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Pharmacokinetics and Pharmacogenetics

Principles, Applications, and Challenges

Edited by Janko Samardzic



Pharmacokinetics and Pharmacogenetics - Principles, Applications, and Challenges

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Pharmaceutical Science

Volume 15

Aims and Scope of the Series

Pharmaceutical science focuses on the design, synthesis, formulation, targeting, distribution, safety, and efficacy of active compounds as potential therapeutics. It is a large interdisciplinary discipline that aims to integrate the basic principles of physical and organic chemistry, biochemistry, biology, and engineering to discover, develop, and characterize active compounds and to optimize the formulation and delivery of drugs in the body for offering new and improved safe and efficacious therapies against human diseases. The research areas covered by the pharmaceutical sciences range from medicinal chemistry and pharmaceutical technology to pharmacology and toxicology, which represent the preliminary phases of drug development. Medicinal chemistry involves the design and synthesis of pharmaceuticals as well as the isolation of active agents from natural sources. Computer-aided strategies are increasingly involved in this drug discovery process. Pharmaceutics is a multidisciplinary science that examines the relationships between drug formulation, delivery, distribution, and clinical outcomes. Modern clinical approaches are increasingly relying on controlled release strategies and drug delivery and targeting systems, including nanotechnological platforms (nanomedicine). Pharmacology is the science of drug action in biological systems. Pharmacologists also make drugs as tools to explore aspects of cell and tissue functions. Toxicology is the study of the adverse effects of active agents on living organisms and the ecosystem, including the prevention and amelioration of such adverse effects. This book series includes volumes on Drug Discovery, Delivery, and Pharmacology. Their overall aim is to present the latest research in the whole path of drug discovery and development from different points of view of this multidisciplinary and dynamic field.

Meet the Series Editor



Prof. Rosario Pignatello is a Full Professor of Pharmaceutical Technology and Legislation at the University of Catania, Italy. He is the Director of the Department of Drug and Health Sciences. He has nearly 30 years of experience in the research and development of innovative formulations for the controlled release and targeting of bioactive molecules, through chemical approaches as well as nanotechnological carriers, aimed at treating different disorders. Prof. Pignatello has coauthored about 180 papers and edited a series of textbooks on biomaterials and their application in medicine. The main areas of his research are polymeric and lipid-based micro- and nanoparticles as modified drug delivery systems; vesicular nanocarriers (liposomes, micelles); lipophilic prodrugs and conjugates; synthesis and evaluation of new polymeric biomaterials for drug delivery and tissue regeneration. In particular, Prof. Pignatello works actively in the field of ocular drug delivery, leading the Research Centre for Ocular Nanotechnology, within the NANOMED Centre (Centre for Nanomedicine and Pharmaceutical Nanotechnology) at the University of Catania.

Meet the Volume Editor



Professor Janko Samardzic is a distinguished pharmacologist at the Medical Faculty of the University of Belgrade. He holds a Ph.D. in Medical Pharmacology and has extensive experience and expertise in Basic and Clinical Pharmacology. Prof. Samardzic completed postdoctoral studies at the University of KU Leuven in Belgium and postgraduate specialty courses at the Erasmus University Medical Centre in Rotterdam. Prof. Samardzic is the author of numerous peer-reviewed papers and book chapters, and a regular speaker at international scientific conferences. He is the winner of the prestigious "Professor Vladislav Varagic" award of the Serbian Pharmacological Society for outstanding results achieved in scientific research. Since 2023, Prof. Samardzic has been a Special Advisor and Assistant Minister for international cooperation at the Ministry of Education of the Republic of Serbia.

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Preface

The fields of pharmacokinetics and pharmacogenetics are among the most dynamic and rapidly evolving disciplines in modern pharmaceutical and biomedical sciences. Over the past several decades, both have advanced remarkably—pharmacokinetics by elucidating how the body handles drugs through absorption, distribution, metabolism, and excretion; and pharmacogenetics by uncovering how inherited genetic variations shape individual drug responses. The book was conceived to bridge these interrelated domains and to provide a consolidated academic resource that highlights not only the underlying scientific principles but also the practical and methodological advances that enable their application in clinical and research settings. As medicine continues its shift from population-based treatment paradigms toward precision-based approaches, the synergy between pharmacokinetics and pharmacogenetics has never been more relevant. This book aims to illuminate that convergence, emphasizing both the opportunities and the challenges inherent in translating these concepts into practice.

The introductory chapter, "Integrating Pharmacokinetics and Pharmacogenetics in the Era of Personalized Medicine", outlines the principles of pharmacokinetics and pharmacogenetics, emphasizing how their integration enables more precise dosing strategies, optimization of clinical trials, and development of safer therapeutics. The chapter also highlights the growing role of artificial intelligence, machine learning, and pharmacogenetic databases in predicting individual drug responses. However, despite rapid progress, it recognizes ongoing barriers such as testing standardization, ethical concerns, and disparities in access to pharmacogenetic resources. The message is clear: sustained research, equitable implementation, and technological innovation are essential to fully realize the promise of personalized medicine.

Building upon this conceptual framework, Chapter 2, "Pharmacokinetic Considerations for Drugs Targeting the Central Nervous System (CNS)" explores one of the most complex and clinically relevant areas of pharmacokinetics. It explores the intricate relationship between drug properties, the blood–brain barrier (BBB), and CNS pharmacodynamics. The chapter provides a structured overview of how factors such as lipid solubility, protein binding, active transport, and pathological alterations of BBB permeability influence CNS drug disposition. It also discusses novel strategies to overcome these barriers, such as nanocarrier-based systems and the emerging potential of the nose-to-brain route for direct CNS targeting. By elucidating these mechanisms, the chapter bridges molecular pharmacology with clinical neurotherapeutics.

Chapter 3, "Ocular Pharmacokinetic Studies: Challenges and Best Practices", addresses another organ system where unique anatomical and physiological features profoundly affect drug disposition. Unlike systemic circulation, the eye presents multiple compartmental barriers and limited lymphatic clearance, necessitating specialized PK methodologies. This chapter highlights the limitations of extrapolating systemic PK data to ocular exposure and offers a comprehensive discussion of experimental and computational techniques designed for ocular pharmacokinetic

evaluation. It provides best practices for experimental design, sample collection, and modeling approaches, while emphasizing the translational importance of understanding intraocular drug kinetics to ensure both efficacy and safety in ophthalmic drug development.

Chapter 4, "Development and Validation of an HPLC–PDA Method for Quantitative Bioanalysis of Curcuminoids and Its Application to Preclinical Pharmacokinetics", focuses on the analytical dimension of pharmacokinetic research. It presents the development of a robust, cost-effective HPLC–photodiode array (PDA) method for simultaneous quantification of curcuminoids—bioactive compounds of significant therapeutic potential but limited bioavailability. The chapter details method validation in accordance with international regulatory guidelines, demonstrating the reliability of HPLC–PDA as an accessible alternative to mass spectrometry-based assays in preclinical settings. It further illustrates how bioanalytical validation is integral to pharmacokinetic studies, enabling accurate interpretation of absorption and systemic exposure data. The application of the method to a self-emulsifying drug delivery system exemplifies the iterative nature of formulation optimization in translational pharmacokinetics.

Chapter 5, "Comprehensive Pharmacogenetic Allele Landscape from Whole Exome Sequencing: Single-Center Cohort Analysis in the Population of North Macedonia", expands the focus from molecular analysis to population-level pharmacogenetics. Using whole-exome sequencing data, this study provides the first detailed population-wide PGx landscape, revealing both common and novel pharmacogenetic variants with potential clinical significance. The findings underscore the importance of region-specific allele frequency data, particularly in smaller or underrepresented populations, for the accurate interpretation of pharmacogenetic tests. By demonstrating the limitations of targeted genotyping and the added value of whole-exome sequencing, the chapter highlights the role of population genomics in informing precision medicine and guiding local healthcare policies.

The final chapter, "The State of the Art: Pharmacogenomics, Multi-Omics, and Translational Barriers in Antipsychotic Therapy", brings the discussion full circle, applying PK and PGx concepts to one of the most challenging areas of clinical pharmacology — neuropsychiatric drug therapy. Variability in response to antipsychotics remains a significant clinical problem, influenced by genetic variation in both pharmacokinetic and pharmacodynamic pathways. This chapter synthesizes current knowledge on key metabolic enzymes, receptor polymorphisms, and emerging multi-omics data that together shape therapeutic outcomes. It also addresses systemic barriers to clinical implementation, including the need for robust clinical evidence, standardized testing protocols, and enhanced education for healthcare providers. In doing so, it provides a candid appraisal of the gap between scientific capability and clinical reality.

Together, the chapters in this volume demonstrate that integrating pharmacokinetics and pharmacogenetics is not merely a theoretical construct but a necessary evolution toward precision pharmacotherapy. Each contribution, while focused on a specific domain — from CNS and ocular drug delivery to bioanalytical validation and population genomics — reflects the same guiding principle: that understanding the interplay between drug behavior and genetic diversity is essential to optimizing therapeutic efficacy and safety.

This book aspires to provide academic researchers with a comprehensive yet practical reference that connects fundamental principles with translational relevance. By presenting multidisciplinary perspectives, it invites collaboration between pharmacologists, geneticists, clinicians, and other scientists. Ultimately, *Pharmacokinetics and Pharmacogenetics – Principles, Applications, and Challenges* seeks to advance the shared goal of contemporary biomedical science — to move from generalized treatment paradigms toward genuinely individualized medicine, where therapy is guided not by averages but by the biology of each patient.

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Chapter 1

Introductory Chapter: Integrating Pharmacokinetics and Pharmacogenetics in the Era of Personalized Medicine

Janko Samardzic

1. Basic prinicples of Pharmacokinetics

Pharmacokinetics is a branch of pharmacology that uses mathematical models to quantitatively describe biological processes affecting the movement of drugs through the body. The main processes include absorption, distribution, metabolism, and excretion (ADME) [1]. Understanding these processes is essential for determining the optimal dosing regimens, ensuring therapeutic efficacy, and minimizing adverse effects. Absorption is the transfer of a drug from its site of administration into the bloodstream. It depends on both the drug's properties and the characteristics of the administration site. For instance, lipophilic drugs are absorbed more rapidly, whereas hydrophilic drugs and those that ionize face slower absorption. The rate of absorption is expressed by the pharmacokinetic parameter Tmax (time to reach the maximum plasma concentration - Cmax), while the extent of absorption is measured by bioavailability, which represents the proportion of the administered dose that reaches systemic circulation. Distribution occurs once the drug enters the bloodstream and spreads throughout the body's tissues and organs. A significant portion of the drug binds transiently to plasma proteins, while the remainder distributes into tissues. Distribution is influenced by regional blood flow, drug characteristics, and tissue membrane properties. The volume of distribution (Vd) quantifies this process, defined as the ratio of the administered dose to the plasma drug concentration. Elimination encompasses metabolism and excretion. Metabolism, or biotransformation, mainly occurs in the liver but can also involve the lungs, plasma, intestinal wall, kidneys, and brain. This process can either activate or deactivate the drug, influencing its therapeutic effect. Enzymes such as cytochrome P450 play a significant role in drug metabolism. Furthermore, their genetic variations in these enzymes can lead to significant interindividual differences in drug response. Drugs are primarily excreted via the kidneys, with smaller amounts eliminated through bile, lungs, feces, or other secretions. The elimination half-life ($t_{1/2}$), which depends on both metabolism and excretion, indicates the time required for the plasma concentration of a drug to decrease by half. It is the key pharmacokinetic parameter for determining dosing regimens. Renal function, along with factors such as age, can affect the rate of excretion, influencing drug levels and potential toxicity.

Pharmacokinetic principles are integral to drug development. They guide the design of dosing regimens, formulation strategies, and the prediction of drug–drug interactions. For example, physiologically based pharmacokinetic (PBPK) modeling uses mathematical representations of the body's physiology to predict the absorption and distribution of drugs, aiding in the design of new therapies and the evaluation of potential interactions.

2. Basic prinicples of pharmacogenetics

Pharmacogenetics analyzes the genetic factors that cause differences in how patients respond to medications, focusing on how individual genes and genetic variations influence both the pharmacokinetics and pharmacodynamics of drugs [2]. Its primary goal is to customize drug therapy according to a patient's genetic profile, improving treatment effectiveness while minimizing side effects. Variations in genes can impact drug-metabolizing enzymes, transport proteins, and molecular drug targets. By identifying these genetic differences, pharmacogenetics has become a key component in the development and implementation of personalized medicine.

According to the U.S. Food and Drug Administration (FDA), personalized medicine involves tailoring disease prevention and treatment strategies to the unique characteristics of each individual, considering factors such as environmental exposures, lifestyle choices, and increasingly, genetic profile. Within this framework, pharmacogenetics emerges as a key element, with the FDA currently recommending genetic testing for more than 50 medications in clinical settings. However, implementing these tests routinely faces several obstacles, including limited accessibility and the complexity involved in interpreting genetic data. Drug responses are influenced by multiple factors, and variations in single genes alone cannot account for all individual differences [3]. While pharmacogenetics holds significant promise for optimizing drug therapy and guiding drug development, additional research is necessary to fully integrate its potential into everyday clinical practice.

3. Integrating pharmacokinetics and pharmacogenetics

The integration of pharmacokinetics and pharmacogenetics offers an innovative and comprehensive approach to personalized medicine [4]. Combining pharmacokinetic data with pharmacogenetic information allows for the development of personalized dosing regimens. For example, in the case of drugs metabolized by cytochrome P450 enzymes, genetic testing can identify individuals who may require dose adjustments to achieve therapeutic drug levels. Furthermore, incorporating pharmacogenetic data into the drug development process can lead to more efficient clinical trials and the development of safer, more effective therapies. By identifying genetic factors that influence drug response early in the development process, pharmaceutical companies can design drugs that are more likely to succeed in diverse patient populations. To fully realize the potential of integrating pharmacokinetics and pharmacogenetics, several challenges must be addressed. These include the development of robust biomarkers, standardization of testing procedures, and ensuring equitable access to genetic testing.

4. Future directions

The future of pharmacokinetics and pharmacogenetics lies in the continued advancement of technology and research. Emerging fields such as artificial intelligence and machine learning hold promise for enhancing the prediction of drug responses based on genetic and pharmacokinetic data [5]. Additionally, the expansion of pharmacogenetic databases and the development of global standards for genetic testing will facilitate the broader application of personalized medicine. Artificial intelligence (AI) can be a powerful tool to analyze a vast amount of data to identify patterns and predict outcomes. In pharmacogenetics, AI can assist in the detection of genetic variants associated with drug responses, leading to more targeted therapies. Similarly, AI can enhance pharmacokinetic modeling, improving the prediction of drug behavior in various populations. The establishment of global standards for pharmacogenetic testing and data sharing is crucial for the widespread implementation of personalized medicine [6]. International collaborations can lead to the development of comprehensive genetic databases, aiding in the identification of population-specific genetic variations and facilitating the development of tailored therapies. As pharmacogenetics becomes more integrated into clinical practice, ethical considerations regarding genetic privacy, consent, and potential discrimination must be addressed. Establishing clear guidelines and policies will ensure that the benefits of pharmacogenetics are realized without compromising patient rights.

Conflict of interest

No conflict of interest.

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Chapter 2

Pharmacokinetic Considerations for Drugs Targeting the Central Nervous System (CNS)

Elvia Mera Jiménez, Martín Martínez Rosas, Martha Edith Macías Pérez, Rodrigo Romero Nava, Karla Aidee Aguayo Cerón and Maricarmen Hernández-Rodríguez

Abstract

Pharmacokinetic considerations need to be taken into account for drugs targeting the central nervous system (CNS). For this reason, in the present chapter, the general pharmacokinetic considerations are described. The CNS comprises the brain and the spinal cord. Both structures are surrounded by a highly selective barrier that protects them, the blood-brain barrier (BBB). However, the permeability of the BBB can be modified in several pathological conditions. To reach the CNS, the free form of the drug must be able to cross the BBB and achieve adequate concentrations, which can be a challenge. Characteristics to consider include the drug's lipid solubility, active transport mechanisms, such as P-glycoprotein, and protein binding. These factors will influence the absorption, distribution, metabolism, and elimination (ADME) properties of the drug, as well as its ability to cross the BBB. To enhance brain penetration, in addition to systemic methods for administering drugs that target the CNS, the development of delivery systems that enhance the transport of drugs to the brain has emerged as a promising approach for overcoming the challenges of transport across the BBB. Among these methods, the nose-to-brain route has demonstrated effectiveness, then gained considerable interest recently. Finally, *in vitro* assays to assess the BBB permeability are briefly described. Understanding these factors is essential for optimizing drug delivery, achieving the desired therapeutic effect, and minimizing potential toxicities.

