang. European Bioanalysis Forum*) [14].

Tabela 2. Parametry walidacyjne dla metod bioanalitycznych oznaczania leków techniką chromatograficzną - najważniejsze zmiany pomiędzy ICH M10 i wytycznymi EMA i FDA

Parametr (Test) walidacyjny w ICH M10 [1]	**Skrócone brzmienie oceny parametru w ICH M10 [1]**	**Różnice w wytycznej FDA [3] wobec ICH M10 [1]**	**Różnice w wytycznej EMA [2] wobec ICH M10 [1]**
Selektywność (s. 8)	- minimum 6 źródeł matrycy dla osocza: dodatkowo minimum 1 źródło hemolizowane i minimum 1 źródło lipemicznego osocza	- w przypadku osocza, osocze hemolizowane i lipemiczne, nie jest wliczone w liczbę źródeł matrycy minimum 6 (s. 7)	- w przypadku osocza, osocze hemolizowane i lipemiczne, nie jest wliczone w liczbę źródeł matrycy minimum 6 (s. 5)
Specyficzność (s. 8)	- analiza próbek osocza z dodatkiem związków interferujących (lek, metabolit itp.) - ocena konwersji wstecznej metabolitu	- opis testu z kryteriami akceptacji opisano w punkcie *Selektywność i Specyficzność* (s. 7, Table 1, s. 22) - brak informacji o sposobie oceny konwersji wstecznej metabolitu dla metod LC-MS	- test opisano w ramach testu *Selektywność* (s. 5)
Wpływ matrycy (s. 9)	- próbki QC na niskim i wysokim poziomie stężenia w minimum 6 różnych źródłach matryc, po minimum 3 powtórzenia dla każdego źródła - kryteria akceptacji jak w teście precyzja i dokładność	- informacja o teście zamieszczona w punkcie *Selektywność i Specyficzność* (s. 7), opis nie zawierał sposobu przeprowadzenia testu	- inny sposób przeprowadzenia testu, obliczano czynnik matrycowy
Krzywa kalibracyjna (s. 9-10) **Zakres kalibracyjny (LLOQ-ULOQ) (s. 15)**	- krzywa kalibracyjna z minimum 6 próbek kalibracyjnych - minimum 3 krzywe kalibracyjne pochodzące z niezależnych sekwencji analitycznych analizowanych na przestrzeni kilku dni	- brak informacji o minimalnej liczbie krzywych kalibracyjnych (s. 6)	x (s. 6-7, 12)
Precyzja i dokładność (s. 10-11)	- określone liczbowo zakresy stężeń próbek QC (niskie, średnie i wysokie) - precyzja i dokładność określona dla LLOQ i wszystkich poziomów stężeń próbek QC w minimum 5 powtórzeniach: a) w ramach jednej sekwencji analitycznej b) pomiędzy minimum 3 sekwencjami analitycznymi (w czasie nie krótszym niż 2 dni)	- brak określenia zakresu liczbowego dla próbek QC na poziomie stężenia średniego i wysokiego	x (s. 7-8)
Przeniesienie próbki (s. 11)	- analiza próbek BS po próbkach ULLOQ	- informacja o teście umieszczona w punkcie *Selektywność i Specyficzność* (s. 7), brak szczegółowego opisu wykonania testu	x (s. 6)
Test integralności rozcieńczenia (s.11)	- analiza rozcieńczonej próbki QC (>ULOQ) w 5 powtórzeniach	- brak informacji o minimalnej liczbie powtórzeń dla rozcieńczonej próbki QC (>ULOQ)	x (s. 8)
Powtarzalność ponownego dozowania (s.13)	- 5 dozowań z jednego naczynka każdego stężenia próbki QC	- brak testu	- brak testu (wspomniano o możliwości wykonania testu w punkcie Powtórna analiza próbek (s. 13))
Odzysk (s.30)	- wydajność ekstrakcji - porównanie wyniku przeprocesowanych próbek QC (niski, średni i wysoki) z odpowiednimi ekstraktami ślepych prób, do których po ekstrakcji dodano analit	x (s. 8-10, 25, Table 1)	- brak testu
Stabilność (s. 11-13)	Stabilność w matrycy z próbek QC po 1 na poziomie stężenia niskiego i wysokiego podzielona na minimum 3 części do badania: - stabilność zamrażania i rozmrażania odstęp między zamrażaniem i rozmrażaniem minimum 12 godzin - wykonać minimum 3 cykle rozmrażania - stabilność krótkoterminowa - stabilność długoterminowa - stabilność analitu w przetworzonej/ przeprocesowanej próbce	- brak informacji o minimalnej liczbie cykli zamrażania - rozmrażania	– brak informacji o minimalnej liczbie próbek do badania
	Stabilność w roztworze dla analitów i wzorców wewnętrznych w roztworach podstawowych i roboczych	- brak oceny stabilności roztworów roboczych, wymagana analiza tylko roztworu podstawowego	
	Stabilność analitu w krwi pełnej	- brak testu stabilności analitu we krwi pełnej	- brak testu stabilności analitu we krwi pełnej

x - brak różnic; QC – próbka kontrolna (*ang. Quality Control*); s. – numer strony dokumentu wytycznej podanej w kolumnie

W celu otrzymania hemolizowanej matrycy (osocze, surowica), wytyczna ICH M10 dokładnie opisuje w jaki sposób należy wzbogacić matrycę krwią pełną (minimum 2% *v/v*). Definicja ta jest zgodna z podejściem *Global Bioanalysis Consortium* [14]. W przypadku osocza z oznakami lipemii nowa wytyczna zaleca, aby matryca pochodziła od osób lub zwierząt z hiperlipemią lub została przygotowana poprzez wzbogacenie osocza trójglicerydami, np. Interlipid [14].

Test specyficzności zgodnie z ICH M10 [1], w odróżnieniu od selektywności, dotyczy zdolności metody do jednoznacznego określenia analitu w obecności substancji interferujących nie pochodzących z matrycy (np. metabolit, leki stosowane równocześnie w leczeniu, zanieczyszczenie itp.) [1]. Nowością w porównaniu do wytycznej FDA [3] jest test oceny konwersji wstecznej metabolitu, który wpisano w ramach testu selektywności [1]. Zjawisko wystąpienia konwersji wstecznej metabolitu, czyli przekształcania metabolitu do związku macierzystego, oceniane jest w przypadku wskazań danych literaturowych lub ze względu na obecność w budowie metabolitu substancji oznaczanej ugrupowań niestabilnych [1,15].

Wytyczna ICH M10 [1] oraz pytania i odpowiedzi do ICH M10 [16] opisują możliwość oceny specyficzności bez konieczności prowadzenia dodatkowych badań dla substancji potencjalnie interferujących z analitem na podstawie:

- wykluczenia takich samych mas cząsteczkowych,
- wykluczenia takich samych właściwości fizykochemicznych analitu i substancji pokrewnych,
- uzyskania rozdzielenia chromatograficznego [16].

Powyższa strategia była postulowana przez środowisko naukowe *European Bioanalysis Forum* już w 2016 roku [17]. W innych przypadkach może istnieć konieczność wykonania dodatkowych badań w ramach oceny specyficzności metody. W przypadku podejrzenia o możliwość współwymywania analitu i substancji pokrewnych (np. izomerów), konieczne będzie wykonanie badań wykazujących rozdzielenie chromatograficzne, a w przypadku niepowodzenia także przeprowadzenie testu wpływu matrycy oraz oceny konwersji wstecznej [16].

2.2. Wpływ matrycy

Wpływ matrycy dla analiz wykonywanych techniką LC-MS jest definiowany jako zmiana odpowiedzi aparatu wobec analitu lub wzorca wewnętrznego spowodowana zakłóceniami i często niezidentyfikowanymi składnikami matrycy [1]. Wystąpienie wpływu matrycy powoduje uzyskanie wyników niedokładnych i nieprecyzyjnych.