Keywords: central nervous system (CNS), blood-brain barrier (BBB), pharmacokinetics, drugs, brain disposition, *in vitro* models

1. Introduction

Despite the increasing incidence of neurological disorders worldwide, developing drugs for the CNS remains an unresolved issue. This is due to high costs, lengthy pathways to clinical use, and significant failure rates, which are often linked to

pharmacokinetic factors, including particularly challenges in achieving adequate drug concentrations in the brain [1]. These challenges arise mainly from specialized biological barriers, including the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB), which limit CNS penetration, as well as other factors, including metabolism, drug efflux transport, and the physicochemical properties of the drugs.

The BBB is constituted mainly by the endothelial cells (ECs) of the brain capillaries. In contrast, the BCSFB is constituted of a tight epithelium that lines the brain's ventricles located at the choroid plexus [2]. Due to the size difference, the BBB is considered the main barrier for the exchange of molecules between the blood and the CNS [3]. Since the BBB determines the passage of nutrients to the CNS and the efflux of metabolites, it also limits the penetration of most drugs to the brain, creating a significant limitation to developing new treatments that target the CNS [4]. These result in particular challenges for polar drugs with a high molecular weight, unlike non-polar drugs with a low molecular weight, which can effectively cross this barrier [5]. However, in certain circumstances, the BBB integrity can be compromised due to neurological and systemic diseases. Then, changes in BBB permeability allow the passing of drugs into the CNS, as well as potentially harmful substances [6].

In addition to systemic methods for administering drugs that target the CNS, the development of delivery systems that enhance the transport of drugs to the brain has emerged as a promising approach for overcoming the challenges of transport across the BBB. Among these methods, the nose-to-brain route has demonstrated effectiveness then gained considerable interest recently [7–9].

During the drug design process, several assays can be performed to assess the penetration of the drug into the CNS. Despite their limitations, *in vitro* models can be valuable for developing drugs aimed at targeting the CNS [10]. As a result, established *in vitro* BBB models for evaluating brain penetration that can be implemented in laboratories without requiring extensive bioengineering expertise are described.

The present review aims to offer a general vision of the pharmacokinetic considerations for drugs designed to target the CNS.

2. The global burden of neurological disorders

The growing need for the design of drugs targeting the CNS is attributed to the rising number of deaths and disabilities caused by neurological diseases.

Neurological diseases are the leading cause of disability and the second leading cause of mortality worldwide. Furthermore, a significant burden of these cases is found in developing countries [11]. The rising prevalence of neurological diseases has been associated with global population growth and aging, in addition to an increase in risk factors, including diabetes, hypertension, and obesity, as well as an increase in neurodevelopmental conditions.

The most significant increases in the age-standardized incidence rates of neurological disorders worldwide from 1990 to 2019 have been reported in China, Ecuador, and Italy, with Parkinson's disease, idiopathic epilepsy, bipolar disorder, and dementias being the leading causes [12].

As shown in **Figure 1**, several differences in age patterns of neurological disorders have been reported. For instance, in children younger than 5 years, infectious conditions (meningitis, encephalitis, and tetanus) and epilepsy are the leading causes of neurological diseases. In contrast, migraine and cerebrovascular disease affect young

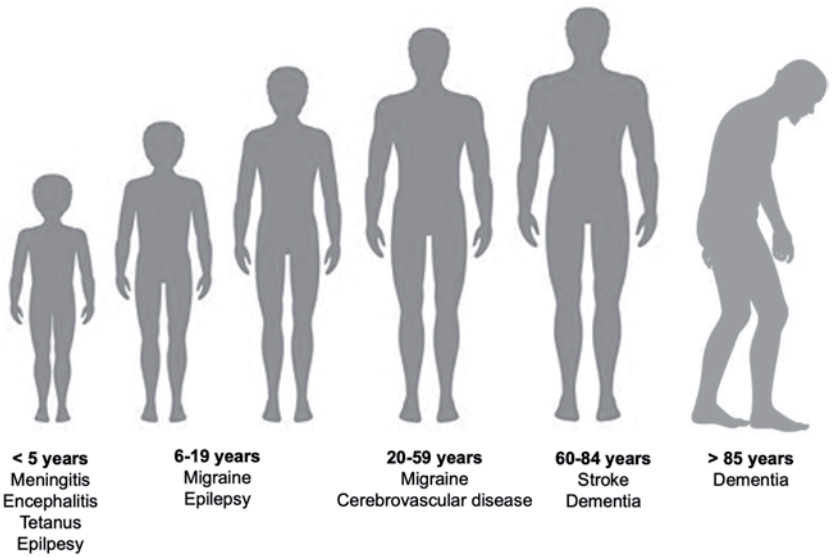


Figure 1.
Neurological disorders vary in incidence based on age. In children under 5 years old, the most common neurological diseases are infectious conditions and epilepsy. In contrast, migraine and cerebrovascular diseases are more prevalent among young and midlife adults. The impact of stroke is most significant in individuals aged 60 to 84 years. Meanwhile, Alzheimer's disease (AD) and other dementias primarily affect those aged 85 and older, with the most pronounced effects seen in individuals over 95 years.

and middle-aged adults. The burden of stroke is most pronounced among individuals aged 60 to 84 years. In comparison, dementia, including Alzheimer's disease (AD) as the firstly cause, primarily affects those aged 85 years and older, with the most significant impact seen in individuals over 95 years [13].

In addition, although progress in the neurosciences has attempted to decrease the burden of neurological diseases, significant hurdles remain in the drug development pipeline [14].

The drug development pipeline for drugs targeting the CNS is a lengthy, complex, and costly process, characterized by a high failure rate. Challenges include the unknown pathophysiology of many CNS disorders, difficulties in identifying and validating drug targets, and issues of toxicity and inadequate efficacy, which are primarily regulated by pharmacokinetics.

The failure rate for general drug discovery and development rises to 90%. However, the failure rate for new drugs targeting the CNS could be higher than 90% and indeed, in neurodegenerative conditions such as AD, the clinical failure rate for treatments has been nearly 100% [15]. While the failure rates related to drug-like properties have decreased due to advancements in understanding the anatomical aspects of the CNS and drug properties, they still account for a high rate of failure cases [16]. For this reason, pharmacokinetic considerations must be addressed to favor brain disposition.

3. Anatomical considerations of CNS system

The brain and spinal cord, collectively known as the CNS, act as the command center for the human body, directing everything from our thoughts and feelings to

our movements and vital organ functions. The CNS requires a continuous supply of energy substrates—primarily oxygen and glucose—for its proper functioning, delivered through a rich network of blood capillaries, probably the most complex in the body [17]. However, and seemingly paradoxically, the internal environment of the CNS is isolated from the bloodstream by a structure that is also unique in the body: the so-called BBB. The BBB is not just a simple wall; it is a highly specialized and active boundary. This barrier becomes apparent to clinical specialists when a pathology affects the CNS and requires pharmacological treatment. The BBB prevents the drug from reaching its therapeutic target [18]. In this regard, several antibiotics that could be ideal for fighting the germ that causes meningitis cannot be selected because they cannot pass through the BBB, or many cytostatic agents, which are of no use in the fight against primary CNS tumors, for the same reason. In this section, the BBB structure and function will be briefly reviewed.

3.1 The BBB

The BBB was once considered simply a barrier that prevented the entry of certain dyes and potentially harmful substances into the brain. It is currently considered a highly complex selective filtration system that maintains the cellular environment and the proper functioning of the CNS. The BBB is a specialized biological system composed of ECs of the cerebral microvasculature, and the interplay with surrounding cells, such as pericytes, astrocytes, basement membrane (BM), microglia, and neurons, forms the neurovascular unit (NVU) [19]. Its central function is to protect the brain from toxic substances while allowing the passage of essential molecules such as oxygen and glucose [20]. However, the BBB is the main obstacle to the delivery of drug compounds from blood to the brain.

3.1.1 Cellular and molecular components of the BBB

The BBB is primarily composed of ECs that line the blood vessels of the brain, forming a continuous monolayer around the lumen of capillaries. The ECs are connected by tight junctions that limit the paracellular diffusion of solutes between them [21, 22]. Tight junctions are formed through the interaction of proteins such as claudins, occludins, and junctional adhesion molecules [23]. These proteins maintain BBB integrity, as they are connected intercellularly and interact with cytoplasmic proteins zonula occludens (ZO)-1 and – 2, and cingulin, to the actin cytoskeleton (**Figure 2**) [23, 24]. ECs have few endocytic vesicles, which limit the transcellular flow and transform the vascular endothelium into a single interface between circulating blood and the cerebral extracellular space [25, 26], providing the first line of defense against potentially harmful substances (**Figure 2**). ECs are very rich in mitochondria, which can occupy up to 11% of the inner cytoplasmic volume and are thought to ensure the energy production necessary to maintain the structural and biochemical characteristics of the cerebral capillary endothelium (**Figure 2**) [27].

Pericytes, located on the abluminal surface of microvessels, are separated from ECs and astrocytes by a BM (**Figure 2**) [28]. Pericytes project extensions that penetrate the BM and cover 20–30% of the vascular circumference [29]. Morphologically, the contact between pericytes and ECs consists of pinching and crowding of the membranes of the two cell types [30]. Furthermore, the exchange of molecules between pericytes and ECs is facilitated by the presence of gap junctions between the two cell types [31].

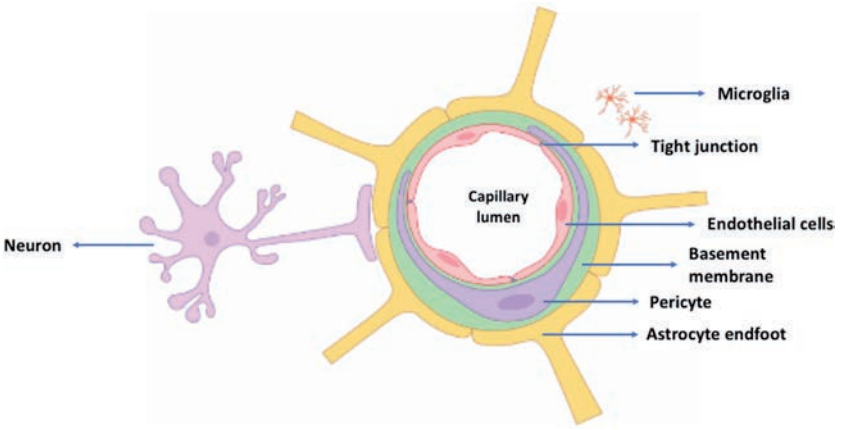


Figure 2.
The anatomical structure of the blood-brain barrier (BBB) consists of brain capillary walls made up of a thin monolayer of specialized brain microvascular endothelial cells. These cells are tightly connected by junctions that serve as a physical, transport, and metabolic barrier. Surrounding the endothelial cells, there is a basement membrane, pericytes, and astrocyte endfeet, all of which contribute directly or indirectly to the barrier function of the BBB.

Astrocytes, on the other hand, also project extensions, called "endfeet," toward ECs that line blood vessels, covering a significant portion of the microvascular surface, and provide metabolic support to ECs (**Figure 2**) [20]. A three-dimensional reconstruction of astrocytic endfeet shows that they have a characteristic rosette structure and that a single astrocyte can contact several ECs or that a single EC can be contacted by several astrocytes [32]. A morphometric study of human cerebral vessels showed that only 11% of the cortical vessel perimeter was free of astrocytic endfeet [33].

3.2 Changes in the BBB in neurodegenerative diseases

Although the BBB has a key role in the protection and homeostasis of the CNS, its integrity can be compromised in some pathologies. Disruption of the BBB increases its permeability, allowing the entry of potentially harmful substances into the brain, which can trigger or intensify neuroinflammation and contribute to the pathogenesis of various neurological diseases [20].

On the other hand, the characteristics of the BBB are thought to be partly responsible for the difficulties in acting pharmacologically in diseases as diverse as AD and Huntington's disease, ataxia, stroke, and brain tumors, or in preventing the slow neurodegeneration associated with Parkinson's disease or multiple sclerosis [6, 34]. One of the main reasons is that more than 98% of potentially active molecules to treat these diseases cannot cross the BBB. Therefore, in addition to research programs aimed at understanding the pathophysiological mechanisms of chronic diseases affecting the CNS, programs aimed at promoting drug penetration into the brain have become essential [34].

3.2.1 Stroke and ischemia

In ischemic stroke, the lack of blood flow to affected brain regions leads to endothelial dysfunction and the disruption of tight junctions, increasing the BBB permeability [35]. The augmented BBB permeability allows plasma proteins, such as fibrinogen

and albumin, to leak into the brain parenchyma, contributing to edema and neuroinflammation [24]. Inflammatory mediators, such as cytokines and matrix metalloproteinases (MMPs), further degrade the structural integrity of the BBB, creating a vicious cycle of BBB degradation and neuronal damage. Therefore, understanding the mechanisms involved in BBB disruption and how they contribute to disease progression is critical for developing therapeutic strategies aimed at restoring BBB integrity and preventing or treating the neurological damage induced by ischemia.

3.2.2 AD and BBB dysfunction

In AD, the BBB becomes progressively compromised, contributing to disease pathogenesis. The BBB disruption facilitates the extravasation of harmful peripheral substances into the brain parenchyma, including immune cells, proinflammatory cytokines, and neurotoxic molecules, which exacerbate neuroinflammation and neuronal damage [36, 37]. Key mechanisms include alterations in tight junction proteins (e.g., claudins and occludins), impaired transport systems like P-glycoprotein, and the involvement of astrocytes and pericytes in maintaining BBB integrity [38]. Studies have shown that amyloid-beta (Aβ) plaques, a hallmark of AD, disrupt BBB integrity by interfering with the expression of tight junction proteins and inducing apoptosis of ECs [39]. The accumulation of Aβ in the brain parenchyma increases the BBB permeability [40], and the decreased Aβ clearance due to BBB dysfunction accelerates plaque accumulation and exacerbates the neurodegenerative process [41]. Therefore, the BBB dysfunction is a pivotal event in AD pathogenesis, contributing to neuroinflammation, impaired waste clearance, and neuronal damage. Therapeutic approaches aimed at restoring BBB integrity, modulating its transport mechanisms, and leveraging its potential as a direct target represent promising avenues for future AD treatments.

3.2.3 Multiple sclerosis, BBB disruption, and immune cell infiltration

In multiple sclerosis (MS), the loss of BBB integrity is one of the first steps in the pathophysiology of the disease and can be influenced by genetic and environmental factors [37]. The BBB disruption allows immune cells, such as T cells, B cells, and macrophages, to cross it, leading to demyelination, axonal damage, and neuroinflammation [37]. BBB degradation leads to the activation of immune cells and the production of proinflammatory cytokines, which compromise ECs' integrity [42]. Chronic inflammation in MS leads to progressive neurodegeneration and permanent neurological disability.

4. Key pharmacokinetic principles applied to the CNS

Pharmacokinetics is defined as the kinetics of drug absorption, distribution, metabolism, and elimination (ADME). The drug's chemical properties influence these four key phases, as well as the method of administration and individual host factors. When developing therapies for the CNS, it is necessary to consider specific pharmacokinetic characteristics that may modify their effectiveness.

4.1 Absorption

As a rule, a drug must reach an adequate concentration at its site of action to produce a therapeutic effect. In the case of neurological conditions, this implies that

the drug must cross the BBB to target the CNS effectively. Since only the free form of the drug can cross this barrier, its concentration and protein binding can significantly limit its absorption [43]. In the context of CNS pharmacokinetics, absorption refers to the process by which a drug enters the CNS. Thus, the relation between the free drug concentration in the brain and the free plasma concentration is known as brain penetration [44]. However, cerebrospinal fluid or brain tissue over time reveals distinct patterns of absorption into the CNS. Thus, quantifying drug absorption in the CNS is more complex due to the dynamic interactions between drug concentrations in the plasma, cerebrospinal fluid, interstitial fluid, and brain tissue [45].

Transport into the CNS can occur through either passive or active mechanisms. As expected, low molecular weight and lipophilicity are key physicochemical properties that enable molecules to diffuse directly through cell membranes, unlike larger or more hydrophilic molecules. However, the passive transport of specific molecules may be hindered by efflux pumps located on the ECs of the BBB [45]. In contrast, polar molecules, such as amino acids, glucose, and specific regulatory proteins, cross the BBB through active transport, utilizing symporters or specific transport proteins found in the endothelial cells [46]. Interestingly, the expression of efflux pumps on ECs located at the BBB can be modified due to environmental factors [47]. Therefore, these factors must be considered to predict how effectively a specific molecule can penetrate the CNS.

4.2 Distribution

In the CNS, the distribution of a drug depends both on physiologic exchange between fluids and the extracellular and intracellular spaces. In this sense, molecules with low hydrogen bonding potential distribute within the extracellular fluid (ECF), allowing them to interact with both neurons and glial cells [48]. Once in the ECF, drugs can also enter the intracellular space through passive or active mechanisms. This may lead to the accumulation of substances within a specific type of cell [49]. In addition, as it has been described previously, pathological conditions can modify the brain disposition of drugs as well as the drug distribution in the CNS. For example, although ischemia and acidosis can limit drug distribution due to reduced blood flow, the design of nanocarriers that release their drug in response to acidic tissue pH ensures the selective pharmacological reach of a tissue at risk, thereby minimizing undesirable effects [50].

4.3 Metabolism

Drug metabolism, also known as biotransformation, refers to the chemical modification of a drug to facilitate its elimination from the body. Nevertheless, the chemical modification can be approached to generate more active metabolites or by administering a prodrug [51]. Although the liver is the primary organ involved in the biotransformation of drugs, in the brain, the enzymes involved in biotransformation are also expressed in the ECs of cerebral blood vessels [52]. The cytochrome superfamily of enzymes (CYP) plays a critical role in brain drug biotransformation by performing reduction and oxidation reactions. Within this family, CYP2B6 is particularly important for the biotransformation of chemotherapeutic agents, ifosfamide and cyclophosphamide, into their active metabolites, being of clinical significance for the treatment of medulloblastoma [53]. In addition to the CYP family, glutathione S-transferases and catechol O-methyltransferase, which are involved in

the detoxification of drugs and the regulation of neurotransmitters, are more highly expressed in the brain than in the liver [54].

4.4 Elimination

For drugs targeting the CNS, elimination implies the removal of a drug from this site to avoid its effect. Elimination involves various mechanisms. Firstly, a drug can enter the intracellular space or bind irreversibly to a cell surface receptor, avoiding its further action. In addition, chemical biotransformation into inactive compounds can be achieved by the CYP superfamily, glutathione S-transferases, and catechol O-methyltransferase, among others, as previously described. Drugs can be eliminated from the CNS by transporting into the blood or CSF through diffusion or the glymphatic system. The latter utilizes the perivascular spaces around blood vessels to facilitate CSF flow throughout the brain's tissue, acting as a pathway for ECF. This system serves two primary purposes: it delivers CSF constituents to the brain tissue and allows for the elimination of drugs and metabolites from the brain. In this way, CSF enters the brain through the perivascular space, which are fluid-filled compartment surrounding blood vessels in the brain and spinal cord, mixes with ECF, and then exits *via* the bloodstream through subarachnoid granulations and meningeal lymphatics [55]. Thus, the rate of CSF turnover directly affects the drug elimination, and consequently, its alterations affect drug pharmacokinetics [56]. Thus, pharmacological interactions are relevant. For example, commonly used medications such as acetazolamide and omeprazole decrease CSF production, hence potentially modifying the pharmacokinetics of drugs targeting the CNS [57].

5. Factors affecting pharmacokinetics of drugs in the CNS

Pharmacokinetics is a set of processes and reactions of the drug inside the organism, from the compound being delivered from the formulation until it reaches the target organ or tissue. However, as described, all these processes are more complicated in the CNS, and delivering drugs to this site is a significant challenge due to several factors that can affect pharmacokinetics in the CNS. The main characteristics are described above.

5.1 Protein binding

In the CNS, there are some mechanisms of transport that endogenous molecules use to cross the BBB: (a) active transport for small molecules such as glucose; (b) paracellular and transcellular transport for proteins and peptides; (c) paracellular diffusion in which molecules pass through gaps between the endothelial cells [58]. To deliver and transport drugs, receptor-mediated transcytosis is the most commonly used mechanism. This mechanism involves the binding of molecules to different receptors, accumulation in intracellular spaces (invaginations), and then the molecules enter the ECs using a clathrin complex that helps the transport of the molecule [59]. The use of exosomes is considered the best option for crossing the BBB [60]. However, it has been described that in inflammatory conditions, the trafficking of drugs through the BBB is enhanced [61].

Changes in the permeability of the BBB are caused by several factors, including the increase in reactive species of nitrogen and oxygen and decreased endogenous

antioxidant mechanisms (oxidative stress), inflammatory mediators such as IL-1β, IFN-γ, and TNF-α, lipid mediators, vasogenic agents, and pathogens (bacteria, parasites, fungi, and viruses) [62]. Nevertheless, as it has been previously described, some pathological conditions modify the BBB, including AD and ischemia, and brain tumors [63]. Another factor influencing the permeability of the BBB is aging [64].

It has been described that protein binding with drugs could be a strategy to cross the BBB [65]. The mechanism for this type of transport involves conformational changes in the protein when the protein-drug complex comes into contact with the membrane; after that, the drug is released from the complex and can begin to diffuse in the CNS [66]. Although albumin is the most common protein to which a drug can bind, it has been described that it cannot cross the BBB under physiological conditions.

Exist some options to increase the permeability of drugs using nanotechnology based on the association of one compound without activity with the drug. According to the composition of the compound, there are three types of nanocarriers: lipid-based, polymer-based, and metal-based. Those use transcytosis as transport [67].

5.2 Drug efflux transporters

Drug efflux transporters, such as Multidrug Resistance Protein 1 (MDR1 or P-glycoprotein), are another type of drug transport protein that facilitates efflux across the BBB, which is part of a large family of transporter proteins. MDR1 is a membrane-bound ATP transporter that affects the absorption, distribution, metabolism, and elimination of drugs [68]. Many drugs are transported using this protein, and the activity of drugs depends on their penetration into the CNS [69]. It has been described that polymorphisms of P-glycoprotein can lead to numerous changes and differences in the potential toxicity and pharmacokinetics of drugs [70]. The primary concerns associated with this are neurotoxicity and teratogenesis. Another factor is that the functional activity of P-glycoprotein is saturable [71].

5.3 Physicochemical properties

Passive diffusion is one of the most common transport mechanisms for CNS drugs. The molecular weight of drugs used in the CNS that easily pass through the BBB is around 150–500 Da [72]. This is because the distance of the brain capillary ECs is 20 μm and allows small molecules to permeate the membrane [73]. The mechanism of diffusion is divided into paracellular and transcellular. The first process depends on tight junctions and adherent junctions, where molecules move from a higher concentration to a lower one. It has an essential limitation, as only tiny and hydrophilic molecules can permeate.

The second factor influencing the permeability of the drug, thus the molecules which have <500 Da, lipophilicity, and weak hydrogen bonds are the best option of drugs to reach the CNS, but in the case of endogenous compound, it is necessary that even the last characteristics the molecules must possess an amphipathic structure [73, 74]. However, the research in this field has developed some strategies to improve the passive diffusion of drugs into the CNS. Cell-penetrating peptides, which are covalent or non-covalent molecules, could be one option to facilitate the penetration of CNS drugs.

6. Drug delivery to the central nervous system

Many potentially effective treatments fail in clinical practice not because of a lack of potency, but because of problems with administration. The treatment of CNS diseases is particularly complex, as numerous obstacles can hinder the delivery of these drugs to the target site. Consequently, in addition to systemic administration, various delivery strategies have been developed, as described below.

6.1 Systemic delivery to CNS

Drugs administered systemically need to cross the BBB to reach the CNS. To achieve this purpose, a drug designed to reach the CNS needs to accomplish several characteristics that include low molecular weight, lipophicity, low protein binding, and low affinity for BBB efflux pumps [75], as described below.

Generally, as was previously described, it is accepted that lipophilic drugs with a molecular weight below 400–500 Daltons are more likely to cross the BBB by passive diffusion. In contrast, larger and polar molecules often require specific transport systems to enter the CNS. However, it should be noted that extraordinarily high lipid solubility is usually accompanied by a high degree of protein binding, which decreases the amount of free drug. This amount can diffuse into the CNS. Lipid solubility decreases if the drug is ionized in the medium's pH. Thus, for those molecules that can exist in both ionized and non-ionized forms (e.g., weak acids such as penicillin or cephalosporins), their penetration into the CNS is pH-dependent, so they penetrate more easily through lipid bilayers in their non-ionized form. In the context of meningitis, the pH may drop below 7, so the drug will be less ionized within the CSF, creating a backflow into the blood.

Finally, the concentration of a drug targeting the CNS can be altered if it acts as a ligand for a transport system. P-glycoprotein is a protein transporter present throughout the BBB and a clear example of the strong impact that a pumping system can have on drug concentrations in the CNS. Although most published studies are *in vitro*, and it is difficult to predict based solely on physicochemical properties which drugs will be potent ligands for this system, it has been shown that treatment with rifampicin or protease inhibitors causes overexpression of P-glycoprotein, which can decrease the final concentration of other drugs.

However, most of the drugs that target the CNS are polar, high molecular weight molecules. For this reason, some approaches have been designed for delivering drugs to the CNS across the BBB. One strategy, employed nowadays in clinical practice, consists of modifying the BBB permeability by the administration of hyperosmotic solutions or vasoactive molecules, including leukotriene C4, bradykinin, serotonin, and histamine, which is particularly employed to treat brain tumors. Additionally, the increase in BBB permeability as a non-specific method for drug release can also lead to heightened permeability for potentially toxic substances, such as albumin. This situation makes the brain more vulnerable to infections and toxins, even if exposure occurs for a short time [76].

6.2 Invasive local delivery

The different techniques for bypassing the BBB can be classified into three categories: non-invasive, invasive, and relatively invasive strategies. Every

technique presents advantages and limitations. Invasive techniques are sometimes necessary to deliver drugs in sufficient quantities directly to the CNS (e.g., anti-cancer drugs and neurotrophic factors) [77]. The degree of invasiveness of the techniques varies and includes cutting or puncturing the skin or internal organs, inserting instrumentation, or producing a mechanical or chemical shock [78]. The invasive techniques are listed in **Table 1**, along with their respective advantages and disadvantages.

Invasive local delivery	Advantages	Disadvantages
Interstitial administration: Direct intracerebral administration through injections *via* intrathecal (IT), intracisternal magna (ICM), or intracerebroventricular (ICV). The administered drug is rapidly extracted from the CSF outflow tract by the arachnoid villi into the general circulation, from where it reaches the cerebral circulation and crosses the BBB. The drug can also reach the brain parenchyma from the CSF through two other pathways; however, these are very limited for effective drug distribution.	Drugs are administered directly into the CSF; however, this technique is considered equivalent to slow IV administration of the drug [34, 79].	Although it was considered the most attractive route of administration for the treatment of primary brain tumors, it has disadvantages, such as higher risk of CNS infection, catheter obstruction, minimal penetration into the brain parenchyma, and inadequate drug distribution. These techniques do not apply to all brain pathologies, especially in lesion sites that are located deeply within the brain [34].
Convection-enhanced delivery (CED). Catheters are stereotactically placed through cranial burr holes into the deep brain regions, and microinfusion pumps are used to improve therapeutic delivery into the brain using specially formulated infusate [78–80].	Catheters stereotactically placed into the brain through microinfusion pumps improve therapeutic delivery into the brain. Not produce cerebral edema or increases in intracranial pressure [78].	The procedure is very invasive, and misplaced catheters could lead to tissue damage and infection risks [80, 81].
Intracerebral implantation. Placing of therapeutic agents in direct contact with lesioned sites using a biodegradable polymeric matrix [82]. Currently, there is one FDA-approved implant, the Gliadel® wafer, impregnated with bis-chloroethylnitrosourea for the treatment of glioblastoma multiforme, which is implanted at the resection site to eliminate infiltrating cells during the tumor resection [67, 82–84].	Placing of drugs using a biodegradable polymeric matrix releases the drug directly and in a controlled manner into the lesioned region [82].	This procedure could be very invasive, with additional tissue damage and infection risks, poor drug penetration, and a diminished safety profile [85]. The main adverse effects are: cerebral edema, intracranial hypertension, meningitis, impaired wound healing, CSF leak, wafer migration, and seizures. Its clinical use has been limited [86].
Biological tissue delivery. Autologous transplantation of chromaffin cells from the adrenal medulla into the brain was initially used to restore dopamine production in the striatum and other neurotrophic factors in the caudate nucleus for Parkinson's disease [87, 88].	To reestablish a physiologic source of dopamine to the striatum [88].	The transplanted tissue could not survive due to the lack of neovascular innervation and, due to the occurrence of other complications, its clinical use was discontinued [87, 89].

Invasive local delivery	Advantages	Disadvantages
BBB disruption (BBBD) by relatively invasive strategies		
Transient osmotic disruption of the BBB.	Hyperosmolar mannitol solution (25%) is the most commonly used to induce the shrinkage of cerebrovascular endothelial cells. This shrinkage induces a disruption of inter-endothelial tight junctions for several hours [90, 91]. It has been successfully used in several clinical studies [92, 93].	There is an unfavorable toxic/therapeutic ratio in clinical studies. However, this technique should be carefully used [94].
Biochemical BBBD strategy. Intracarotid administration of autacoids (bradykinin, histamine, leukotrienes C4 and D4) and alkylglycerols induces opening of the BBB by several mechanisms [95, 96].	It is the least invasive and most reliable technique, primarily targeting the affected vasculature.	Induces: neurological disorders [95]. Pulmonary vasoconstriction and bronchoconstriction [96] and increased ischemic regions and BBB dysfunction [97].
Magnetic resonance-guided focused ultrasound (MRgFUS) for BBBD. Ultrasound pressure waves (frequencies of 20 kHz or greater) can be focused, reflected, and refracted through a medium, and the use of magnetic resonance allows a more precise application of ultrasound waves.	It is a novel method under real-time imaging and thermographic guidance, which is used to deliver drugs to the targeted regions in the brain through the intact skull in clinical trials with encouraging results [98, 99].	Too great force of oscillation to open the BBB can produce hemorrhage. Oscillations induce stretching, acoustic streaming, and shear forces on the vasculature, which can affect the permeability of tight junctions and the function of efflux transporter proteins in the long term [100].

Table 1.
Invasive techniques for disruption of the BBB and drug delivery into the CNS.

6.3 Intranasal administration

Most drugs could pass through the BBB and penetrate easily and quickly into the brain, so in this case, not only the desired effects, but also the side effects can be observed in a very short period, due to the necessity to find an alternative route of administration to decrease that risk. The intranasal administration could be considered a perfect option, specifically to deliver drugs to the CNS [101, 102]. The nose-brain delivery system is a valuable method that allows for the administration of drugs, offering advantages that promote the delivery of drugs and avoid metabolism and specific reactions that decrease the biodisponibility of the drug. This system is used for intranasal administration of drugs, which is a method that does not require special knowledge or the help of another person to administer the drug. To avoid the problems of taste and odor, reducing the systemic concentration of the drug in the system is crucial, as this helps avoid adverse or off-target effects.

Additionally, easy and fast access of the drug to the nervous system is one of the main advantages of this type of drug administration [103]. The nasal route is easily accessible and has a high vascular epithelium, which favors the absorption of drugs and fewer side effects [104]. There are various formulations, including nasal drops,

spray, nanoemulsions or microemulsions, nanogels, powders, nanostructured lipids, liposomes, microspheres, and nanoparticles [105]. In general, the intranasal drug delivery systems are targeted for neurodegenerative diseases, psychiatric disorders, headache and its varieties, sleep disturbances, and brain injuries [106].

Many structures participate in the distribution of the drug during intranasal administration, which involves more than one type of system. In the olfactory mucosa, nerve fibers and branches of the trigeminal nerve facilitate communication from the nose to the brain. However, the complete description of these pathways remains unknown due to the variety of routes by which drugs can reach the brain. In general, two main pathways can be described: (1) communication between the olfactory nerve and the olfactory bulb, and (2) cerebrospinal, both of which distribute in the parenchyma to different brain areas [103]. Furthermore, one of the main limitations is the ability of drugs to permeate the BBB. Still, the intranasal route avoids not only the BBB, but also the gastrointestinal tract, hepatic first pass metabolism, renal clearance, and some other reactions that reduce the biodisponibility of drugs [107]. However, one of the most significant advantages of this type of route administration is the reduction in plasma concentration of the drug, as this approach prevents systemic distribution of the drug [107]. According to the polarity of the molecules, lipophilic drugs are well absorbed, and hydrophilic ones are too; however, the latter sometimes require an enhancer to improve absorption [104].