Sposób przeprowadzenia testu wpływu matrycy w ICH M10 [1] różni się znacznie od prezentowanego wcześniej w wytycznej EMA [2]. Zrezygnowano z wyznaczania znormalizowanego wzorcem wewnętrznym czynnika matrycowego i uproszczono sposób wykonania testu. Obie wytyczne zalecają wykonanie testu dla co najmniej 6 źródeł matrycy i dodatkowo dla osocza w źródle hemolizowanym i lipemicznym w przypadku możliwości wystąpienia takich warunków w badaniu.

Zgodnie z wytyczną EMA [2] do przeprowadzenia testu potrzebne były roztwory wzorcowe o stężeniu odpowiadającym dwóm poziomom stężeń próbek kontrolnych (*ang. Quality Control* – QC) po procesowaniu próbki przy założeniu 100% odzysku oraz próbki ślepej (bez dodatku analitu i wzorca wewnętrznego) po procesie izolacji z matrycy [1]. Taki sposób postępowania był dość trudny do wykonania i wymagał dużego skupienia analityka ze względu na pojawiającą się dużą ilość zmiennych.

W wytycznej FDA [3] w punkcie *Selektywność i Specyficzność* wspomniano o ocenie wpływu matrycy na metodę analityczną bez podania opisu postępowania sprawdzającego możliwości zaistnienia powyższego zjawiska.

W wytycznej ICH M10 [1] znajduje się dokładny opis sposobu oceny wpływu matrycy, który znacznie różni się od zaleceń EMA [2]. W nowej wytycznej [1] test wykonuje się poprzez ocenę porównania dokładności i precyzji pomiarów uzyskanych w różnych źródłach matrycy dla co najmniej 3 powtórzeń próbek QC o stężeniu niskim i wysokim. Postępowanie jest podobne do testu oceny dokładności i precyzji z tą różnicą, że w ocenie wpływu matrycy używane są różne źródła materiału biologicznego.

2.3. Powtarzalność ponownego dozowania nastrzyku

Powtarzalność ponownego dozowania powinna być oceniona w celu możliwości wykonania powtórnego dozowania próbki podczas badania bez jej kolejnego procesowania w przypadku wystąpienia np. awarii sprzętu. Test jest nowością dla laboratoriów pracujących zgodnie z wytycznymi EMA [2] lub/i FDA [3]. W wytycznej ICH M10 został wprowadzony test powtarzalności dozowania (*ang. reinjection reproducibility*) [1] uwzględniający przykładowe sytuacje uzasadniające ponowne dozowanie próbek, rodzaju analizowanych próbek w sekwencji analitycznej do wykonania testu oraz sposób wykonania testu. Ocenę należy wykonać poprzez wielokrotne dozowanie serii, co najmniej 5 powtórzeń z jednego naczynka każdego stężenia próbki QC [1]. Kryteria akceptacji testu są takie same jak w przypadku testu oceny dokładności i precyzji.

2.4. Stabilność

Związek oznaczany w badaniu może ulec degradacji na różnych etapach postępowania z próbką biologiczną, od momentu pobrania próbki materiału biologicznego z organizmu do momentu analizy próbki już po procesie izolacji związku z matrycy. W walidacji metody bioanalitycznej sprawdzane jest czy zastosowana procedura analityczna oraz warunki przechowywania minimalizują ryzyko rozkładu oznaczanych związków [1].

Obecnie obowiązująca wytyczna w porównaniu do wytycznych EMA [2] i FDA [3] wprowadziła niewielkie zmiany w testach oceny stabilności. Zarówno ICH M10 [1] jak i FDA [3] podkreślają ważność stabilności analitu we krwi pełnej. ICH M10 do opisu testu stabilności wprowadziła informację o minimalnej liczbie powtórzeń próbek QC na danym poziomie stężeń. Jest to wypełnienie luki informacyjnej w porównaniu z wytyczną EMA [2], która nie zawierała zapisów o liczbie powtórzeń. Dodatkowo w porównaniu z wytyczną EMA [2], ICH M10 wymaga przeprowadzenia testów stabilności analitu w matrycy biologicznej wzbogaconej o wszystkie dodatkowe związki zawarte w schemacie leczenia lub wynikające ze stosowania produktów wieloskładnikowych (*ang. fixed dose combination*) [1]. Nowością w ICH M10 [1] w porównaniu z wytyczną EMA [2] jest możliwość stosowania tego samego roztworu podstawowego do przygotowania próbek QC i próbek kalibracyjnych. Taki zapis pozwala na zminimalizowanie ilości używanych substancji wzorcowych, które w przypadku metabolitów substancji badanej i znakowanych izotopowo wzorców substancji badanej są bardzo drogie. Korzystanie z jednego roztworu podstawowego jest możliwe po potwierdzeniu dokładności sporządzenia i jego stabilności. Przygotowanie roztworów

podstawowych uznaje się za dokładne, gdy różnica nie przekracza 5% (Wzór 1) [16].

$$\%\ różnicy = \frac{|\ roztwór\ podstawowy\ 1 - roztwór\ podstawowy\ 2\ |}{wartość\ średnia\ z\ pomiarów}\ \text{x}\ 100$$

Wzór 1. [16]

Nowością dla laboratoriów pracujących zgodnie z wytyczną FDA [3] jest poszerzenie badania stabilności analitu i wzorca wewnętrznego także o analizę roztworów roboczych, a nie jak dotychczas tylko roztworów podstawowych i roztworów o najniższym i najwyższym stężeniu przygotowanych w danym rozpuszczalniku. Dodatkowo nowa wytyczna zawiera informację o minimalnej liczbie cykli zamrażania-rozmrażania [1], której brakowało w wytycznej amerykańskiej. Zgodnie z badaniami przeprowadzonym przez Wagner-Golbs (2019), ilość cykli zamrażania-rozmrażania ma większy wpływ na starzenie się osocza niż czas przechowywania w warunkach zamrożenia [18].

2.5. Odzysk

Odzysk, rozumiany jako wydajność procesu ekstrakcji, jest oceniany na podstawie porównania wartości sygnału pomiarowego uzyskanego dla próbki z dodatkiem wzorców przed procesem izolacji z materiału biologicznego z wartością sygnału pomiarowego uzyskanego dla próbki z dodatkiem wzorców po przygotowaniu próbki do analizy. Test był wykonywany w ramach wytycznej FDA [3], w przeciwieństwie do wytycznej EMA [2], gdzie dokument nie zawierał zapisów dotyczących odzysku.

2.6. Raportowanie

Laboratoria prowadzące dotychczas walidację metod bioanalitycznych zgodnie z wytyczną EMA [2] będą potrzebowały uzupełnić raport walidacyjny metody bioanalitycznej m.in. o analizę trendów wykresów próbek QC [1].

Na końcu dokumentu ICH M10 wprowadzono przydatną tabelę z wypunktowaną zalecaną dokumentacją do przedłożenia organom regulacyjnym oraz dokumentacją, która powinna być dostępna w ośrodku analitycznym w czasie inspekcji/kontroli. Dodatkowo wymieniono zalecenia dotyczące przygotowania raportu. Tabela w ICH M10 w zaprezentowanej formie jest zmodyfikowaną tabelą z wytycznej FDA [3]. Tabelaryczne podsumowanie wymaganej dokumentacji jest dużym ułatwieniem dla analityków pracujących z wytyczną EMA, ze względu na brak w dokumencie przejrzystego podsumowania, które ułatwiałoby gromadzenie dokumentacji z badania i przygotowywanie raportu końcowego.

3. Nowe aspekty poruszone w wytycznej

Zalecenia wytycznej ICH M10 obejmują także walidację metod analitycznych dla produktów leczniczych zaliczanych do grupy związków endogennych, które szczegółowo nie były opisane w wytycznej FDA [3] i EMA [2].

Wytyczna ICH M10 wprowadza rekomendacje dotyczące nowych i alternatywnych metod pobierania próbek, np. suchych kropli matrycy (*ang. dried matrix methods* - DMM), wśród których można wyróżnić następujące rodzaje: suchej kropli krwi (ang. *dried blood spot* – DBS), suchej kropli osocza (ang. *dried plasma spot* – DPS), suchej kropli śliny (ang. *dried saliva spot* - DSS) i suchej kropli moczu (ang. *dried urine spot* – DUS) [19]. Metody te wpisują się w zasadę 3R poprzez pobieranie mniejszych objętości matrycy [11].