Finally, it is essential to consider some factors that could affect the nasal absorption, and they could be classified as follows:

1. Patient

    a. Biological facts: changes in the anatomy of cavities or biochemical changes, specifically the presence of enzymes that could degrade the drug

    b. Physiological: some pathological conditions that affect the blood supply, membrane permeability, and local secretions [108]

2. Drug

    a. Physicochemical properties: it include size, polarity, solubility, chemical structure, etc.

    b. Physicochemical properties of formulation: viscosity and osmolarity. Those are properties that change the absorption and sometimes improve the storage of the drug, which could damage the nasal membrane.

Respecting the mechanism described for the transport of drugs using the nasal cavity [105, 109], we can classify according to the neuron:

1. Intracellular: First, the drug is transported by endocytosis or using receptors, and the main structures that participate in this process are the olfactory neurons. The drug is then transported inside the cell by an endosome until it reaches the synapse. After that, the drug is transported to the CNS by exocytosis, but other kinds of transport are necessary to enter the CNS.

2. Extracellular: In this case, the drug passes throughout the space between the epithelial cells to reach the lamina propria. In this site, the drugs could be transported *via* the perineural space by bulk flow.

In general, drugs reach the CNS within 5–30 minutes, depending on the distance, and the maximal concentration is related to the brain region. However, the most useful mechanism of transport is extracellular, and in this case, some studies have demonstrated that it can take 0.7 hours.

One of the main steps in the intranasal route is the absorption of drugs, which means the ability to cross the nasal mucosa to reach an adequate concentration to have an effect in the CNS (bioavailability), according to the characteristics and properties of the drug. All strategies are based on increasing the bioavailability by enhancing the permeability of the drug and reducing its excretion [110, 111]. Some of these strategies are:

a. Prodrug design: to design molecules that use the "natural" reactions of the organism related to the absorption, metabolism, and elimination of drugs.

b. Solubilizing agents: to increase the solubility of the drug to improve the absorption of some formulations and some excipients.

c. Absorption promoter: The primary component that is modulated for this kind of compound is the permeability of the membrane, which could be modified by increasing membrane fluidity and opening junctions. To select the optimal promoter more effectively, it is essential to consider not only the benefits but also the characteristics related to toxicity, allergenicity, and irritant activity.

d. Bioadhesive formulations: to increase the contact time between the drug and the mucosal surface and/or decrease the mucosal drainage.

Many illnesses related to the CNS are treated using drugs by intranasal administration, such as Parkinson's disease, Huntington's disease, and depression. However, there are other kinds of diseases, such as obesity, eating disorders, in which this route of administration has started to be used [112, 113].

7. *In vitro* models of BBB

Since the BBB represents the main barrier to drugs targeting the CNS, several *in vitro* models with varying levels of complexity have been designed to evaluate the permeability across the BBB. However, it is essential to highlight that the highly dynamic nature of the BBB and the diverse factors that can influence its permeability, none of these models perfectly replicate *in vivo* conditions. For this reason, the selection of an appropriate model must match the study's specific aim, and the results must be interpreted in consideration of these limitations. The following section briefly describes the most commonly used BBB *in vitro* models, which can be implemented in most laboratories without requiring extensive bioengineering expertise.

7.1 Parallel artificial membrane permeability assay (PAMPA)

A highly employed cell-free method in the pharmaceutical industry to measure the passive diffusion of drugs targeting the CNS is the parallel artificial membrane permeability assay (PAMPA). This assay was initially developed to predict drug absorption in the gastrointestinal tract [114]. In this assay, an artificial membrane

composed of a porcine brain lipid extract dissolved in n-dodecane (PAMPA-BBB) or on black lipid membranes (PAMPA-BLM) divides two compartments, which are filled with buffer solutions. Since the studied drug is dissolved in one of the compartments, it can cross through the artificial membrane, thus evidencing its BBB permeability [115]. However, it is necessary to highlight that the permeability of the studied drugs can vary in function of the lipid mixture, indicating that lipid composition selection is crucial for assessing passive permeability [116].

The main advantages of PAMPA *in vitro* models arise from their low cost and technical simplicity, which makes them an ideal option for high-throughput screenings [114]. In contrast, as can be expected, the main disadvantages include the impossibility of evaluating the effect of BBB-specific transporters and the changes in permeability induced by pathological conditions.

7.2 Cell-based static models

Although static BBBs do not imitate the shear stress generated by cerebral blood flow, these models are widely employed due to their technical simplicity and low cost. In these models, a layer of brain microvascular ECs is cultured on the upper side of a semipermeable membrane insert (Transwell ®) with a pore size ranging from 0.2 to 0.4 μm. This design facilitates the diffusion of small molecules and avoids cell migration. The insert is placed into a cell culture well, thus replicating the luminal side of the BBB and the parenchymal side, respectively. In this model, the permeability can be determined in relation to the permeability of specific markers, such as radiolabeled sugars, including sucrose or mannitol, and fluorescent molecules such as sodium fluorescein [117]. However, due to a lack of relevant information regarding cell culture conditions, the expression of transporters may produce significant variations among studies.

7.3 The cone-plate BBB apparatus

The cone-plate BBB apparatus was designed to model the effects of cerebral blood flow on monolayer cocultures of brain microvascular ECs and astrocytes [118]. This device is composed of a rotating cone that generates shear forces on the ECs through the surrounding medium. Thus, the cone angle, velocity, and their nature (pulsatile or laminar) produce different levels of shear stress in the cocultures [119]. However, although this model attempts to simulate physiological conditions, a significant limitation of this method is that shear stress is not distributed uniformly throughout the coculture monolayer, which produces substantial differences in the shear stress conditions generated by brain microvascular ECs *in vivo* [120].

8. Conclusions

Pharmacokinetics refers to the processes and reactions that occur within an organism, starting from the delivery of a compound from its formulation until it reaches the target organ or tissue. However, these processes become more complex when dealing with the CNS, making drug delivery a particular challenge. This complexity arises from the intricate anatomy and structures that hinder the free diffusion of a drug into the brain. To enhance brain penetration, several key characteristics of the drug must be considered. These include the drug's lipid solubility, active transport

mechanisms, and protein binding. These factors influence the drug's ADME properties, which directly impact its ability to cross the BBB. In addition to systemic methods of drug administration, alternative delivery systems, such as the nose-to-brain route, have emerged as promising solutions to overcome the challenges associated with crossing the BBB. Furthermore, *in vitro* assays can serve as practical tools to evaluate the permeability of a drug to cross the BBB. Understanding these factors is essential for optimizing drug delivery, achieving the desired therapeutic effect, and minimizing potential toxicities.

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Chapter 3

Ocular Pharmacokinetic Studies: Challenges and Best Practices

Marianna Bacellar-Galdino, Sandeep Jain and Simon Kaja

Abstract

Pharmacokinetic studies are conducted during drug development to increase the understanding of the absorption, distribution, metabolism, and excretion profile of a given drug within the body. As such, these studies offer critical information about drug concentrations in the systemic circulation and target tissues over time, guiding dosing strategies, therapeutic windows, and safety margins. However, systemic PK data often fail to accurately predict ocular exposure. This is due to the unique anatomy and physiology of the eye, including the presence of a strong blood-ocular barriers, numerous levels of compartmentalization, and limited lymphatic drainage. Therefore, ocular PK studies are specifically designed to evaluate the distribution and clearance of drugs within the sub-tissues of the eye. In this chapter, technical challenges associated with the design and execution of ocular PK studies will be highlighted, and we will present best practices, and provide examples highlighting the need for ocular PK studies to ensure drug safety.

Keywords: ocular, ophthalmic, pharmacokinetic, anterior segment, posterior segment

1. Introduction

Pharmacokinetic (PK) studies are a critical component of the drug discovery and development process. The evaluation of the tissue levels of a therapeutic agent, or test article, in the eye and in the different ocular sub-tissues as well as the analyses of the ocular structure are essential components in ocular studies [1].

Animal use in preclinical research has been the gold standard for drug development and the advancement of knowledge. Animal research is premised on the fact that there are many similarities between animal models and human beings [2–5]. Preclinical animal studies contribute to the understanding and advancement of new therapies, guide dosages and exposure effects in humans as well as the creation of new surgical techniques [4].

In vision research and ocular drug discovery, the importance of animal models cannot be overstated as they provide an opportunity to understand biological mechanisms in a controlled setting. Due to the invasive sampling from the human eye and ethical restrictions, ocular PK relies on animal models [6]. As such, studies in laboratory animals help ensure the safety of novel treatments for the many ophthalmic conditions with a high unmet clinical need.

One of the many challenges is translational relevance, where anatomical, physiological, and genetic differences are major factors to be considered. Even though animal models are an indispensable tool, it is also important to keep in mind the use of the "3Rs" of animal research. In this chapter, we will discuss the technical challenges associated with the design and execution of ocular PK studies, present best practices that will guide to reduce and refine study designs and provide examples highlighting the need for ocular PK studies to ensure drug safety.

2. Experimental design considerations

Little specific guidance is provided by the Food & Drug Administration (FDA) or the International Conference on Harmonization (ICH) pertaining to the regulatory expectations for the design of preclinical PK studies required for ophthalmic drugs. This is in part due to a variety of factors that affect the appropriate design of PK studies, including anatomical and physiological considerations, the route of administration, and physiochemical properties of the test article. In addition, the anticipated dose and dosing frequency, disease indication, and targeted patient population determine the most appropriate experimental design.

To ensure quality and reliability of Investigational New Drug (IND)-enabling studies, adherence to Good Laboratory Practice (GLP) is generally required for toxicology studies. In contrast, primary ocular PK studies do not need to be conducted in compliance with GLP standards to meet FDA requirements [7].

Best practices involve a well-structured sampling schedule that should include time points that capture at least three, but preferably five elimination half-lives in the target tissue to adequately characterize the terminal phase. Additionally, the study should aim to cover at least 80% of the area under the concentration–time curve (AUC). Such a design can ensure robust estimation of ocular PK parameters. As for toxicokinetic (TK) analyses, dense sampling during the early phase is important to define absorption and peak concentrations, while extended sampling is necessary for long-acting formulations, such as intravitreal injections or sustained-release medical devices. Generally, parallel sampling from relevant ocular compartments or ocular sub-tissues is conducted, as discussed below.

3. Factors influencing ocular drug distribution

3.1 Anatomical and physiological considerations

The eye presents with complex anatomy and physiology that present significant challenges to effective drug delivery and pharmacokinetic analysis and modeling. Compared with other organs, the eye shows a high degree of compartmentalization with highly restricted permeability across tissue boundaries (for review, see [8]). These barriers are both static and dynamic and include the tear film, the corneal epithelium, conjunctiva, sclera, and intraocular barriers, including the retina [9].

The corneal epithelium represents the major barrier to topically administered test articles, as tight junctions between corneal epithelial cells prevent entry of hydrophilic molecules and preferentially permit passive diffusion of small lipophilic molecules [10]. In contrast, the blood-ocular barriers, which comprise the blood-aqueous and blood-retinal barriers, effectively limit penetration from the systemic

circulation into intraocular compartments. This substantially reduces the biodistribution of systemically administered agents to ocular tissues [8, 11].

Modeling of ocular PK properties is further complicated by the presence of active drug transporters (e.g., P-glycoprotein) and metabolizing enzymes (e.g., cytochrome P450 isoforms) in ocular tissues [12–14]. Activity of these proteins modifies drug absorption, distribution, and elimination contributing to the non-linear kinetics and inter-individual variability observed when studying ocular PK properties.

Collectively, these anatomical and biochemical barriers result in ocular sub-tissue specific pharmacokinetics that cannot be well predicted using standard compartmental models. Ongoing efforts focus on the development of novel compartmental and physiologically-based PK models and finite element models aided by the use of artificial intelligence [15].

3.2 Routes of administration

The route of ocular drug administration significantly influences the PK properties of test articles and can—in part—overcome the limitations of achieving therapeutic tissue levels by differentially affecting anatomic barriers and ocular fluid dynamics. When designing ocular PK studies, it is critical to determine the most appropriate preclinical laboratory species. This selection is driven by anatomical and physiological considerations as well as by volumes that can be safely administered (**Table 1**; for review, see [1]).

Topical administration is the most common route for treating anterior segment diseases such as dry eye or glaucoma. However, ocular tissue distribution is limited by significant precorneal loss as a result of tear turnover, blinking, and drainage via the nasolacrimal duct. Estimates suggest that less than 5% of the administered dose reaches intraocular tissues [16]. Permeation of test articles following topical application can occur through the lipophilic corneal pathway or the hydrophilic conjunctival–scleral route. However, conjunctival vascular absorption often leads to systemic exposure rather than intraocular delivery (**Figure 1**) [17].

Intracameral injections are used for delivery of test articles, including sustained release devices for the anterior segment diseases and antimicrobial prophylaxis associated with ocular surgery.

For diseases affecting the posterior segment of the eye, such as age-related macular degeneration or diabetic retinopathy, intravitreal injections deliver highest posterior

	Mouse	Rat	Rabbit	Minipig	Cat	Dog	NHP
Topical	5 µl	10 µL	50 µL	50 µL	50 µL	50 µL	50 µL
Intracameral	2 µL	5 µL	70 µL	50 µL	70 µL	70 µL	50 µL
Subconjunctival	2 µL	5 µL	100 µL	100 µL	100 µL	100 µL	100 µL
Periocular	2 µL	5 µL	0.5 mL	2 mL	0.5 mL	4 mL	4 mL
Intravitreal	2 µL	5 µL	100 µL	200 µL	200 µL	200 µL	250 µL
Subretinal	1 µL	2 µL	100 µL	200 µL	200 µL	200 µL	200 µL
Suprachoroidal	3 µL	5 µL	100 µL	200 µL	200 µL	200 µL	200 µL

Table 1.
Dosing volumes for different route of ocular drug delivery across the most commonly used preclinical laboratory species. Typical maximal volumes are listed. It is important to note delivery volumes may need to be adjusted in the case of repeated drug delivery.

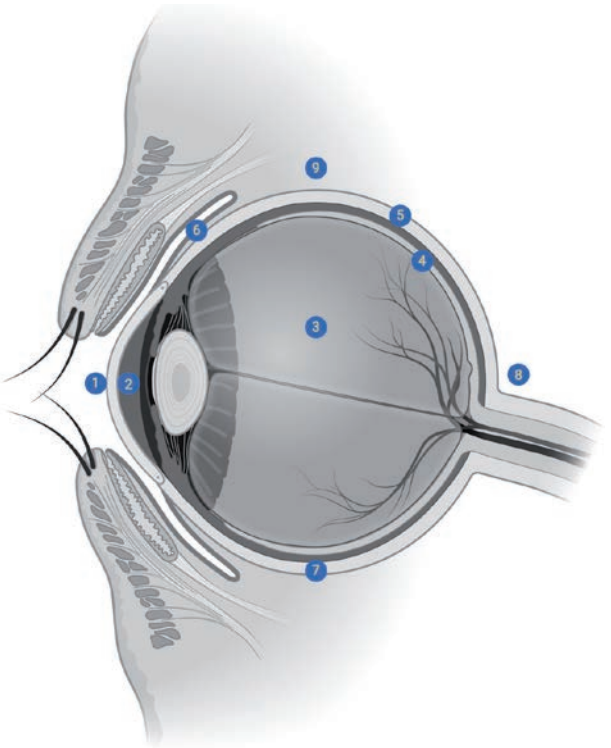


Figure 1.
Common routes of ocular drug delivery. Schematic illustration of the principal ocular drug delivery routes: (1) topical, (2) intracameral, (3) intravitreal, (4) subretinal, (5) suprachoroidal, (6) subconjunctival, (7) intrascleral, (8) posterior sub-Tenon's, (9) anterior sub-Tenon's. Created in BioRender. Kaja, S. (2025) https://BioRender.com/gsnq6ps.

segment tissue distribution and have become daily clinical routine [18]. Intravitreal injections bypass the external and vascular barriers, enabling direct delivery into the vitreous humor, from where test articles can diffuse to the retina [9]. The major contributors to elimination from the vitreous include aqueous outflow via the anterior route and efflux into the choroidal circulation via the posterior route. Importantly, intravitreal injections can be performed in all preclinical laboratory species. However, the anatomical differences between species, especially the relative size of the lens and vitreous, affect tissue distribution of test articles, ocular clearance, and thus, translational relevance. Therefore, rabbits and non-human primates offer the most translatable models for evaluating PK parameters of intravitreally delivered test articles.