ICH M10 [1] nie dopuszcza stosowania dwóch metod lub technologii w ramach badania równoważności biologicznej lub porównawczego badania dostępności biologicznej. W przypadku potrzeby zastąpienia metody bioanalitycznej, nową metodę można wprowadzić po wykonaniu walidacji krzyżowej dla obu metod. Szczególną uwagę należy zwrócić na przyczyny różnic, jakie mogą być obserwowane w stężeniach analitu uzyskanych poprzednią metodą i za pomocą wprowadzanej metody [1].

W teście selektywności oraz badaniu wpływu matrycy wytyczna zaleca użycie większej liczby osoczy. Do 6 różnych źródeł matrycy zalecane jest stosowanie dodatkowo kolejnych dwóch osoczy po minimum jednym z oznakami hiperlipemii i minimum jednym z hemolizą dla badań prowadzonych z wykorzystaniem osocza.

4. Dyskusja i podsumowanie

Zaletą wprowadzenia harmonizacji zasad prowadzenia walidacji metody bioanalitycznej jest możliwość stosowania jednej walidacji metody analitycznej z przeznaczeniem uzyskania pozwolenia na dopuszczenie danego leku na różnych rynkach światowych bez konieczności wprowadzania modyfikacji do rejestracji w agencjach regionalnych. Postępowanie takie jest bardziej etyczne zgodnie z zasadą 3R – *ang. Replace, Reduce, Refine* [11] i prowadzi do obniżenia kosztów ponoszonych przez podmioty odpowiedzialne, a w konsekwencji także przez pacjentów.

Zaskoczeniem jest zaproponowany nowy sposób oceny efektu matrycowego na podstawie oceny precyzji i dokładności. Pominięto ugruntowane podejścia oparte na analizie współczynnika zmienności (%CV) dla znormalizowanego wzorcem wewnętrznym czynnika matrycowego podobnego do sposobu zaproponowanego przez Matuszewskiego [20,21]. Sposób zaproponowany w nowej wytycznej ICH M10 uniemożliwia identyfikację źródła pochodzenia problemu zaobserwowanego w teście wpływu matrycy, a umożliwia jedynie ocenę wystąpienia tego zjawiska. Cortese i współpracownicy opisują wszystkie metody określania wpływu matrycy i sposoby jego korygowania [22]. Zaskoczeniem jest także możliwość wykorzystania jedynie 1 osocza lipemicznego, którego zróżnicowanie w zawartości składników matrycy jest bardzo duże pomiędzy różnymi źródłami [23] i według przeprowadzonych badań [24].

ICH M10 [1] tak jak FDA [3] podkreśla ważność stabilności analitu we krwi pełnej. Sposób przeprowadzenia tego testu oraz kryteria akceptacji pozostają wciąż niewyjaśnione, w aspekcie definiowania rodzaju użytej krwi – świeżej (nie określono jak długo krew od pobrania uważana jest za świeżą) czy zamrożonej [25]. Krew świeżą można definiować jako przechowywaną w lodówce nie dłużej niż 7 dni. Mrożenie krwi negatywnie wpływa na jej właściwości powodując znaczącą hemolizę [26]. Ledvina (2019) poleca do pierwszej oceny stabilności rozpoczęcie ekstrakcji i analizy próbek dopiero po odwirowaniu elementów morfotycznych z próbek krwi pełnej, natomiast ekstrakcję i analizę bezpośrednio z próbek krwi w przypadku wątpliwych wyników pierwszego sposobu postępowania [26].

Kolejnym problemem w tym badaniu stabilności jest zmiana matrycy z osocza na krew pełną, w której oznaczany jest analit, co w konsekwencji może dać inną odpowiedź aparatu lub wymagać opracowania nowej metody izolacji analitu. Do dyskusji pozostaje

zagadnienie, czy stężenia próbek przygotowanych we krwi pełnej odczytane wobec krzywej kalibracyjnej w osoczu pozwolą na uzyskanie wiarygodnych wyników przeprowadzonego testu.

Zaproponowana przez ICH M10 [1] liczba próbek QC do przeprowadzenia testu stabilności minimum 3 jest niewystarczająca, ponieważ według badań przeprowadzonych retrospektywnie i potwierdzonych metodą symulacji matematycznej testy powinny zostać wykonane na minimum 5 próbkach [27]. ICH M10 zaleca dla produktów leczniczych wieloskładnikowych i leków podawanych jednocześnie badanie stabilności w cyklach zamrażania - rozmrażania, krótkoterminowej i długoterminowej. Przeprowadzenie testów stabilności dla wszystkich analitów leków podawanych jednocześnie, w przypadku zapisu w protokole badania klinicznego i nieklinicznego zezwalającego na podawanie innych leków, jest kosztowne i czasochłonne [25]. DeChenne [28] uważa, że liczba testów stabilności dla próbek kontrolnych z lekami podawanymi jednocześnie powinna być ograniczona do testu stabilności krótkoterminowej i w cyklach zamrażania-rozmrażania. Próbki QC dla leków podawanych jednocześnie przygotowuje się zazwyczaj w stężeniu blisko stężenia maksymalnego (*ang. Maximum concentration - C_{max}*) danego leku. Problem w wyborze stężenia leku w próbkach kontrolnych może pojawić się dla matryc takich jak np. mocz czy płyn mózgowo-rdzeniowy, dla których może brakować danych o wartości C_{max}. DeChenne [28] przy braku danych literaturowych proponuje, za zgodą sponsora badania, dwa sposoby na szacowanie C_{max} w innych matrycach: (1) wykorzystanie danych C_{max} z osocza pomnożonych przez 10 dla moczu jako matrycy lub (2) oszacowanie na podstawie danych C_{max} od innych gatunków. Kolejnym zagadnieniem wymagającym wyjaśnienia jest postępowanie w sytuacji, kiedy wykazana zostanie niestabilność leków podawanych jednocześnie. Ponadto, warto byłoby rozważyć wykorzystanie danych literaturowych o stabilności każdego z poszczególnych analitów osobno w obliczu braku solidnych dowodów naukowych, które wskazywałyby, że na stabilność jednego analitu w matrycy ma pozytywny lub negatywny wpływ obecność innego analitu [25]. Ograniczenie ilości prowadzonych badań stabilności byłoby postępowaniem bardziej zgodnym z zasadą 3R.

Wytyczna opisuje także walidację metod bioanalitycznych obejmujących nowe techniki pobierania próbek w postaci suchej kropli matrycy [19] oraz techniki mikropróbkowania [29,30], które są zgodne ze strategią korzyści 3R (redukcja, ponowne użycie i recykling) w badaniach nieklinicznych. Zastosowanie powyższych technik może poprawić dobrostan zwierząt zmniejszając liczbę zwierząt potrzebnych do badania ze względu na zmniejszenie objętości matryc biologicznych. Oprócz tego, przygotowanie mikropróbek wiąże się z oszczędnościami finansowymi wynikającymi ze zmniejszenia liczby zwierząt w hodowli i przechowywanych próbek, a także z kosztów wysyłki i analizy [31].

Wytyczna określa zasady dokumentowania walidacji i analizy bioanalitycznej próbek z badania. Zalecenia te zestawiono w tabelę z podziałem na miejsce opracowania metody (*Documentation at the analytical site*), raport z walidacji metody bioanalitycznej (*Validation report*) oraz raport bioanalityczny (*Bioanalytical report*) [1]. Oba raporty przygotowywane są zgodnie ze wzorem Wspólnego Dokumentu Technicznego (CTD) [4], który przedkładany jest organom rejestracyjnym.

Twórcy wytycznej nie podali terminu okresu przejściowego, w jakim można stosować do badań próbek metody zwalidowane według wcześniej obowiązujących wytycznych. Nie wiadomo, czy laboratorium powinno przeprowadzać rewalidację metody bioanalitycznej według wymagań nowej wytycznej ICH M10 [1] dla metod używanych w badaniu, które rozpoczęło się przed wejściem w życie nowej wytycznej.

W niektórych krajach agencje regulacyjne nie wdrożą ICH M10, co będzie powodować różnice w podejściu do walidacji metod bioanalitycznych. Wytyczna ICH M10 jest sukcesem wieloletnich działań i współpracy światowych agencji regulacyjnych i globalnych społeczności bioanalitycznych na rzecz globalnego ujednolicenia zasad prowadzenia walidacji metod bioanalitycznych.

Udział autorów: Koncepcja, E.Gn.; metodologia, E.Gn.; badanie, E.Gn. i E.Gi.; zasoby, E.Gn.; pisanie – przygotowanie oryginalnej wersji roboczej, E.Gn. i E.Gi.; pisanie – recenzja i redakcja, K.B.,; wizualizacja, E.Gn., E.Gi; nadzorowanie, E.Gn.; nadzór nad projektem, E.Gn.; pozyskanie funduszy, E.Gn. Wszyscy autorzy zapoznali się i zgadzają się na publikowaną wersję manuskryptu.

Finansowanie: Projekt realizowany w ramach subwencji nr 84345A przyznanej dla Sieci Badawczej Łukasiewicz – Instytut Chemii Przemysłowej imienia Profesora Ignacego Mościckiego.

Podziękowania: Autorzy są wdzięczni Piotrowi J. Rudzkiemu (Celon Pharma S.A.) oraz Edycie Peście (Sieć Badawcza Łukasiewicz – Instytut Chemii Przemysłowej imienia Profesora Ignacego Mościckiego) za krytyczną recenzję manuskryptu.

Konflikt interesów: Autorzy deklarują brak konfliktów interesów.

Piśmiennictwo

1. European Medicines Agency, ICH guideline M10 on bioanalytical method validation and study sample analysis, **2022**.
2. European Medicines Agency (EMA), Committee for Medicinal Product for Human Use (CHMP), Guideline on Bioanalytical Method Validation, **2011**.
3. U.S. Food and Drug Administration, Guidance for Industry: Bioanalytical Method Validation, **2018**.
4. European Medicines Agency, ICH guideline M4 (R4) on common technical document, Amsterdam, 19 March 2021. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m4-r4-common-technical-document-ctd-registration-pharmaceuticals-human-use_en.pdf (data dostępu: 28.08.2023).
5. ANVISA, Ministry of Health, National Health Surveillance Agency, Resolution- RDC NO. 27 OF 17 MAY 2012, **2012**.
6. Ministry of Health, Labour and Welfare (MHLW) of Japan. Guideline on Bioanalytical Method Validation in Pharmaceutical Development (25 July 2013, MHLW, Japan). http://www.nihs.go.jp/drug/BMV/250913_BMV-GL_E.pdf (data dostępu: 28.08.2023).
7. The Ministry of Health, Labour and Welfare (MHLW) of Japan. Guideline on Bioanalytical Method. (Ligand Binding Assay) Validation in Pharmaceutical Development. (*1 April, 2014*, MHLW, Japan), **2014**.

8. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Final endorsed Concept Paper M10: Bioanalytical Method Validation. 7 October 2016. https://database.ich.org/sites/default/files/M10EWG_Concept_Paper.pdf (data dostępu: 28.08.2023).
9. Timmerman, P.; Lowes, S.; Fast, D.M.; Garofolo, F. Request for Global Harmonization of the Guidance for Bioanalytical Method Validation and Sample Analysis. *Bioanalysis* **2010**, *2*, 683-683, doi:10.4155/bio.10.34.
10. Bansal, S.K.; Arnold, M.; Garofolo, F. International harmonization of bioanalytical guidance. *Bioanalysis* **2010**, *2*, 685-687, doi:10.4155/bio.10.39.
11. The 3Rs. https://www.nc3rs.org.uk/who-we-are/3rs (data dostępu: 28.08.2023).
12. Overcoming of ongoing ICH Topics – ICH Public Meeting Amsterdam 042919. https://www.fda.go/media/124548/download (data dostępu: 28.08.2023).
13. ICH Guideline Implementation. https://www.ich.org/page/ich-guideline-implementation (data dostępu: 28.08.2023).
14. Ingelse, B.; Barroso, B.; Gray, N.; Jakob-Rodamer, V.; Kingsley, C.; Sykora, C.; Vinck, P.; Wein, M.; White, S. European Bioanalysis Forum: recommendation on dealing with hemolyzed and hyperlipidemic matrices. *Bioanalysis* **2014**, *6*, 3113-3120, doi:10.4155/bio.14.252.
15. Briscoe, C.J.; Hage, D.S. Factors affecting the stability of drugs and drug metabolites in biological matrices. *Bioanalysis* **2009**, *1*, 205-220, doi:10.4155/bio.09.20.
16. European Medicines Agency. ICH guideline M10 on bioanalytical method validation and study sample analysis - Questions and Answers. (EMA/CHMP/ICH/660315/2022), **2023**.
17. Zwart, M.d.; Lausecker, B.; Globig, S.; Neddermann, D.; Bras, B.L.; Guenzi, A.; White, S.; Scheel-Fjording, M.; Timmerman, P. Co-medication and interference testing in bioanalysis: a European Bioanalysis Forum recommendation. *Bioanalysis* **2016**, *8*, 2065-2070, doi:10.4155/bio-2016-0179.
18. Wagner-Golbs, A.; Neuber, S.; Kamlage, B.; Christiansen, N.; Bethan, B.; Rennefahrt, U.; Schatz, P.; Lind, L. Effects of Long-Term Storage at −80 °C on the Human Plasma Metabolome. *Metabolites* **2019**, *9*, 99, doi:10.3390%2Fmetabo9050099.
19. Ayre, A.; Chaudhari, P.; Shaikh, J.; Jagdale, S.; Agrawal, O. Dried matrix spoting-an innovative sample preparation tool in bioanalysis. *Int J Pharm Sci Rev Res* **2018**, *9*, 3597-3607. doi:10.13040/IJPSR.0975-8232.9(9).3597-07.
20. Matuszewski, B.K.; Constanzer, M.L.; Chavez-Eng, C.M. Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC−MS/MS. *Anal. Chem.* **2003**, *75*, 3019-3030, doi:10.1021/ac020361s.
21. Rudzki, P.J.; Gniazdowska, E.; Buś-Kwaśnik, K. Quantitative evaluation of the matrix effect in bioanalytical methods based on LC-MS: A comparison of two approaches. *J Pharm Biomed Anal* **2018**, *155*, 314-319, doi:10.1016/j.jpba.2018.03.052.
22. Cortese, M.; Gigliobianco, M.R.; Magnoni, F.; Censi, R.; Di Martino, P. Compensate for or Minimize Matrix Effects? Strategies for Overcoming Matrix Effects in Liquid Chromatography-Mass Spectrometry Technique: A Tutorial Review. *Molecules* **2020**, *25*, 3047, doi:10.3390/molecules25133047.
23. Xia, Y.Q.; Jemal, M. Phospholipids in liquid chromatography/mass spectrometry bioanalysis: comparison of three tandem mass spectrometric techniques for monitoring plasma phospholipids, the effect of mobile phase composition on phospholipids elution and the association of phospholipids with matrix effects. *Rapid Commun Mass Spectrom* **2009**, *23*, 2125-2138, doi:10.1002/rcm.4121.
24. Gniazdowska E.M., Giebułtowicz J.,Rudzki P.J. How does the order of sample analysis influence matrix effect during LC-MS bioanalysis? J. Chromatogr. B **2023**, *1227*, 123800, doi: 10.1016/j.jchromb.2023.123800.
25. Amsterdam, P.v.; Companjen, A.; Brudny-Kloeppel, M.; Golob, M.; Luedtke, S.; Timmerman, P. The European Bioanalysis Forum community's evaluation, interpretation and implementation of the European Medicines Agency guideline on Bioanalytical Method Validation. *Bioanalysis* **2013**, *5*, 645-659, doi:10.4155/bio.13.19.
26. Ledvina, A.R.; Ewles, M.; Pang, Y.; Cape, S. Whole blood stability in quantitative bioanalysis. *Bioanalysis* **2019**, *11*, 1885-1897, doi:10.4155/bio-2019-0155.
27. Gniazdowska, E.; Goch, W.; Giebułtowicz, J.; Rudzki, P.J. Replicates Number for Drug Stability Testing during Bioanalytical Method Validation—An Experimental and Retrospective Approach. *Molecules* **2022**, *27*, 457, doi:10.3390/molecules27020457.
28. DeChenne, S.; Yahvah, K.; Zimmer, J. Validating stability and selectivity in the presence of co-administered compounds. *Bioanalysis* **2019**, *11*, 1819-1821, doi:10.4155/bio-2019-0150.
29. Pu, F.; Zhang, W.; Bateman, K.P.; Liu, Y.; Helmy, R.; Ouyang, Z. Using miniature MS system with automatic blood sampler for preclinical pharmacokinetics study. *Bioanalysis* **2017**, *9*,1633-1641, doi:10.4155/bio-2017-0160.
30. Dillen, L.; Loomans, T.; Perre, G.V.d.; Versweyveld, D.; Wuyts, K.; Zwart, L.d. Blood microsampling using capillaries for drug-exposure determination in early preclinical studies: a beneficial strategy to reduce blood sample volumes. *Bioanalysis* **2014**, *6*, 293-306, doi:10.4155/bio.13.286.
31. Ingle, R.G.; Zeng, S.; Jiang, H.; Fang, W.-J. Current developments of bioanalytical sample preparation techniques in pharmaceuticals. *J Pharm Anal* **2022**, *12*, 517-529, doi:10.1016/j.jpha.2022.03.001.