In recent years, subretinal delivery has gained significant interest, especially as a means to deliver retinal gene therapy [19]. Subretinal injections offer the precise delivery between the retinal pigmented epithelium and the photoreceptor layers, causing a bleb and subsequent diffusion of the test article.

Suprachoroidal injections are an emerging method for targeted drug delivery to the posterior segment involving the administration of test articles into the suprachoroidal space [20]. The suprachoroidal space is best described as a *potential* space between the sclera and choroid. Suprachoroidal delivery minimizes exposure to structures of the anterior segment and lowers the potential of risk of intraocular complications compared to intravitreal injections. However, suprachoroidal injections

have not yet been widely adopted due to their technically challenging nature and the limited availability of tools to confirm successful injection, especially in rodents.

Another aspect driving selection of the route of administration for ocular PK studies is based on the physiochemical properties of the test article. The maximal permissive volumes for intraocular delivery are further complicated by differential tolerance of ocular sub-tissues for excipients, pH, osmolarity, or viscosity.

It should also be noted that preclinical laboratory animal species, especially rabbits, are highly sensitive to endotoxin. While the United States Pharmacopeia (USP) General Chapter <85> [21] and the ICH Q6A Specifications [22] mandate a maximum allowable endotoxin limit of 0.2 EU/mL, rabbits and rodents may present with inflammatory responses at levels of >0.01 EU/eye. For example, the no-observable effect level (NOEL) was determined as 0.01 EU/eye in pigmented Dutch Belted rabbits for intravitreal injection [23]. Therefore, it is important to conduct endotoxin testing even at early stages in the drug discovery process to not mistake endotoxin responses for test article related toxicity. This is particularly important as endotoxin responses can affect tissue distribution in PK studies.

Taken together, different routes of ocular drug administration can improve targeted delivery of test articles to specific ocular sub-tissues by avoiding or minimizing the contribution of anatomical and functional barriers. However, specialized ocular delivery further complicates pharmacokinetic modeling and simulations.

4. Considerations for tear PK analysis

While the concentration of test articles in the tears is of therapeutic relevance, the assessment of tear PK properties is challenging due to sampling variability and the small volume of tears in most preclinical laboratory animal species. Furthermore, determination of tear PK properties must take into account the route of administration, physiochemical properties of the test article, and the species tested.

For systemically delivered drugs, the concentration of free drug in the plasma is a primary determinant for excretion into the tears, however, this typically only occurs for unionized drugs that have some lipid solubility [24]. In contrast, ionized drugs (acidic or basic), the level of excretion into tears is highly dependent on tear pH, which may show high inter-individual and diurnal variation [24–26]. For example, tear pH is significantly more acidic after prolonged eye closure [25]. Tear pH can further be impacted by topical medications e.g. [27].

One additional factor affecting ocular PK of ophthalmic drugs is their ability to bind melanin pigment, which is expressed in the iris and ciliary body. One prominent example of a topically administered drug with significant melanin binding is the beta-adrenoreceptor agonist, timolol [28, 29]. Melanin binding can be exploited to enhance the efficacy of long-acting drugs, as the releases kinetics of the drug from melanin is generally slow [30]. Ocular PK properties of test articles exhibiting melanin binding should therefore be tested in pigmented species, such as Dutch Belted rabbits.

Species differences in tear film composition, thickness and blink rate (**Table 2**) further contribute to the challenges in tear PK studies. This is particularly noteworthy for the rabbit which represents the most used species for initial ocular toxicology and PK studies.

Rabbits do not produce a large quantity of tears and that tear volume is highly variable [31]. Extrapolation of tear PK from rabbits to humans is confounded by the significant differences in inter blink time of around 10 min compared with humans (5–8 s), which results in significantly longer tear break up times (TBUT) in rabbits

Species	Tear film thickness	Tear turnover rate	Tear film break-up time (BUT)	Blink rate
Mouse	~5 μm	High	Short	High (~10–20/min)
Rat	~5 μm	High	Short	High (~10–20/min)
Rabbit	~10 μm	Moderate	~30 minutes	Low (~1 blink/10 min)
Cat	~10 μm	Moderate	Moderate	Moderate (~1–2/min)
Dog	~10 μm	Similar to humans	Moderate	Moderate (~3–5/min)
Non-Human Primate	~7–10 μm	Similar to humans	8–30 seconds	Most similar to humans (~15–20/min)

Table 2.
Species differences in tear film thickness, tear turnover, tear film break-up time and blink rate.

compared with humans (**Table 2**; [32]), and which is the result of rabbits having a much more stable tear film.

Accurate sampling of tears poses additional challenges beyond the physiochemical and pharmacokinetic properties of test articles. Tear volume collection is inherently variable requiring substantial dilution for bioanalytical analysis. Collection of tears is performed using phenol-red coated threads, Schirmer's tear test strips, or microcapillaries. However, collection of tears for PK sampling depletes non-stimulated tears and elicits production of stimulated (reflex) tears, thereby diluting residual tears. As a consequence, any tear PK measurement would not reflect an accurate measurement of the concentration of test article on the surface. Furthermore, this dilution effect will make repeated sampling in the same animal prohibitive, as test article is removed from the surface during sampling.

In rodents, tear volume quantification and sampling are most commonly performed using phenol red coated threads. However, as of recently, these are no longer available commercially and research efforts have been made to develop custom-made threads and determine their accuracy and variability [33].

In rabbits, typical mean Schirmer's tear test (STT) volumes have been reported to be around 5 mm, which corresponds to an approximate 3 μl volume [31, 34], with a range of 3–15 mm for a 60 s test. While the clinical utility of STT for diagnostic purposes in clinical veterinary practice remains undisputed, the suitability for systematic PK evaluation remains questionable.

In large animals and humans, tears can be collected as ocular surface washes (OSW), which involves instilling a small volume of sterile saline, buffer, or lubricating eye drops onto the ocular surface and then recovering the fluid using a microcapillary or micropipette. OSW collection is a minimally invasive and a more reproducible method for obtaining tear samples, which is particularly useful in species or conditions with low tear volumes. OSW collection enables the efficient recovery of soluble tear film components while minimizing reflex tearing and mechanical stimulation, reducing variability associated with tear collection. As such, OSW collection is best suited for pharmacokinetic analysis and related downstream biochemical analyses, including proteomics and cytokine profiling.

5. Considerations on eye dissection and fixation for histology

Compared to systemic PK or TK studies, each time point assessed in an ocular PK study requires a separate cohort of animals, as repeated sampling is prohibitive.

Therefore, minimizing the variability in sample collection and processing is critical for the accurate quantification of the concentration of test articles in the ocular sub-tissues, the analysis of the ocular structure, and minimization of animal numbers. Sub-tissue dissections, however, require expertise and experience due to the eye size and its unique structure.

The selection of the best species is usually driven by the type and amount of tissue needed to support bioanalytical analysis and/or species similarities to targeted tissue. The use of rodents, especially mice, in PK studies is limited due to their size. While mice offer the study of genetic mutations and represent an important tool for drug development, a larger animal sample size is required to yield the tissue amount needed for bioanalytical analysis [35]. The rabbit is still the most used species in ocular PK studies as many ocular disease models have been developed in rabbits allowing for pharmacodynamic (PD)/PK studies [36–38]. Lately, minipigs have been used more frequently due to their similar eye size and retinal anatomy to humans. Larger eyes are usually better suitable as the ocular sub-tissues are more easily isolated with lower contamination rates; many sub-tissues cannot routinely be dissected or sampled from rodents, e.g. the trabecular meshwork or the vitreous humor (**Table 3**).

Two techniques involving either fresh or frozen eyes are being extensively used. Frozen dissections allow for tissue preservation and later separation with less anticipated cross-contamination between ocular sub-tissues. However, the freezing process may create some obstacles including freezing artifacts, challenges during tissue separation, and a decrease in the amount of tissue collected. Fresh dissection is considered best practice for ocular PK studies as it provides better separation of tissues as well as less contamination. Furthermore, gross macroscopy observation can be recorded during dissection.

Even though most PK studies focus on sub-ocular tissue collection for test article concentrations, ocular examination and pathology increases the understanding of safety and toxicity of test articles. As discussed above, ocular PK, safety assessment, and histopathological evaluations may be incorporated into study designs to minimize

	Ocular sub-tissue
Posterior segment	Retina
	RPE*
	Choroid*
	Sclera
	Vitreous humor**
Anterior segment	Cornea
	Conjunctiva
	Lens
	Iris/ciliary body
	TM***
	Aqueous humor***

**RPE and choroid are sampled together with sclera in rodents, however, RPE/choroid can be separated from the sclera by scraping. RPE can be separated from the choroid only in pigmented animals, not in albinos (such as NZW rabbits).*
***Vitreous humor can be collected from rats, but typical collection volumes are <5 μl.*
****Trabecular meshwork (TM) and aqueous humor cannot readily be samples from rodents.*

Table 3.
Ocular sub-tissues collected for ocular PK studies.

animal use. Ophthalmic examinations, as reviewed previously [1], are a macroscopic tool to ensure safety and guide further histological assessments. These exams help build the clinical knowledge of in vivo test article effects.

Whole eye collection, trimming of tissue, and fixation are extremely important as artifacts derived from those steps can compromise the histopathologic interpretation and potentially result in false-positive indications of toxicity.

Generally, processing for histopathological analysis begins with enucleation at necropsy. To maintain the correct orientation during trimming, the globe is typically marked with a suture (or tissue ink) at the 12 o'clock position. Unless needed, conjunctivae and muscles are trimmed from the globe, and the eye is fixed whole to minimize artifacts [39]. Fixative selection is dependent on target tissue, eye size, and downstream analysis. For example, if the tissue of interest is the retina, 10% neutral buffered formalin may not be an appropriate fixative as it produces a less optimal retinal preservation and artifactual retinal detachments; on the other hand, it is well suited for anterior segment studies.

Booler et al. advise the use of Davidson's fixative or Modified Davidson's fixative for a period of 24 h (up to 48 h for larger eyes) as the primary choice for immersion fixation for light microscopy in toxicity studies. The fixation is followed by a rinse and transfer to 70% ethanol or 10% neutral buffered formalin (NBF) for storage [39]. The authors also suggest changes in fixation protocols when working with immunohistochemistry, *in situ* hybridization, and electron microscopy.

6. Conclusions

Ocular PK studies are an integral component of IND-enabling studies for ophthalmic drugs. With minimal regulatory guidelines, adhering to best practices in the experimental design of non-GLP ocular PK studies is critical to ensure the safety of novel drug candidates.

An in-depth understanding of ocular anatomy and physiology, including the role of ocular barriers and drug transporters, technical skills in delivering test articles to the eye, and expertise in dissection of ocular sub-tissues are critical to generate high-quality data for regulatory submission that can be translated to humans. Ultimately, understanding the challenges and best practices associated with ocular PK models will aid the development of better therapies.

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Conflict of interest

Sandeep Jain holds equity ownership in Selagine, Inc., Advaite, Inc. Dr. Jain is a consultant for Neutrolis, Inc. and Amgen, Inc. Marianna Bacellar-Galdino is an employee of Altasciences. Dr. Kaja is the principal of K&P Scientific LLC.

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Chapter 4

Development and Validation of an HPLC-PDA Method for Quantitative Bioanalysis of Curcuminoids and Its Application to Preclinical Pharmacokinetics

Manish Issar

Abstract

Curcuminoids, including curcumin (CUR), demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC), are polyphenolic compounds from *Curcuma longa* with diverse pharmacological activities but limited clinical translation due to poor bioavailability and instability. Sensitive bioanalytical methods are therefore essential to quantify curcuminoids in plasma for pharmacokinetic and formulation studies. While LC-MS/MS is the bioanalytical gold standard, its high cost and limited accessibility underscore the need for validated HPLC-photodiode array (PDA) assays in academic and preclinical settings. Here, we describe the development and validation of an HPLC-PDA method for the simultaneous quantification of CUR, DMC, and BDMC in mouse plasma, in accordance with FDA and EMA bioanalytical guidelines. Validation included calibration model selection, accuracy, precision, recovery, and stability. Weighted $1/x^2$ regression with intercept, applied to analyte/IS area ratios, provided the most reliable calibration performance, minimizing bias at lower concentrations. Intra- and inter-day accuracy and precision were within acceptance limits ($\leq$15% CV, $\pm$15% bias; $\leq$20% at the LOQ). Recovery was moderate (20–35%) but reproducible, and freeze-thaw studies demonstrated stability after 1 cycle, with significant losses for CUR and DMC after 2 cycles at -20°C, whereas BDMC showed greater resilience. Finally, the validated method was applied to a pharmacokinetic study in mice given a curcuminoid-loaded self-emulsifying drug delivery system (SEDDS). Plasma concentrations at 30 minutes post-dose confirmed systemic absorption and enabled screening of prototype formulations. This robust and accessible assay provides a cost-effective alternative to LC-MS/MS and supports translational research on curcuminoid pharmacokinetics and formulation development.

Keywords: Curcuminoids, HPLC-PDA, validation, accuracy, precision, matrix stability, mouse plasma, oral, SEDDS, pharmacokinetics

1. Introduction

Curcuminoids, the polyphenolic pigments of turmeric (*Curcuma longa*), have long been used in Indian cuisine and Ayurveda. Their pharmacological profile includes anti-inflammatory, antioxidant, and anticancer effects, and the three main constituents—curcumin (CUR), demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC)—are still the focus of biomedical research.

Curcumin has also been studied as a potential imaging probe for Alzheimer's disease due to its affinity for β-amyloid plaques in the retina [1, 2]. While this provides a non-invasive way to monitor disease progression, its usefulness is limited by very low systemic exposure after oral dosing, which restricts transport across the blood-retinal barrier. Animal and human studies consistently show poor absorption, with oral doses up to 340 mg/kg rarely producing more than 250 ng/mL of free curcumin in plasma [3]. Higher levels often consist of conjugated metabolites such as curcumin O-glucuronide, which are polar and unable to cross neurological barriers.

Improving systemic exposure has therefore been a central challenge. One approach is lipid-based self-emulsifying drug delivery systems (SEDDS), which promote lymphatic uptake. Prototype formulations were screened *in vitro* and then evaluated in mice by measuring plasma concentrations 30 minutes after oral gavage.

A reliable bioanalytical method is essential for such studies. Despite the sensitivity advantages of LC-MS/MS, its use is constrained by expense and instrument access. For practical reasons, many laboratories continue to rely on HPLC with PDA detection in preclinical studies. However, curcuminoids present specific obstacles: poor solubility in gastrointestinal fluids (4–11 ng/mL at 25°C), hydrophobic character (log P $\sim$ 3.2), and rapid degradation at pH $\geq$6.8 through hydrolysis, oxidation, and metabolism [4, 5]. Light exposure further accelerates their breakdown. In vivo, they are also converted to metabolites such as tetrahydrocurcumin and glucuronides, which can overlap chromatographically with parent compounds or matrix components.

Despite these hurdles, curcuminoids are strongly chromogenic (λ_m 420–430 nm), and by optimizing chromatographic conditions at this wavelength, HPLC-PDA can still provide selective and reproducible quantification. In this chapter, we describe the development and validation of such a method, and its application to a mouse pharmacokinetic study following oral administration of curcuminoids in a SEDDS formulation.

2. Materials and methods

2.1 Chemicals and reagents

Curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) reference standards (>99% purity) were purchased from ChromaDex Inc. (a Niagen Bioscience subsidiary). The total curcuminoids, containing 82.24% curcumin, 9.7% DMC, and 0.99% BDMC—totaling 92.93%—were bought from Total Herb Solutions (THS) in Mumbai. Clozapine, used as the internal standard, was obtained from Fischer Scientific. HPLC-grade acetonitrile, methanol, and formic acid were purchased from Millipore and Sigma, USA. De-ionized water for HPLC was purified using a Milli-Q system (Millipore, Bedford, MA, USA). The Impact™ rapid protein precipitation plates, vacuum manifold, and F5 Kinetex® column were acquired from

Phenomenex, Torrance, CA, USA. Mouse plasma for validation was obtained from Equitech-Bio, Inc., Texas, USA.

2.2 Preparation and characterization of curcuminoid-loaded SEDDS formulations

Curcuminoid-loaded SEDDS (4% w/w CUR, DMC, BDMC) were prepared by dissolving the drugs in lipid excipients (Gelucire® 48/16, castor oil, Caproyl 90, Croduret®) at 60°C with trituration until fully solubilized. Selected formulations were fortified with a solid amorphous dispersion (ASD) of curcuminoids in Kollidon® VA64 (SEDDS-ASD). All formulations were stored in amber vials and assayed for drug content after 24 hours using HPLC. Particle size and zeta potential were measured by dynamic light scattering to confirm dispersion stability. For *in vitro* characterization, the solubility of curcuminoids in oils was evaluated, and the aqueous dispersibility and stability of SEDDS were assessed. Forced lipolysis studies with pancreatin were performed to mimic gastrointestinal digestion. Subsequently, formulations were tested *in vivo* for plasma exposure after oral gavage in mice.