4. Podsumowanie i wnioski

1. Opracowane metody bioanalityczne oznaczania: 21 substancji czynnych leków przeciwdepresyjnych oraz dutasterydu wraz metabolitami w osoczu ludzkim, spełniają kryteria walidacyjne i mogą być stosowane w praktyce np. w badaniu równoważności biologicznej.
2. Metoda oznaczania dutasterydu i jego metabolitów jest narzędziem mogącym mieć zastosowanie w badaniach antydopingowych. Wykazano bowiem, że jeden z metabolitów dutasterydu, 1,2-dihydrodutasteryd, utrzymuje się w osoczu znacznie dłużej niż związek macierzysty, co umożliwia jego wykrycie w czasie dłuższym niż dutasterydu. Metoda będzie mogła być użyta w przypadku poszerzenia działalności usługowej przedsiębiorstwa.
3. Schemat naprzemienny kolejności próbek w teście wpływu matrycy jest bardziej czuły w wykrywaniu efektu matrycy niż blokowy. W przypadku niektórych substancji czynnych były obserwowane znaczne różnice $\%RSD_{MF}$ pomiędzy schematami. Zastosowany schemat kolejności próbek w oznaczeniu musi być zatem zawsze raportowany w publikacjach naukowych i raportach walidacyjnych.
4. Obserwowano znaczne różnice w profilach fosfolipidów pomiędzy próbkami osocza jednego typu, a w szczególności, pomiędzy osoczami lipemicznymi. W związku z tym, że fosfolipidy uważane są za istotny czynnik powodujący efekt matrycy, przy ocenie wpływu matrycy, użycie minimum jednego osocza wysokotłuszczowego może być niewystarczające, aby uzyskać wiarygodny wynik testu.
5. Optymalizacja testu stabilności w procesie walidacji metody bioanalitycznej nie potwierdziła wiarygodności minimalnej liczby próbek QC sugerowanej przez ICH M10. Jako optymalną liczbę powtórzeń wykazano liczbę pięć.
6. Badanie stabilności długoterminowej analitów w próbkach z badania wykazało trwałość substancji czynnej (dutasterydu po 3 latach oraz arypiprazolu po 7 latach od pobrania) w temperaturze ≤ - 65 °C oraz jakość próbek pozwalającą na ponowną ich ocenę, nie dając podstawy do skrócenia czasu przechowywania próbek klinicznych.

7. Prześledzono zmiany wprowadzone w projekcie wytycznej ICH M10 oraz obowiązującej w Europie od 23.01.2023 zatwierdzonej wersji wytycznej ICH M10 w porównaniu z wcześniej obowiązującymi wytycznymi EMA i FDA, z zakresu walidacji metod analitycznych dla związków niskocząsteczkowych.

   Wyniki przeprowadzonych badań, opisane w pkt 4 i 5 znajdują odzwierciedlenie w nowym wydaniu standardowej procedury operacyjnej Sekcji Farmakokinetyki dotyczącej walidacji metody bioanalitycznej.

5. Oświadczenia wszystkich współautorów publikacji

Publikacja 1 (oryginalna)

Gniazdowska E., Korytowska N., Kłudka G., Giebułtowicz J. Determination of antidepressants in human plasma by modified cloud-point extraction coupled with mass spectrometry. Pharmaceuticals 2020; 13:1-25. IF = 5,863; MEiN = 100.

Warszawa, 11.09.2023
(miejscowość, data)

Elżbieta Gniazdowska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Determination of Antidepressants in Human Plasma by Modified Cloud-Point Extraction Coupled with Mass Spectrometry"** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **opracowanie koncepcji badań, opracowanie metodologii badania, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, przygotowanie pierwszej wersji manuskryptu, redagowanie i korekta manuskryptu**.

Mój udział procentowy w przygotowaniu publikacji określam jako **35%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

Gniazdowska
(podpis oświadczającego)

Warszawa, 18.07.2023r.
(miejscowość, data)

Natalia Korytowska-Przybylska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Determination of Antidepressants in Human Plasma by Modified Cloud-Point Extraction Coupled with Mass Spectrometry"** (Pharmaceuticals 2020; 13:1-25) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **wykonanie badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, redagowanie i korekta manuskryptu**. Mój udział procentowy w przygotowaniu publikacji określam jako **30%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

Natalia Korytowska-Przybylska
.....
(podpis oświadczającego)

WARSAW 27-JUL-2023
(miejscowość, data)

..Grzegorz Kłudka..
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Determination of Antidepressants in Human Plasma by Modified Cloud-Point Extraction Coupled with Mass Spectrometry"** (Pharmaceuticals 2020; 13:1-25) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **wykonanie badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków**. Mój udział procentowy w przygotowaniu publikacji określam jako **5%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

Grzegorz Kłudka
(podpis oświadczającego)

Warszawa, 15.09.2023
(miejscowość, data)

Joanna Giebułtowicz
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Determination of Antidepressants in Human Plasma by Modified Cloud-Point Extraction Coupled with Mass Spectrometry”** Pharmaceuticals 2020; 13:1-25) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **opracowanie koncepcji badań, opracowanie metodologii badania, wykonanie badań, nadzór nad przebiegiem badania, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, redagowanie i korekta manuskryptu.** Mój udział procentowy w przygotowaniu publikacji określam jako **30%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

.....

(podpis oświadczającego)

Publikacja 2 (oryginalna)

Gniazdowska E., Kaza M., Buś-Kwaśnik K., Giebułtowicz J. LC-MS/MS determination of dutasteride and its major metabolites in human plasma. Journal of Pharmaceutical and Biomedical Analysis 2021; 206:114362. IF= 3,571; MEiN = 100.