2.3 Animal housing and blood collection

All procedures involving animals in this study were approved by the Western University of Health Sciences IACUC and adhere to the NIH Guide for the Care and Use of Laboratory Animals. All animals were housed in the vivarium in groups of five, following standard husbandry policies established by the institute. They were acclimated before the study and fasted overnight, with water provided *ad libitum* throughout. The animals ranged in age from 4 to 6 weeks and weighed between 22 and 30 grams. A dose of 100 mg/kg of total curcuminoids was administered orally via gavage. Terminal blood samples were collected 1 hour after dosing following euthanasia with isoflurane anesthesia. Four microliters of 0.5 M EDTA were added to each blood collection tube. All samples were centrifuged at 2800 *x g* for 10 minutes, and the plasma was aliquoted and analyzed on the same day as collection.

2.4 Stock and standard solution preparation

Individual stock solutions of CUR, DMC, BDMC, and clozapine were prepared in DMSO at a concentration of 1 mg/mL. Combined working stock solutions (WSS) were then made with concentrations of 250 μg/mL CUR, 125 μg/mL DMC, and 50 μg/mL BDMC in DMSO. A 100 μg/mL working stock solution of clozapine was also prepared in DMSO to serve as an internal standard. Calibration standards were created in mouse plasma by spiking 10 μL of the curcuminoid WSS into 0.5 mL of blank mouse plasma, followed by serial dilution to achieve CUR/DMC/BDMC concentrations ranging from 25 to 2500 ng/mL (25, 50, 100, 200, 500, 1250, and 2500 ng/mL). For DMC and BDMC, the calibration curves ranged from 12.5 to 1250 ng/mL and from 6.25 to 625 ng/mL, respectively. Similarly, standards for the analytes were prepared by serial dilution in methanol. All spiked plasma solutions were analyzed by HPLC on the day they were prepared.

2.5 Sample preparation

To both blank and spiked plasma (50 μL), 10 μL of clozapine WSS was added and vortexed for 5 seconds. The plasma samples were then precipitated with 150 μL of

methanol and vortexed for 45 seconds. The mixture was transferred to a 2 mL square, 96-well Impact protein precipitation cartridge (Strata-X polymeric SPE) mounted on a vacuum manifold to filter the contents into a 96-well deep-well plate. A low-grade vacuum was applied for 10 minutes, allowing the fluid to collect in the bottom chamber of the acceptor plate. The total volume was then pipetted out and transferred to a low-volume auto-injector vial for immediate HPLC analysis.

2.6 Apparatus and chromatographic conditions

A binary pump (Flexar Series, PerkinElmer, paired with Chromera communication software, USA) was used to pump the mobile phase (pump A: acetonitrile with 0.1% formic acid; pump B: ultrapure water with 0.1% formic acid) at a flow rate of 0.2 mL/min. Chromatographic separations were performed on an F5 reversed-phase column (F5 Kinetex® 2.6 μm, 150 × 3 mm i.d., Phenomenex Inc., Torrance, CA, USA), with optimal separation achieved through gradient elution. The initial solvent concentrations in pump A (organic phase) and pump B (aqueous phase) were 50% and 50%, respectively. The organic phase increased to 60% over 10 minutes, then decreased linearly to 50% in 3 minutes. A 10 μL sample was injected using an autosampler (Flexar series, PerkinElmer) with a 20 μL loop, employing a partial filling technique. The analytes eluting from the column were detected with a photodiode array detector (Nexera X2 SPDM30A, Shimadzu, Japan) set at 425 nm. Chromatographic peaks were integrated using LabSolutions chromatography software (Shimadzu, Japan).

2.7 Validation

The optimized HPLC-PDA method was validated following international bioanalytical method validation guidelines, including those from the U.S. Food and Drug Administration [6] and the European Medicines Agency [7]. Validation parameters included selectivity, sensitivity, calibration model performance, accuracy, precision, recovery, matrix effects, and stability. Acceptance criteria were set at ±15% for accuracy and ≤ 15% for precision at all QC levels (±20% at the LLOQ), with at least two-thirds of replicates required to meet these limits.

2.7.1 Selectivity

Selectivity was assessed by analyzing blank mouse plasma samples (n = 6 lots) to confirm the absence of endogenous interference at the retention times of curcumin (CUR), demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC), and the internal standard (clozapine). Chromatograms were examined at 425 nm (curcuminoids λ_{max}) and 384 nm (IS λ_{max}). To verify selectivity, blank plasma samples were compared with plasma spiked at the LLOQ (50 ng/mL) and mid-level QC concentrations. The absence of co-eluting peaks at the analyte or IS retention times indicated sufficient selectivity.

2.7.2 Lowest limit of detection (LOD) and quantitation (LOQ)

The HPLC assay detection limits for CUR, DMC, and BDMC in mouse plasma were determined after sample cleanup, based on a signal-to-noise ratio (S/N) of three times the baseline noise (S/N > 3). The lowest limit of quantification (LLOQ) is defined as

the lowest sample concentration that can be measured with acceptable accuracy and precision, typically with less than 20% deviation from the theoretical lowest concentration.

2.7.3 Calibration curve, model selection, and verifications

Calibration curves were developed on each of six analytical days (n = 6 days) by spiking blank mouse plasma with curcumin (CUR), demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) across the assay range. Curves were created by plotting analyte-to-internal standard (IS) peak area ratios against nominal concentrations. Various weighted linear regression models were evaluated, including ordinary least squares (unweighted), $1/x$, $1/\sqrt{x}$, $1/\log(x)$, and $1/\tan(x)$ schemes. Model performance was assessed based on the correlation coefficient (r^2), residual distribution, and the accuracy of back-calculated concentrations.

The $1/x^2$ weighted linear regression consistently minimized heteroscedasticity and provided the most reliable fit across days, making it the primary calibration model. Daily calibration curves were verified by back-calculating standards, with acceptance defined as ≤15% deviation from nominal values (≤20% at the lower limit of quantification, LLOQ), in accordance with FDA [6] and EMA [7] guidelines. Unknown sample concentrations were determined using calibration curves prepared in the same matrix on the same day as the analysis.

2.7.4 Validation: Accuracy and precision

Intra- and inter-assay accuracy and precision were assessed in mouse plasma using quality control (QC) samples prepared at four levels: low (LLOQ, 50 ng/mL), medium (200 ng/mL), intermediate (1250 ng/mL), and high (2500 ng/mL). Each QC level was analyzed in quadruplicate across six independent runs. Accuracy was calculated as %Bias = 100 × (measured – nominal)/nominal, and precision was expressed as %CV = 100 × (SD/mean). Intra-day (repeatability) was determined across replicate QC samples within each day. Inter-day (reproducibility) was evaluated by pooling data across valid days, with Day 5 excluded due to systematic calibration failure. Acceptance criteria followed FDA [6] and EMA [7] guidance, with an accuracy of ±15% (±20% at the LLOQ) and a precision of ≤15% (≤20% at LLOQ). At least two-thirds of QC replicates needed to meet these limits.

2.7.5 Validation: Recovery

Recovery of CUR, DMC, and BDMC from spiked quality control samples was assessed by comparing the peak response (peak height) from a post-extracted plasma sample to the peak response obtained after analyzing the corresponding standard dilutions in methanol, injected directly at concentrations of 50, 200, 1250, and 2500 ng/mL.

$$\%\ Recovery = \left[\frac{Peak\ response\ from\ extracted\ spiked\ standard}{Peak\ response\ from\ unxtracted\ mobile\ phase\ standard}\right] \times 100 \quad (1)$$

Where the response represents the peak height of the analyte of interest.

2.7.6 Validation: Effect of freeze-thaw cycles

Freeze-thaw stability of curcumin (CUR), demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) in mouse plasma was evaluated at four QC levels (LLOQ/Low, Medium, Intermediate, and High). QC aliquots were stored at −20°C and were subjected to repeated freeze-thaw (FT) cycles. For the first cycle, samples were thawed at room temperature, processed, and analyzed (Day 6). For the second cycle, separate aliquots were thawed, re-frozen, and thawed again before analysis (Day 7). On each day, freshly prepared calibration standards and QC samples were processed in parallel to serve as the reference baseline.

Stability was determined by comparing the percentage of analyte-to-IS peak area ratios in FT samples with those of freshly prepared QCs from the same day. Acceptance criteria were set as mean concentrations within 85–115% of the nominal value (±20% at the limit of quantification, LLOQ) and precision (%CV) within ≤15% (≤20% at LLOQ), following guidelines from the FDA [6] and EMA [7].

3. Results and discussion

3.1 Optimization of chromatographic conditions

The widely cited method by Li *et al.* [8], which assayed curcumin using UV detection, was not designed to quantify all three curcuminoids—CUR, DMC, and BDMC—simultaneously. Because our assay aimed to measure these three analytes in one run, the chromatographic conditions described by *Li et al.* were unsuitable due to the similar physicochemical properties (pKa and logP) of the curcuminoids. To achieve proper baseline separation of CUR, DMC, and BDMC, we systematically optimized the chromatographic conditions.

Baseline separation was improved by carefully choosing column material, length, pore size, and elution method to balance sensitivity and resolution. To maintain a detection limit (LOD) of 1 ng/mL and prevent high back pressures, the flow rate for all narrow-bore (3 mm i.d.) columns was set at 0.2 mL/min.

During the first round of column testing, we compared C_{18} biphenyl and F_5 Kinetex phases (2.6 μm, 3 mm i.d.) under isocratic conditions. Across a 40–70% acetonitrile range, the chromatograms exhibited poor separation: two of the curcuminoids consistently overlapped, and even the internal standard (clozapine) was not well resolved. This confirmed that isocratic elution alone was inadequate for our system. This aligns with the known limitations of isocratic elution when analyzing mixtures containing analytes with different polarities or retention behaviors.

In contrast, gradient elution using the same C_{18} biphenyl column produced sharp, baseline-resolved peaks for CUR, DMC, BDMC, and IS. These results align with chromatographic literature, which consistently shows that gradient elution reduces band broadening and enhances resolution for complex analyte sets, where the relative elution strength is methodically adjusted over time.

The elution behavior of curcuminoids was highly sensitive to mobile-phase pH. Across a gradient increase from pH 3.8 to 6.8, retention time for curcumin decreased markedly, and at pH values above 6.8, peak splitting was evident, confirming that curcuminoids elute more effectively under acidic conditions. Because curcuminoids are polyphenolic diketones with ionizable phenolic hydroxyl groups, they ionize in alkaline environments, leading to peak broadening. Addition of formic acidic into the

mobile phase causes the suppression of ionization of analytes like CUR, DMC and BDMC, leaving them predominantly in their neutral and hydrophobic states. This enhances their interaction with the stationary phase, producing sharper chromatographic peaks. Our findings are consistent with those of Treesinchai *et al.* [9], who reported that curcumin stability and HPLC peak quality deteriorate progressively as pH shifts from acidic toward neutral-basic conditions.

Binary mobile phases composed solely of acetonitrile and water resulted in pronounced peak tailing. Furthermore, the proportion of acetonitrile at the start of the gradient strongly influenced retention behavior. Increasing the initial acetonitrile content enhanced analyte sensitivity but compromised chromatographic resolution, leading to shorter retention times and insufficient baseline separation.

The close logP values of CUR, DMC, and BDMC (3.29, 3.15, and 3.16, respectively) meant their separation was primarily dependent on subtle hydrophobic and polarity differences. Removal of one methoxy group in DMC increased polarity relative to CUR, causing earlier elution. In contrast, the absence of both methoxy groups in BDMC made it even more polar and thus eluted earlier than both CUR and DMC.

The F_5 Kinetex® column showed better selectivity and resolution than the C_{18} column. Unlike the C_{18} phase, which mainly relies on hydrophobic interactions, the pentafluorophenyl phase offers multiple types of interactions, including hydrophobic, π-π stacking, hydrogen bonding, and dipole-dipole interactions, thereby improving differential selectivity among similarly structured curcuminoids. In the end, the optimized HPLC method employed gradient elution on the F_5 Kinetex® column, using an initial mobile phase of 50% organic solvent and 50% aqueous solvent, which resulted in sharp peaks and baseline separation of CUR, DMC, BDMC, and IS.

3.2 Selectivity

The selectivity of the optimized HPLC-PDA method was evaluated by comparing chromatograms of blank mouse plasma, plasma spiked with curcuminoids (CUR, DMC, BDMC), and the internal standard (clozapine).

In blank plasma extracts, no endogenous peaks were observed at the retention times of the curcuminoids (BDMC, 6.9 min; DMC, 7.3 min; CUR, 7.9 min) or the internal standard (4.7 min). This confirmed the absence of interfering matrix components at both 425 nm (curcuminoid λ_{max}) and 384 nm (IS λ_{max}). In spiked calibration standards, CUR, DMC, and BDMC eluted as sharp, baseline-resolved peaks in the expected order (BDMC < DMC < CUR), while the internal standard eluted earlier at 4.7 min. The curcuminoids and IS were well separated, with no evidence of co-elution.

Figure 1 shows representative chromatograms of blank plasma, a spiked QC sample at the LLOQ, and a study sample collected 0.5 hours post-dose of a curcuminoid-loaded SEDDS formulation. This demonstrates that the method successfully discriminated the three analytes (CUR, DMC, BDMC) from potential matrix interferences in both spiked and study samples. Several studies have reported that tetrahydrocurcumin (THC) is a major in vivo metabolite of CUR [10, 11]. Based on its physicochemical properties, THC is expected to elute earlier than BDMC. Specifically, THC has a slightly lower logP than CUR, reduced π-conjugation, and fewer π-electrons available for aromatic stacking, resulting in weaker π-π interactions with the F5 stationary phase [12, 13]. Since phenolic and diketone groups remain neutral in acidic mobile phases, retention is mainly governed by hydrophobic and π/π/dipole interactions. Consequently, THC is predicted to elute at ~6.0–6.5 min, which is before BDMC and does not interfere with either the IS or parent curcuminoid peaks.

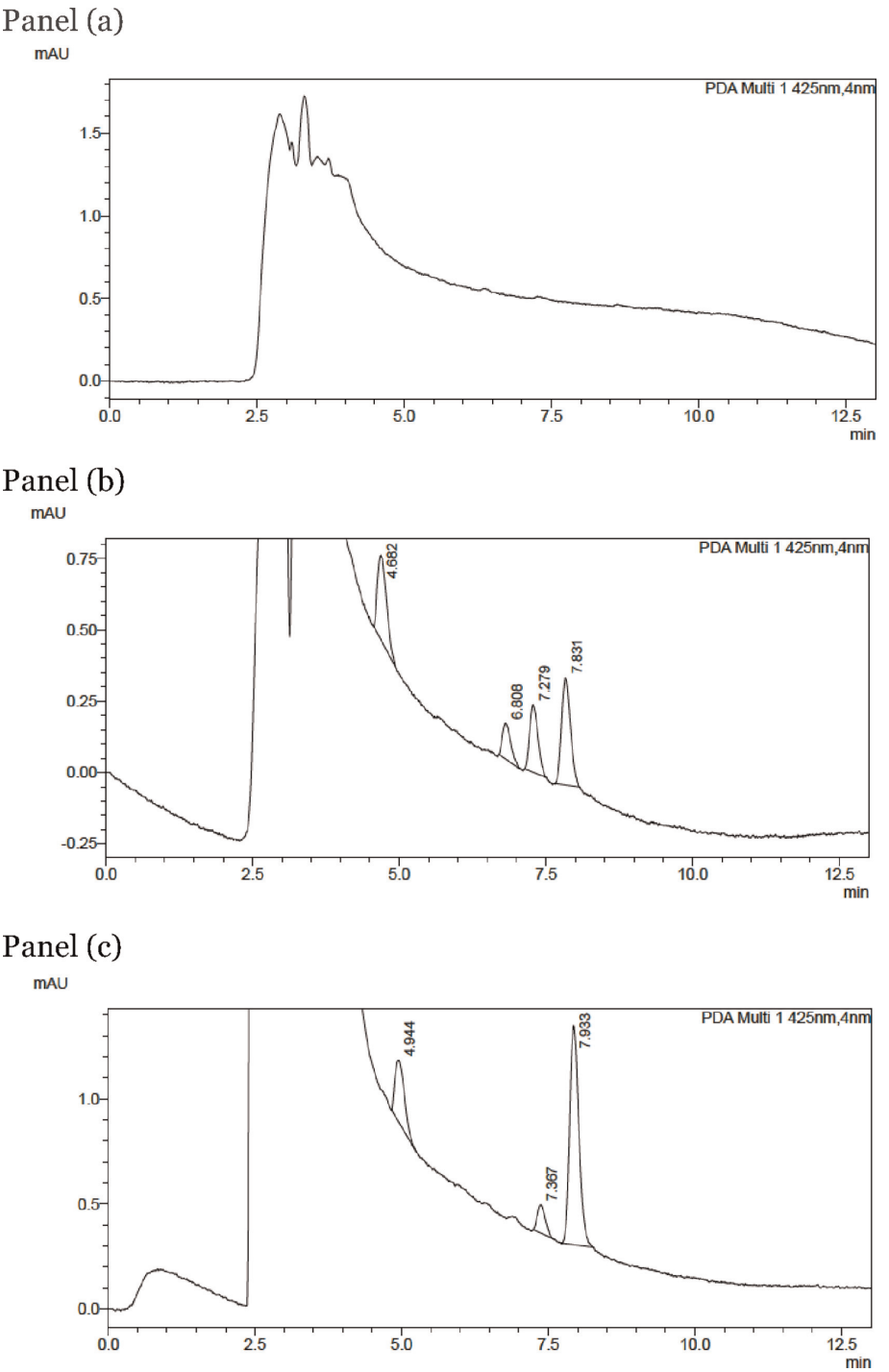


Figure 1.
Representative chromatograms of (a) blank plasma, (b) spiked QC sample (100 ng/mL), and (c) in vivo *plasma sample collected 1 hour after oral administration of a curcuminoid-loaded SEDDS formulation to mice.*

3.3 Sample clean-up

Several approaches were evaluated to optimize plasma sample clean-up for accurate recovery of curcuminoids and the internal standard (IS). Liquid-liquid extraction (LLE): Multiple organic solvents were tested. Although LLE produced consistent recoveries of 70–80%, the method was not suitable for high-throughput workflows, as it required dual extraction steps, was labor-intensive, and offered limited throughput capacity [10, 11].

To develop a more efficient method, protein precipitation (PPT) was evaluated. PPT is commonly used for bioanalytical assays due to its simplicity and compatibility with direct injection [14]. However, when applied to narrow-bore HPLC columns (3 mm i.d.), unfiltered PPT supernatants risked clogging the columns. They caused rapid column fouling after a small number of injections, consistent with previous reports of matrix-related column instability [15].

To overcome this, the PPT supernatant was further purified using a 96-well Strata-X polymeric SPE Impact™ cartridge. This additional step efficiently removed residual particulates and matrix components like phospholipids that usually interfere with the analytes of interest. The processed samples were free from interfering peaks at the detection wavelengths of interest (389 nm and 426 nm), ensuring assay robustness and reproducibility for routine application.

3.4 Calibration curve, model selection, and verifications

Calibration curves for curcumin (CUR), demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) were developed in mouse plasma over six analytical days. Multiple regression models were tested, including ordinary least squares (OLS) and weighted least squares using factors of $1/x$, $1/\sqrt{x}$, and $1/x^2$, with and without intercepts constrained to zero. Although OLS achieved slightly higher correlation coefficients ($R^2 > 0.99$), it exhibited less accuracy at the LLOQ. The best performance across days was achieved with a linear regression using $1/x^2$ weighting and a free intercept on analyte/IS peak-area ratios, which reduced heteroscedasticity and enhanced back-calculated accuracy at lower concentrations.

Residual analysis verified that the $1/x^2$ model was appropriate. As shown in **Figures 2–4** for CUR, DMC, and BDMC, the residuals were symmetrically spread around zero, with most within ±15% of the nominal value, and all LLOQ points within ±20%. Some deviations were observed at the upper QC levels, but they did not affect the calibration fit. These deviations align with the natural linearity

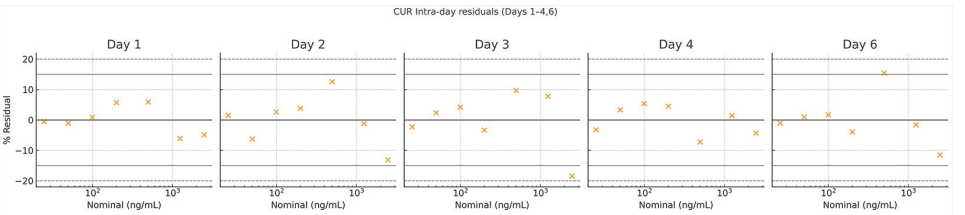


Figure 2.
Residual plots for curcumin (CUR) calibration curves (Days 1–4, 6) using $1/x^2$ weighted regression.

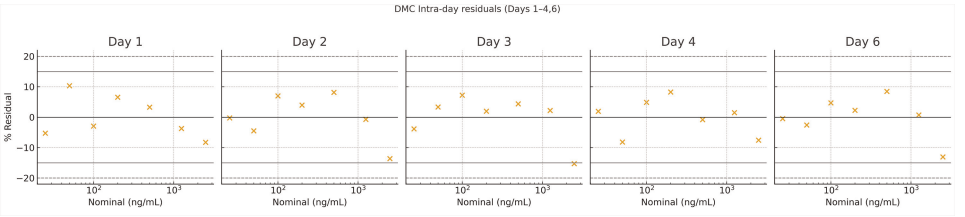


Figure 3.
Residual plots for demethoxycurcumin (DMC) calibration curves (Days 1–4, 6) using $1/x^2$ weighted regression.

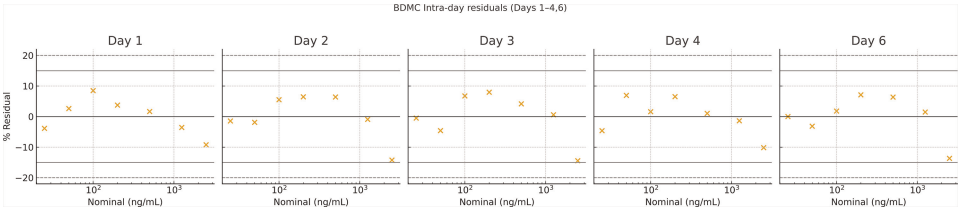


Figure 4.
Residual plots for bisdemethoxycurcumin (BDMC) calibration curves (Days 1–4, 6) using $1/x^2$ weighted regression.

limits of PDA detection at higher concentrations, rather than deficiencies in the regression model.

The robustness of the $1/x^2$ weighted regression was demonstrated across six separate analytical days. Individual-day regression parameters for CUR, DMC, and BDMC (slopes, intercepts, and R^2) are summarized in the supplementary data provided. Pooled data showed a slight decrease in correlation compared to individual fits, reflecting the expected inter-day variability; however, overall accuracy and precision remained within regulatory acceptance limits. Based on these findings, the validated calibration model was defined as a linear regression with $1/x^2$ weighting and an intercept, applied to analyte-to-internal standard (IS) peak-area ratios. The validated range for CUR was 50–200 ng/mL, while DMC and BDMC were validated over the full QC range (50–2500 ng/mL). This calibration model was used for all subsequent analyses of accuracy, precision, recovery, stability, and pharmacokinetics.

3.5 Accuracy and precision (intra- and inter-day)

Accuracy and precision of the HPLC-PDA method were evaluated for curcumin (CUR), demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) in mouse plasma at four QC concentrations (50, 200, 1250, and 2500 ng/mL). Inter-day calculations excluded Day 5, which showed clear calibration drift consistent with instrument-related instability.

For CUR, the daily runs showed good repeatability, with precision remaining under 15% CV and accuracy generally within the ±15% target (±20% at the LLOQ). Looking across Days 1–4 and 6, the combined inter-day data confirmed acceptable performance at the LLOQ (50 ng/mL; +2.4% bias, 8.9% RSD). At the medium QC (200 ng/mL), however, the pooled precision slipped slightly above the 15% cut-off (16.4% RSD), a deviation mainly attributable to the high variability seen on Day 1 (−34.5% bias).

QC Level	Nominal (ng/mL)	Scenario	n	Mean Back-Calc (ng/mL)	%Bias	% RSD	Result
LLOQ	50.0	Pooled (Days 1–4, 6)	19	40.59	−18.8	9.5	Pass
Medium	200.0	Pooled (Days 1–4, 6)	19	171.32	−14.3	16.4	Fail
Medium	200.0	Pooled (Excl Day 1)	16	178.88	−10.6	12.7	Pass
Intermediate	1250.0	Pooled (Days 1–4, 6)	20	1027.47	−17.8	15.0	Fail
High	2500.0	Pooled (Days 1–4, 6)	20	1853.71	−25.9	13.0	Fail

Acceptance limits: ±15% for bias and ≤ 15% RSD (±20% at LLOQ).

Table 1.
CUR — Accuracy and precision (area ratios, $1/x^2$; pooled Days 1–4 & 6).

Removing Day 1 improved the pooled estimate to −10.6% Bias and 12.7% RSD, which is fully compliant. The intermediate and high QC levels (1250 and 2500 ng/mL) displayed a consistent negative bias (−17.9% to −18.2%), suggesting limits of PDA linearity at high concentrations. On this basis, the validated range for CUR was defined as 50–200 ng/mL (**Table 1**). At least two-thirds of QC replicates per level still met criteria (Supplementary Tables S1–S3).

DMC met intra-day acceptance limits across all QCs except for sporadic outliers. Inter-day precision, excluding Day 5, was ≤25% CV, and accuracy was within ±20% (**Table 2**). More than two-thirds of replicates per QC complied with standards (Supplementary Tables S4–S6), supporting the validated range across all tested concentrations.

BDMC likewise showed ≤15% CV intra-day and accuracy within ±15%, with few exceptions. Pooled inter-day results (excluding Day 5) remained within ±20% accuracy and ≤ 16% CV (**Table 3**), with at least two-thirds of replicates per QC passing (Supplementary Tables S7–S9). The validated range extended across the whole concentration series.

When performance was examined by study day (Days 1–4 and 6), the lowest (50 ng/mL, LLOQ) and medium (200 ng/mL) QC levels consistently fell within guideline limits. Days 2–4 maintained acceptable accuracy and precision across the entire QC panel, but variability appeared on Days 1 and 6 at the higher concentrations

QC Level	Nominal (ng/mL)	Scenario	n	Mean Back-Calc (ng/mL)	%Bias	%RSD	Result
LLOQ	50.0	Pooled (Days 1–4, 6)	19	47.07	−5.9	6.5	Pass
Medium	200.0	Pooled (Days 1–4, 6)	19	192.03	−4.0	7.9	Pass
Medium	200.0	Pooled (Excl Day 1)	16	196.46	−1.8	6.0	Pass
Intermediate	1250.0	Pooled (Days 1–4, 6)	20	1068.68	−14.5	25.1	Fail
High	2500.0	Pooled (Days 1–4, 6)	20	1991.29	−20.3	7.5	Fail

Acceptance limits: ±15% for bias and ≤ 15% RSD (±20% at LLOQ).

Table 2.
DMC — Accuracy and precision (area ratios, $1/x^2$; pooled Days 1–4 & 6).

QC Level	Nominal (ng/mL)	Scenario	n	Mean Back-Calc (ng/mL)	% Bias	% RSD	Result
LLOQ	50.0	Pooled (Days 1–4, 6)	19	48.12	−3.8	16.1	Pass
Medium	200.0	Pooled (Days 1–4, 6)	19	202.61	1.3	4.9	Pass
Medium	200.0	Pooled (Excl Day 1)	16	205.43	2.7	3.8	Pass
Intermediate	1250.0	Pooled (Days 1–4, 6)	20	1164.21	−6.9	7.3	Pass
High	2500.0	Pooled (Days 1–4, 6)	20	2038.74	−18.5	5.1	Fail

Acceptance limits: ±15% for bias and ≤ 15% RSD (±20% at LLOQ).

Table 3.
BDMC — Accuracy and precision (area ratios, $1/x^2$; pooled Days 1–4 & 6).

(1250 and 2500 ng/mL). Importantly, these fluctuations were restricted to the upper calibration range and did not compromise the LLOQ or medium QC values, which are more critical for pharmacokinetic interpretation.

Overall, reproducibility across 5 days was satisfactory, and intra−/inter-day results aligned with FDA [6] and EMA [7] validation guidance. A few outliers were seen, which is not surprising given that PDA systems often show greater variability at higher concentrations when compared with LC-MS/MS [16]. Even so, when the QC results were considered alongside the residual patterns (**Figures 2–4**) and the calibration curve overlays (**Figures 5–7**), the overall picture supported the robustness of the assay for measuring curcuminoids in mouse plasma.

3.6 Recovery

The absolute recovery for CUR, DMC, and BDMC from mouse plasma is presented in **Table 4**. Recovery was assessed by comparing analyte/IS area ratios from extracted plasma samples against post-extraction (mobile phase) standards. Across LLOQ, Intermediate, and High QC levels, recoveries were moderate (20–35%) but consistent, with %CV generally ≤15–20%. Similar modest recoveries have been reported previously for curcuminoids, reflecting their hydrophobic nature and strong binding to plasma proteins and tissues [10, 11].

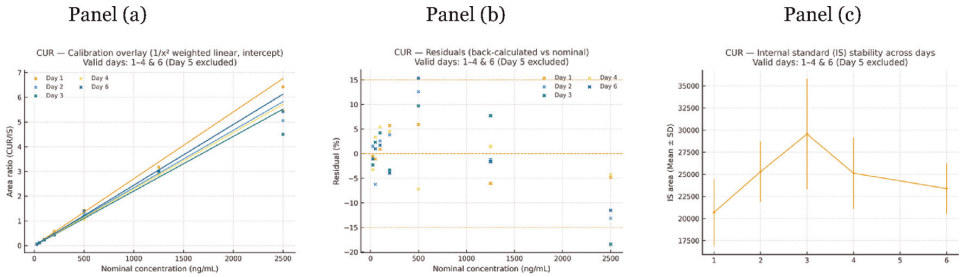


Figure 5.
(a) Representative pooled inter-day Calibration overlay for CUR, (b) residuals for CUR, pooled data: days 1–4,6, (da 54 cluded), and (c) CUR internal standard stability across valid days (1–4, 6).

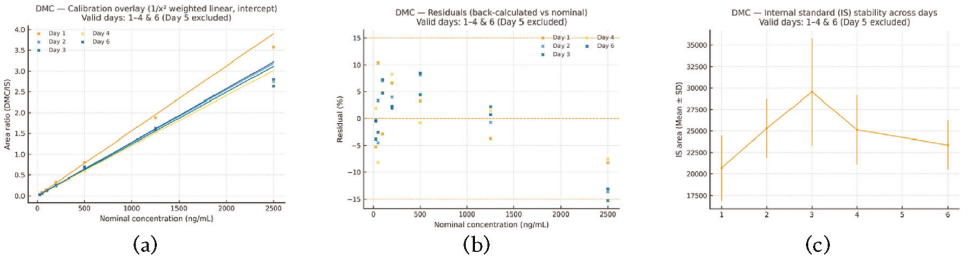


Figure 6.
(a) Representative pooled inter-day Calibration overlay for DMC, (b) residuals for DMC, pooled data: days 1–4,6, (day 5 excluded), and (c) DMC internal standard stability across valid days (1–4, 6).

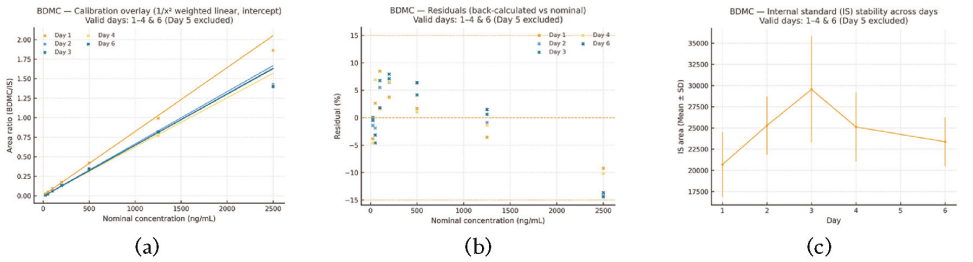


Figure 7.
(a) Representative pooled inter-day Calibration overlay for BDMC, (b) residuals for BDMC, pooled data: days 1–4,6, (day 5 excluded), and (c) BDMC internal standard stability across valid days (1–4, 6).