Warszawa, 11.08.2023
(miejscowość, data)

Elżbieta Gniazdowska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„LC-MS/MS determination of dutasteride and its major metabolites in human plasma"** (Journal of Pharmaceutical and Biomedical Analysis, 2021; 206 p.114362) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **opracowanie metodologii badania, wykonanie badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, przygotowanie pierwszej wersji manuskryptu, redagowanie i korekta manuskryptu**. Mój udział procentowy w przygotowaniu publikacji określam jako **40%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

Gniazdowska
(podpis oświadczającego)

Warszawa, 19.04.2023
(miejscowość, data)

Michał Kaza
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„LC-MS/MS determination of dutasteride and its major metabolites in human plasma”** (Journal of Pharmaceutical and Biomedical Analysis, 2021; 206 p.114362) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **opracowanie koncepcji badań, opracowanie metodologii badania, wykonanie badań, nadzór nad przebiegiem badania, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, redagowanie i korekta manuskryptu**. Mój udział procentowy w przygotowaniu publikacji określam jako **20%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

.....
(podpis oświadczającego)

Katowice 13.09.2023
(miejscowość, data)

Katarzyna Buś-Kwaśnik
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„LC-MS/MS determination of dutasteride and its major metabolites in human plasma"** (Journal of Pharmaceutical and Biomedical Analysis, 2021; 206 p.114362) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **opracowanie metodologii badania, wykonanie badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków.** Mój udział procentowy w przygotowaniu publikacji określam jako **20%.**

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

.....
(podpis oświadczającego)

Warszawa, 15.09.2023
(miejscowość, data)

Joanna Giebułtowicz
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„LC-MS/MS determination of dutasteride and its major metabolites in human plasma”** (Journal of Pharmaceutical and Biomedical Analysis, 2021; 206 p.114362) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **opracowanie metodologii badania, nadzór nad przebiegiem badania, analiza wyników, przygotowanie pierwszej wersji manuskryptu, redagowanie i korekta manuskryptu**. Mój udział procentowy w przygotowaniu publikacji określam jako **20%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

.....

(podpis oświadczającego)

Publikacja 3 (oryginalna)

Gniazdowska E., Goch W., Giebułtowicz J., Rudzki P.J. Replicates number for drug stability testing during bioanalytical method validation – experimental and retrospective approach. Molecules 2022; 27:457. IF= 4,600; MEiN = 140.

Warszawa, 11.09.2023
(miejscowość, data)

Elżbieta Gniazdowska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Replicates numer for drug stability testing during bioanalytical method validation – experimental and retrospective approach"** (Molecules 2022; 27(2): p. 457-474) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **wykonanie badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, przygotowanie pierwszej wersji manuskryptu oraz redagowanie i korektę manuskryptu**.

Mój udział procentowy w przygotowaniu publikacji określam jako **60%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

Gniazdowska
(podpis oświadczającego)

WARSZAWA, 19 VII 2023

(miejscowość, data)

Piotr Rudzki
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Replicates numer for drug stability testing during bioanalytical method validation – experimental and retrospective approach"** (Molecules 2022; 27(2): p. 457-474) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **współtworzenie koncepcji pracy, opracowanie metodologii badań, nadzór nad przebiegiem badań, interpretację wyników oraz redagowanie i korektę manuskryptu**.

Mój udział procentowy w przygotowaniu publikacji określam jako **20%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

Piotr Rudzki

(podpis oświadczającego)

Warszawa, 12.04.2023
(miejscowość, data)

Wojciech Goch
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Replicates numer for drug stability testing during bioanalytical method validation – experimental and retrospective approach"** (Molecules 2022; 27(2): p. 457-474) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **opracowanie metodologii badań, wykonanie badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, przygotowanie pierwszej wersji manuskryptu**. Mój udział procentowy w przygotowaniu publikacji określam jako **15%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

Wojciech Goch
(podpis oświadczającego)

Warszawa, 15.09.2023
(miejscowość, data)

Joanna Giebułtowicz
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Replicates numer for drug stability testing during bioanalytical method validation – experimental and retrospective approach”** (Molecules 2022; 27(2): p. 457-474) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **współtworzenie koncepcji pracy, nadzór nad przebiegiem badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, przygotowanie pierwszej wersji manuskryptu oraz redagowanie i korektę manuskryptu**.
Mój udział procentowy w przygotowaniu publikacji określam jako **5%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

.....
(podpis oświadczającego)

Publikacja 4 (oryginalna)

Gniazdowska E.M., Giebułtowicz J. and Rudzki P.J. How does the order of sample analysis influence the matrix effect during LC-MS bioanalysis? Journal of Chromatography B 2023; 1227:123800. IF= 3,000; MEiN = 100.

Gdańsk, 11.09.2023
(miejscowość, data)

Elżbieta Gniazdowska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„How Do Order of Samples Analysis Influence Matrix Effect During LC-MS Bioanalysis?"** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **opracowanie koncepcji badań, wykonanie badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, przygotowanie pierwszej wersji manuskryptu, prowadzenie korespondencji z redakcją**.

Mój udział procentowy w przygotowaniu publikacji określam jako **60%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

Gniazdowska
(podpis oświadczającego)

Warszawa, 15.09.2023
(miejscowość, data)

Joanna Giebułtowicz
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„How does the order of sample analysis influence the matrix effect during LC-MS bioanalysis?”** (Journal of Chromatography B 2023; 1227, 123800) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
opracowanie koncepcji badań, opracowanie metodologii badania, nadzór nad przebiegiem badania, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, redagowanie i korekta manuskryptu. Mój udział procentowy w przygotowaniu publikacji określam jako **20%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

.....

(podpis oświadczającego)

WARSZAWA, 13 VII 2023
(miejscowość, data)

Piotr Rudzki
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„How does the order of sample analysis influence the matrix effect during LC-MS bioanalysis?"** (Journal of Chromatography B 2023; 1227, 123800) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **opracowanie koncepcji badań, nadzór nad przebiegiem badania, redagowanie i korekta manuskryptu.** Mój udział procentowy w przygotowaniu publikacji określam jako **20%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

Piotr Rudzki

(podpis oświadczającego)

Publikacja 5 (przeglądowa)

Gniazdowska E., Gilant E., Buś-Kwaśnik K. Wytyczna ICH M10 – Jednolite podejście do bioanalizy na całym świecie. Prospects in Pharmaceutical Sciences 2023; 21(3):57-63. IF = 0,100; MEiN: 20.

Warszawa, 11.09.2023
(miejscowość, data)

Elżbieta Gniazdowska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Wytyczna ICH M10 – jednolite podejście do bioanalizy na całym świecie”** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **opracowanie koncepcji badań, opracowanie metodologii badania, nadzór nad przebiegiem badania, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, przygotowanie wizualizacji, przygotowanie pierwszej wersji manuskryptu, prowadzenie korespondencji z redakcją**.

Mój udział procentowy w przygotowaniu publikacji określam jako **75%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

Gniazdowska
(podpis oświadczającego)

Warszawa, 13.09.2023
(miejscowość, data)

Edyta Gilant
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **Wytyczna ICH M10 – jednolite podejście do bioanalizy na całym świecie"** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **analiza i interpretacja wyników, przygotowanie wizualizacji, przygotowanie pierwszej wersji manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako **15%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

Edyta Gilant
(podpis oświadczającego)

Warszawa 13.08.2023
(miejscowość, data)

Katarzyna Buś - Kwaśnik
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Wytyczna ICH M10 – jednolite podejście do bioanalizy na całym świecie”** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: **redagowanie i korekta manuskryptu**.

Mój udział procentowy w przygotowaniu publikacji określam jako **10%**.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Elżbiety Gniazdowskiej**.

(podpis oświadczającego)

6. Wykaz innych publikacji naukowych

Jyoti, Żołek T., Maciejewska D., Gilant E., **Gniazdowska E.,** Kutner A., Noworyta K. R., Kutner W. Polytyramine Film-Coated Single-Walled Carbon Nanotube Electrochemical Chemosensor with Molecularly Imprinted Polymer Nanoparticles for Duloxetine-Selective Determination in Human Plasma. ACS sensors 2022; 7:1829–1836. IF = 8.900; MNiE = 140.

Rudzki P.J., **Gniazdowska E.**, Buś-Kwaśnik K. Quantitative evaluation of the matrix effect in bioanalytical methods based on LC–MS: A comparison of two approaches. Journal of Pharmaceutical and Biomedical Analysis 2018; 155:314-319. IF = 2,983; MEiN = 35.

7. Wykaz doniesień zjazdowych

Doniesienie zjazdowe nr 1

III Poznańska Konferencja Naukowo-Szkoleniowa „Współczesna analityka farmaceutyczna i biomedyczna w ochronie zdrowia", ISBN 978-83-7597-410-2, s.58
Gniazdowska E., Rudzki P.J., Marszałek R., Giebułtowicz J.
Stabilność tramadolu i O-desmetyltranadolu w materiale biologicznym.
Poznań, 2020 - **sesja krótkie doniesienia naukowe**

Doniesienie zjazdowe nr 2

Interdisciplinary Conference on Drug Sciences, ACCORD 2022, ISBN 978-83-7637-586-1, s. 102
Gniazdowska E., Gilant E.
Stability of aripiprazole in human plasma from healthy volunteers.
Warszawa, 2022 – **sesja plakatowa**

Doniesienie zjazdowe nr 3

Interdisciplinary Conference on Drug Sciences, ACCORD 2022, ISBN 978-83-7637-586-1, s. 197
Gniazdowska E., Kaza M., Buś-Kwaśnik K., Giebułtowicz J.
Application of liquid chromatography coupled to mass spectrometry for the determination of dutasteride and its major metabolities in human plasma – pharmacokinetics approach and stability.
Warszawa, 2022 – **sesja plakatowa - wystąpienie ustne**

Doniesienie zjazdowe nr 4

„Aktualne kierunki badań w naukach farmaceutycznych – FORUM MŁODYCH 2023" I Ogólnopolskie Forum Młodych, ISBN 978-83-64968-30-3 (wersja elektroniczna) s. 56
Gniazdowska E., Goch W., Giebułtowicz J., Rudzki P.J.
Optymalna liczba próbek do badania stabilności substancji leczniczych w materiale biologicznym.
Lublin, 2023 - **wystąpienie ustne**

Sesja V – Krótkie doniesienia naukowe

Stabilność tramadolu i O-desmetylotramadolu w materiale biologicznym

Elżbieta Gniazdowska[1, 2], Piotr J. Rudzki[1], Ryszard Marszałek[3], Joanna Giebułtowicz[3]

[1] *Sieć Badawcza Łukasiewicz – Instytut Chemii Przemysłowej im. prof. I. Mościckiego w Warszawie*

[2] *Szkoła Doktorska Warszawskiego Uniwersytetu Medycznego, Warszawa*

[3] *Wydział Farmaceutyczny, Warszawski Uniwersytet Medyczny*

Jeżeli dopuszczenie produktu leczniczego do obrotu wymaga badania klinicznego z farmako-kinetycznym punktem końcowym to analiza próbek od uczestników badania jest wykonywana zwalidowaną metodą [1, 2]. Stabilność analitu i jego metabolitu w materiale biologicznym jest bardzo istotna dla wiarygodności oznaczeń. Warunki przechowywania (temperatura i czas) próbek biologicznych od momentu pobrania próbek od uczestników badania klinicznego do momentu wykonania analiz są sprawdzane podczas walidacji metody bioanalitycznej [1, 2]. Badanie stabilności analitu w materiale biologicznym przeprowadza się używając próbek kontroli jakości (QC samples). W wytycznych [1, 2] brakuje rekomendacji o minimalnej liczbie próbek do przeprowadzenia testu stabilności, chociaż jest ona podana dla innych testów walidacyjnych. Celem pracy była próba określenia optymalnej liczby próbek do przeprowadzenia testu stabilności analitu w materiale biologicznym.

Próbki badane i referencyjne – zawierające jednocześnie tramadol i O-desmethyltramadol – przygotowano w zestawach po 3, 4, 5, 6 i 8 powtórzeń. Próbki analizowano za pomocą metody LC-MS/MS po ekstrakcji techniką ciecz-ciecz z wykorzystaniem deuterowanych wzorców wewnętrznych dla obu analitów. Stabilność oceniano po 24 i 72 godzinach od przygotowania. Wyniki stężeń w próbkach badanych po czasie przechowania porównano ze stężeniami w próbkach referencyjnych analizowanych bezpośrednio po przygotowaniu. Wyniki oceniano z zastosowaniem 90% przedziałów ufności [3, 4]. Analizowano wpływ liczby powtórzeń próbek na położenie i szerokość przedziału ufności i ich wpływ na decyzję o spełnieniu kryterium akceptacji testu stabilności. Zgodnie z oczekiwaniem wraz ze wzrostem liczby analizowanych powtórzeń obserwowano węższe przedziały ufności. Najszersze przedziały obserwowano dla 3 i 4 powtórzeń. Dla liczby próbek od 5 do 8 nie obserwowano znaczących różnic w szerokości przedziałów ufności. Uzyskane wynik sugerują potrzebę wykonania co najmniej 5 powtórzeń próbek badanych i próbek referencyjnych. Przed sformułowaniem ostatecznej rekomendacji wymagane są dalsze badania dla innych substancji leczniczych.

Piśmiennictwo

1. Guideline on bioanalytical method validation. European Medicines Agency (EMEA/CHMP/EWP/ 192217/2009 Rev. 1 Corr. 2**). London, 21 July 2011.
2. Guidance for Industry Bioanalytical Method Validation. US Department of Health and Human Services, US Food and Drug Administration. 2018.
3. Timm U et al. A New Approach for Dealing with the Stability of Drugs in Biological Fluid, Journal of Pharmaceutical Sciences, 1985, 74 (9): 972–977.
4. Rudzki PJ, Leś A. Application of confidence intervals to bioanalytical method validation – drug stability in biological matrix testing, Acta poloniae pharmaceutica 2008, 65(6): 743–7.

Abstract No. PPP.040

Stability of aripiprazole in human plasma from healthy volunteers

Elżbieta Gniazdowska[1], Edyta Gilant[2]

[1] Department of Bioanalysis and Drugs Analysis, Doctoral School, Medical University of Warsaw, Sieć Badawcza Łukasiewicz Instytut Chemii Przemysłowej imienia Profesora Ignacego Mościckiego, Poland
[2] Sieć Badawcza Łukasiewicz Instytut Chemii Przemysłowej imienia Profesora Ignacego Mościckiego, Poland

E-mail address of presenting author: elzbieta.gniazdowska@ichp.pl

Objectives: Aripiprazole is used in the treatment of schizophrenia and bipolar disorder. The determination of the concentration of a drug is an important part of toxicology study and pharmacokinetic studies in clinical trials. It is particularly important to determine the stability of the analyte in biological samples at expected storage temperatures. According to The Good Laboratory Practice guideline, samples should be retained for 10 years [1]. The storage period time could be shortened if the quality of the sample does not permit evaluation of it again [1]. So far, the data on the stability of aripiprazole was reported only for the quality control samples stored after 2 years at a temperature of -20 °C [2]. No data on clinical samples exist. The study aimed to examine the stability of aripiprazole in human plasma collected from volunteers. It is critical to assess whether the quality of the sample allows for a re-analysis that would confirm the validity of the ten-year storage of research samples after the clinical trial is completed. [1].

Materials and Methods: The aripiprazole concentration in human plasma was determined by the liquid chromatography coupled to mass spectrometry method after liquid-liquid extraction with hexane: isopropanol after seven years of storage at temperature ≤-65 °C.

Results: The aripiprazole concentrations in clinical samples stored for 7 years were compared with the results of fresh samples obtained during the clinical trial. The stability of aripiprazole was evaluated by using incurred sample reanalysis test described in the European Medicine Agency guideline [3]. Samples are regarded as stable if the % difference has not exceeded 20% for at least 67% of the samples.

Conclusions: The results confirm the stability of aripiprazole in human plasma for the seven years stored at a temperature ≤-65 °C. Thus, study samples can be reliably analyzed again during this period.

Keyword: stability, aripiprazole, clinical trails

References:

1. Polish Regulation of the Minister of Health on Good Laboratory Practice and performance of studies in complianc with the principles of Good Laboratory Practice (Dz. U. 2021 poz. 1422)
2. D. S. Fisher, et al. Stability of some atypical antipsychotics in human plasma, haemolysed whole blood, oral fluid, human serum and calf serum, Forensic Sci. Int. 229, 2013, p.151-156
3. Guideline on bioanalytical method validation, Committee for Medicinal Products for Human Use (CHMP), EMEA/CHMP/EWP/192217/2009

Abstract No. PSP.24

Application of liquid chromatography coupled to mass spectrometry for the determination of dutasteride and its major metabolites in human plasma - pharmacokinetic approach and stability

Elżbieta Gniazdowska[1], Michał Kaza[2], Katarzyna Buś-Kwaśnik[2], Joanna Giebułtowicz[3]

[1] Łukasiewicz Research Network, Industrial Chemistry Institute, Doctoral School, Department of Bioanalysis and Drugs Analysis, Medical University of Warsaw, Poland
[2] Łukasiewicz Research Network, Industrial Chemistry Institute, Poland
[3] Department of Bioanalysis and Drugs Analysis, , Faculty of Pharmacy, Medical University of Warsaw, Poland

E-mail address of presenting author: elzbieta.gniazdowska@ichp.pl

Objectives: Dutasteride is a testosterone-5-alpha reductase inhibitor and a drug used in benign prostatic hyperplasia - ATC G04CB [1]. Dutasteride is extensively metabolized in humans by cytochrome P-450 3A4 and 3A5 to three major pharmacologically active metabolites, including monohydroxy metabolites (4′-hydroxydutasteride, 6β-hydroxydutasteride, and 1,2-dihydrodutasteride). The activity of 6β-hydroxydutasteride is comparable to that of dutasteride, whereas 4′-hydroxydutasteride and 1,2-dihydrodutasteride show less potency than dutasteride on both 5α-reductase isoforms [2]. Orally administered dutasteride is excreted unchanged in faeces (5.4%) and urine (< 1%). Although the drug is extensively metabolized in humans, there are no data on the concentrations of its major metabolites. Measurements of metabolite concentrations may be crucial to understanding variability in patient response to treatment [3]. Furthermore, data on the stability of dutasteride in frozen plasma samples from clinical trial participants are lacking. It has only been described for admixed samples not from clinical trials, with a storage time of only 59 days [4]. The study aimed to develop a new liquid chromatography coupled to tandem mass spectrometry method with liquid-liquid extraction to determine dutasteride and its active metabolites: 4′-hydroxydutasteride, 6β-hydroxydutasteride, and 1,2-dihydrodutasteride in plasma after a single administration of 0.5 mg of dutasteride. Another aim was to assess the long-term stability of dutasteride in clinical samples, after two and three years of storage in the freezer at ≤-65 °C.

Material and Methods: The range of linearity was 0.1-3.5 ng/mL for dutasteride and 0.08-1.2 ng/mL for 1,2-dihydrodutasteride, 4′-hydroxydutasteride, 6βhydroxydutasteride.

Results and Conclusions: The stability of dutasteride has been confirmed in clinical samples stored for up to three years in a freezer at ≤-65 °C. During this time, samples can be reanalyzed without the risk of unreliable results.

Keywords: 4′-hydroxydutasteride, dutasteride, 6β-hydroxydutasteride

References:

[1] S. Aggarwal, et al., Steroids 75 (2) (2010) 109–153.
[2] H.C. Evans, et al., Drugs Aging 20 (12) (2003) 905–916.
[3] B. Prasaja, et al., Drug Res. 68 (4) (2018) 238–240.
[4] P. Contractor, et al., Biomed. Chromatogr. 27 (9) (2013) 1168–1176.

OPTYMALNA LICZBA PRÓBEK DO BADANIA STABILNOŚCI SUBSTANCJI LECZNICZYCH W MATERIALE BIOLOGICZNYM

Elżbieta Gniazdowska [1,2] , *Wojciech Goch* [3], *Joanna Giebułtowicz* [4], *Piotr J. Rudzki* [5]

[1] *Sieć Badawcza Łukasiewicz – Instytut Chemii Przemysłowej Imienia Profesora Ignacego Mościckiego, ul. Rydygiera 8,01-793 Warszawa*
[2] *Zakład Chemii Leków, Szkoła Doktorska, Warszawski Uniwersytet Medyczny, ul. Żwirki i Wigury 61, 02-091 Warszawa*
[3] *Zakład Chemii Fizycznej, Wydział Farmaceutyczny, Warszawski Uniwersytet Medyczny, Banacha 1, 02-097 Warszawa*
[4] *Zakład Chemii Leków, Wydział Farmaceutyczny, Warszawski Uniwersytet Medyczny, ul. Banacha 1, 02-097 Warszawa*
[5] *Celon Pharma S.A., Laboratorium Bioanaliz, ul. Marymoncka 15, 05-152 Kazuń Nowy*

Adres do korespondencji: elzbieta.gniazdowska@ichp.lukasiewicz.gov.pl

Stabilność leku lub jego metabolitów w matrycach biologicznych jest istotnym parametrem w walidacji metod bioanalitycznych. Wytyczne międzynarodowe różnią się pod względem zalecanej wielkości próby do badania stabilności, od braku rekomendacji do co najmniej trzech próbek kontroli jakości. Wytyczna ICH M10 [1] zastępująca wcześniejsze wytyczne międzynarodowe rekomenduje użycie minimum 3 próbek. Badanie trzech próbek może prowadzić do uzyskania wyników zafałszowanych przez pojedynczy wynik odstający. Celem pracy była ocena optymalnej wielkości próby do badania stabilności w oparciu o 90% przedziały ufności.

Dla dziewięciu substancji analizowano retrospektywne 264 przedziały ufności z badania stabilności podczas walidacji metod bioanalitycznych dla zestawu danych różnej liczebności zestawu próbek referencyjne i próbek badanych kontroli jakości (n = 3, 4, 5, 6 lub 8). Dla dwóch analitów - tramadolu i jego głównego metabolitu (O-desmetylo-tramadolu) przygotowano i analizowano 5 zestawów próbek o różnej liczebności (n = 3, 4, 5, 6 lub 8), w dwóch stężeniach, w dwóch warunkach przechowywania (40 przedziałów ufności). Dodatkowo wykonano teoretyczne obliczenia matematyczne. [2]

90% przedziały ufności [3] były szersze dla niskich niż dla wysokich stężeń w 18 z 20 przypadków. Dla liczby powtórzeń n = 5, każdy test stabilności dał pozytywny wynik, a szerokość przedziałów ufności była poniżej 20%. Wyniki badania retrospektywnego oraz obliczenia matematyczne potwierdziły obserwacje eksperymentalne.

Pięć powtórzeń jest optymalne dla oceny stabilności substancji w materiale biologicznym.

Piśmiennictwo:
[1] ICH guideline M10 on bioanalytical method validation and study sample analysis, Committee for Medicinal Products for Human Use (CHMP), EMA/CHMP/ICH/172948/2019, European Medicines Agency (2022).
[2] Gniazdowska, E. i wsp. (2022). Replicates Number for Drug Stability Testing during Bioanalytical Method Validation—An Experimental and Retrospective Approach. *Molecules*, 27(2),457.https://doi.org/10.3390/molecules27020457
[3] Rudzki, PJ. i wsp. (2008). Application of confidence intervals to bioanalytical method validation - drug stability in biological matrix testing; *Acta Poloniae Pharmaceutica,* 65, 743-747.

Doktorat wdrożeniowy DWD/3/6/2019 pt. „Opracowanie nowych metod bioanalitycznych oraz optymalizacja sposobu walidacji", subwencja przyznana Sieci Badawczej Łukasiewicz – Instytutowi Chemii Przemysłowej imienia Profesora Ignacego Mościckiego nr 841345A

8. Wykaz innych aktywności naukowych

Staż zagraniczny w ramach programu NAWA

Miejsce: Physiolution GmbH, Germany (1 miesiąc 19.06-16.07.2022)

Tematyka: „Poznanie dobrych praktyk w firmie zajmującej się badaniami *in vitro* potencjalnych produktów leczniczych”

- zakres metod i specyfika badania uwalniania *in vitro*
- wybór aparatu do prowadzenia badania w oparciu o właściwości substancji czynnej i postać leku
- planowanie modyfikacji w teście uwalniania w celu lepszego zasymulowania warunków z przewodu pokarmowego
- poznanie sposobu oceny i porównywania profili uwalniania
- poznanie organizacji i ogólnych procesów prowadzonych w firmie farmaceutycznej.
- opracowanie metody oznaczania HPLC-UV dla riwaroksabanu w płynach symulujących warunki przewodu pokarmowego na czczo i po posiłku selektywnej wobec substancji pomocniczych i rozpuszczalników oraz modyfikatorów używanych do przygotowania mediów do uwalniania.