Recovery on **Day 7** was markedly reduced (<12%) and highly variable (CV >35%) across all QC levels. These results were excluded from pooled calculations and attributed to a batch-specific processing or stability artifact, rather than an inherent assay limitation. Pooled recovery was therefore calculated from Days 2 and 3, which met FDA [6] and EMA [7] reproducibility criteria. At the Medium QC (200 ng/mL), recovery was not included in pooled reporting due to consistent non-Gaussian distribution: replicates separated into distinct low- and high-recovery groups across multiple days, yielding persistently high %CVs even after 2 × SD outlier screening. This pattern likely reflects a concentration-specific matrix effect, subtle variability in sample processing, or instability in IS response.

Importantly, since regulatory guidelines require recovery to be reproducible rather than absolute, pooled recoveries at LLOQ, intermediate, and high QCs met precision criteria. Despite anomalies at the Medium QC and Day 7, overall intra- and inter-day accuracy and precision remained within acceptance limits, confirming that quantitative reliability of the method was unaffected. Full day-wise recovery data are provided in Supplementary Tables 10–12.

3.7 Freeze-thaw stability

Freeze-thaw (FT) stability of curcumin (CUR), demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) in mouse plasma was evaluated after storage at −20° C. Fresh QC samples were compared with aliquots subjected to one (Day 6) and two (Day 7) FT cycles. Based on analyte-to-IS ratios, all three analytes remained within FDA/EMA acceptance limits after a single cycle. However, after two FT cycles, instability became evident: (a) CUR recovery decreased to ~50–70% of fresh values at

QC Level (ng/mL)	CUR %Recovery (±%CV)	Pass/ Fail	DMC % Recovery (±%CV)	Pass/ Fail	BDMC %Recovery (±%CV)	Pass/ Fail
LLOQ (50 / 25 / 12.5)	27.8 (13.5)	Pass	24.9 (14.1)	Pass	22.7 (12.8)	Pass
Medium (200 / 100 / 50)	Excluded†	—	Excluded†	—	Excluded†	—
Intermediate (1250 / 625 / 312.5)	32.4 (11.2)	Pass	30.1 (10.9)	Pass	28.6 (11.5)	Pass
High (2500 / 1250 / 625)	30.7 (9.8)	Pass	29.2 (10.7)	Pass	27.5 (10.1)	Pass

†Medium QC excluded due to systematic non-Gaussian recovery pattern. Absolute recovery was moderate but reproducible (20–35% range, %CV ≤15%), consistent with FDA/EMA guidance emphasizing reproducibility rather than absolute yield.

Table 4.
Absolute Recovery of Curcuminoids in Mouse Plasma.

several QC levels, (b) DMC showed <85% stability at multiple levels, and (c) BDMC maintained stability, with most values between 85 and 100%. Across all conditions, precision (%CV) remained acceptable. A pooled summary of stability after one and 2 cycles is presented in **Table 5**.

These findings indicate that CUR and DMC are unstable after two FT cycles at −20°C, whereas BDMC remains sufficiently stable. Although IS-normalized ratios formally met acceptance after 1 cycle, raw area data confirmed analyte loss beyond 1

Analyte	QC Levels (ng/mL)	1FT %Stability (Mean ± CV)	Pass/ Fail	2FT %Stability (Mean ± CV)	Pass/Fail
CUR	LLOQ (50)	172% ± 9.5	Fail	38% ± 12.1	Fail
	Medium (200)	165% ± 11.3	Fail	41% ± 14.7	Fail
	Intermediate (1250)	182% ± 8.7	Fail	43% ± 9.8	Fail
	High (2500)	174% ± 10.2	Fail	52% ± 8.4	Fail
DMC	LLOQ (25)	120% ± 8.4	Pass	60% ± 11.2	Fail
	Medium (100)	118% ± 9.5	Pass	64% ± 10.1	Fail
	Intermediate (625)	135% ± 7.2	Fail	70% ± 9.4	Fail
	High (1250)	138% ± 10.8	Fail	78% ± 7.6	Fail
BDMC	LLOQ (12.5)	129% ± 6.2	Fail	82% ± 8.1	Borderline
	Medium (50)	124% ± 7.9	Fail	85% ± 9.2	Pass
	Intermediate (312.5)	132% ± 8.7	Fail	90% ± 10.4	Pass
	High (625)	130% ± 7.3	Fail	92% ± 9.1	Pass

Acceptance criteria: mean stability within 85–115% of fresh controls and CV ≤15% (≤20% at LLOQ).

Table 5.
Freeze-thaw stability of curcuminoids (CUR, DMC, BDMC) in mouse plasma after 1 and 2 cycles (pooled across QC levels).

cycles. The higher instability of CUR and DMC is consistent with their reactive phenolic diketone structures, which are more susceptible to hydrolysis, oxidation, or binding during repeated freeze-thaw events. In contrast, BDMC's greater stability is explained by its electron-deficient aromatic structure (absence of methoxy groups), which renders it less prone to oxidative degradation [4, 5, 17].

Notably, samples stored at −20°C showed reduced protection compared with ultralow storage (−80°C). Published reports confirm that CUR and its metabolites are stable long-term at −80°C in plasma and tissues, whereas −20°C is more suitable for short-term storage of stock solutions [18]. Our results are consistent with these observations, supporting the recommendation that ultralow storage is required to preserve curcuminoids during extended studies.

3.8 Pharmacokinetic application of the assay

The validated HPLC-PDA method was successfully used to evaluate the pharmacokinetics of curcuminoids after a single oral dose (100 mg/kg) of curcuminoids formulated as self-emulsifying drug delivery systems (SEDDS) in overnight-fasted mice. Representative chromatograms from study plasma samples are shown in **Figure 1, panel c**, which demonstrate that the method is selective and can detect CUR, DMC, and BDMC *in vivo* without interference from endogenous components. Plasma samples collected at 30 minutes post-dose showed measurable concentrations for CUR and DMC curcuminoids. This time point was selected to represent the early absorption phase following oral administration and to provide a quick and effective screen for prototype formulations before conducting a complete pharmacokinetic study. The observed exposure confirmed that the SEDDS formulation delivered measurable systemic levels of free curcuminoids, which are typically poorly absorbed when given as crude suspensions.

These results show that the validated assay not only meets regulatory standards for various validation parameters but is sufficiently robust for application for preclinical pharmacokinetic screening of lipid-based formulations. This use further highlights the assay's potential usefulness in guiding future comparative formulation studies and in connecting preclinical findings to translational research.

4. Conclusions

The validated HPLC-PDA assay for CUR, DMC, and BDMC met FDA/EMA bioanalytical criteria for accuracy, precision, recovery, and stability. Application to a curcuminoid-loaded SEDDS formulation demonstrated reliable quantification in mouse plasma, confirming suitability for preclinical pharmacokinetic studies. Detailed accuracy, precision, recovery, calibration, and stability data are available in the Supplementary Information (Tables S1–S9 and Figures S1–S8).

Acknowledgements

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Conflict of interest

The author declares no conflict of interest.

Availability of data

All validation datasets supporting this study are provided in the Supplementary Information. Additional raw data are available from the corresponding author upon reasonable request.

Appendices and nomenclature

Appendices A: Key equations used in validation

- Accuracy (%Bias):

$$\%Bias. = \frac{(Measured\ concentration - Nominal\ concentration}{Nominal\ concentration} \times 100$$

- Precision (%RSD):

$$\%RSD = \frac{Standard\ Deviation}{Mean\ Concentration} \times 100$$

- Back-calculated concentration:

$$C_{calc} = \frac{(Response - Intercept)}{Slope}$$

Appendices B: Representative chromatograms

- Blank mouse plasma (no interference at analyte or IS retention times).
- Spiked QC sample at LLOQ (50 ng/mL).
- Study sample at 30 min post-dose of curcuminoid-loaded SEDDS.

Appendices C: Supplementary data

Detailed day-wise validation results, recovery breakdown, and freeze-thaw stability data are provided in Supplementary Tables S1–S9 (Excel format). These include:

- Intra- and inter-day accuracy and precision at all QC levels.
- Day-wise recovery relative to mobile-phase standards.
- Freeze–thaw stability across 1 and 2 cycles.

Supplementary information

The supplementary tables can be downloaded from my google drive link below https://drive.google.com/drive/folders/1RopPDQwOCKJlo79b6Lhe4gU8e8kcTHXJ?usp=sharing

Abbreviations

CUR	curcumin
DMC	demethoxycurcumin
BDMC	bisdemethoxycurcumin
THC	tetrahydrocurcumin
LLOQ	lower limit of quantification
QC	quality control
RSD	relative standard deviation
%Bias	percent bias (Accuracy)
PDA	photodiode array detector
HPLC	high-performance liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
SEDDS	self-emulsifying drug delivery system
ASD	amorphous solid dispersion
IS	internal standard

Symbols

R^2	correlation coefficient
λ_{max}	wavelength of maximum absorbance
n	number of replicates

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Chapter 5

Comprehensive Pharmacogenetic Allele Landscape from Whole Exome Sequencing: Single-Center Cohort Analysis in the Population of North Macedonia

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Abstract

Pharmacogenetics (PGx) plays a critical role in optimizing drug therapy by tailoring treatment based on individual genetic profiles, with the potential to improve clinical outcomes. Accurate data on allele and phenotype distributions in specific populations is essential for effective clinical application. This study presents the first comprehensive PGx landscape of the population in North Macedonia, using extended whole exome sequencing (WES) data. All individuals in the cohort had at least one aberrant phenotype across key pharmacogenes, consistent with global findings. Significant differences in haplotype frequencies were observed compared to broader European populations, underscoring the importance of region-specific PGx data. Of the 78 AMP-recommended alleles, 27 were identified, with all of participants carrying at least one Tier 1 allele. Compared to previous targeted studies in our population, WES enabled detection of lower-frequency AMP alleles and rare *CYP2D6* variants not typically captured by local genotyping assays. Based on *in silico* functional impact predictions, 128 novel putative non-functional variants were identified across 19 pharmacogenes, including 17 in AMP-recommended genes such as *DPYD*, *CYP3A4*, and *VKORC1*—where novel variants were sometimes more frequent than those previously reported. These findings highlight the presence of additional clinically relevant variants beyond standard panels and emphasize the limitations of targeted genotyping. However, challenges remain, particularly for complex genes like *CYP2D6*, where short-read WES cannot fully resolve haplotypes. This study reinforces the need for improved sequencing methodologies and guidelines for interpreting novel PGx variants, while demonstrating the value of population-specific profiling to advance precision medicine and inform clinical practice.

Keywords: pharmacogenomics, whole exome sequencing, allele frequency, North Macedonia, novel variants

1. Introduction

Genetic variability in genes involved in the absorption, distribution, metabolism, and excretion of medical drugs can alter gene function, leading to inter-patient differences in drug response. This altered functionality can contribute to therapeutic failure and adverse drug reactions (ADRs), alongside non-genetic factors such as age, sex, reduced organ function, lifestyle, adherence to therapy, and drug interactions. It is estimated that ADRs are between the fourth and sixth leading causes of death [1], and genetic predisposition is being increasingly understood, especially for anticancer, cardiovascular, and neuropsychiatric drugs [2]. One recent study showed that approximately 9% of all reported adverse drug reactions were linked to medications for which the risk of such reactions could be modified based on pharmacogenomic prescribing guidance [3]. Another prospective clinical study demonstrated that implementing a pharmacogenomic panel could lead to a 30% reduction in ADRs in a clinical setting [4, 5], which supports the notion that utilizing pharmacogenetic information can lead to tangible improvements in drug safety. Expert consortia such as the Clinical Pharmacogenetics Implementation Consortium (CPIC) [6], Dutch Pharmacogenetics Working Group (DPWG) [7, 8] or US Food and Drug Administration (FDA) [9] play a crucial role in developing evidence-based, peer-reviewed guidelines that translate pharmacogenetic (PGx) test results into actionable prescribing recommendations for clinicians.

Population-scale studies have demonstrated that a vast majority of individuals harbor at least one genetic variant that has the potential to influence their response to medications, with reported prevalences from large studies higher than 99% [10, 11]. However, there is significant inter-population variability in the frequencies of actionable pharmacogenetic variants [12], and identification of the most common and impactful genetic variations can improve the clinical utility of PGx variants testing.

While genotyping technologies such as SNP arrays, real-time PCR, and Sanger sequencing remain valuable for PGx, whole exome sequencing (WES) has gained prominence for its ability to capture a broad range of genetic variants. Standard WES is limited by its focus on protein-coding regions, potentially missing pharmacogenetically relevant variants in non-coding regions [13]. However, there is emergence of extended WES designs that enhance coverage beyond protein-coding regions to include non-coding variants relevant to pharmacogenomics, providing a more complete genetic profile. Software tools like PharmCat are instrumental in analyzing this comprehensive sequencing data, enabling the construction of star alleles and the annotation of phenotypes based on established guidelines from consortia like CPIC and DPWG, thereby facilitating the translation of complex genetic information into actionable clinical recommendations. Additional value of WES methodology is ability to discover new, potentially population specific functional variants.

The objectives of this research were to identify known and potentially novel actionable PGx variants within our population, to determine their allele frequencies, to compare these findings with the data from other populations, and to provide insights for the development of population-specific pharmacogenetic tests with higher clinical utility.

2. Methods

2.1 Study population and NGS methodology

We have utilized data from a cohort of 1415 patients referred to our institution for clinical genetic testing for rare diseases. Short-read NGS analysis included three different methods for enrichment: 290 subjects were analyzed with clinical exome (TruSight One, Illumina), 599 subjects were analyzed with classical WES (Twist Human Core + RefSeq + Mitochondrial Panel from Twist Bioscience and Illumina Exome Panel v1.2 from Illumina), and 526 subjects were analyzed with extended WES (ExomeXtra from CeGaT GmbH, Tübingen, Germany) panels. Enrichment for extended WES panel (eWES) included coverage of regions containing pharmacogenetically relevant variants, and these 526 subjects were used for comprehensive PGx profiling. Identification of novel PGx variants only in coding regions and regions flanking 25 bp of both sides of exons was performed on all 1415 subjects. The ethnic background and geographic distribution of the participants were resembling the overall population structure of our country according to the latest census of 2022. Of the 526 subjects used for comprehensive PGx profiling, 336 (64%) were Macedonians, 153 (29%) were Albanians, 13 (2.5%) were Roma, 13 (2.5%) were Turks, and 11 (2.0%) of other ethnic origin. Similar ethnic distribution was observed among 1415 subjects. The study was conducted according to the Declaration of Helsinki and informant consent regarding storage of biological specimens and anonymized use of sequencing data was obtained from all participants.

2.2 Data processing, alignment, variant calling, and variant effect prediction

All fastq files from 1415 subjects were processed identically against hg38 "no_alt" version of human reference genome, using in-house developed pipeline. Read alignment was performed using BWA software (v0.7.17), and variant calling was carried out using the Genome Analysis Toolkit (GATK) (v4.1.3.0). The resulting variant call format (VCF) files were merged into a single integrated VCF file using bcftools (v1.9). This integrated file was then annotated using the Ensembl Variant Effect Predictor (VEP) (v112). For identification of novel mutations within genes with established associations in drug metabolism we have used "pharmcat positions 2.15.3.vcf" file (obtained from https://github.com/PharmGKB/PharmCAT) as additional source in VEP annotation in order to distinct between targeted PGx variants and novel variants in relevant pharmacogenes. Further downstream analysis of annotated file was performed using the R software (version 4.2.1).

For *In silico* variant effect prediction of novel missense variants, the following predictive tools were employed: CADD [14], BayesDel_noAF [15, 16], MetaRNN [17], ESM1b [18], AlphaMissense [19], PHACTboost [20], and MutFormer [21], which are well-established most recent algorithms employing deep learning or metascores for variant pathogenicity prediction. Scores were obtained from dbNSFP v.4.8 [22]. For novel splice site variants, SpliceAI [23] predictions were used, leveraging a specialized deep learning algorithm trained specifically on splicing patterns, which not only provides more direct and accurate evidence of splicing disruption compared to the rule-based predictions generated by VEP, but also scores for mutations outside canonical splice sites. Score cut-offs for *In silico* predictors are given in **Table 1**.

In silico **functional predictor**	**Score for pathogenic functional impact**
CADD_phred	> = 25
BayesDel_noAF_score	> = 0.27
MetaRNN_score	> = 0.5
ESM1b_score	<= −7.5
AlphaMissense_pred	P*
PHACTboost_score	> = 0.62
MutFormer_score	> = 0.8838
SpliceAI_score	> = 0.6

**The "P" designation for AlphaMissense_pred means that the prediction is pathogenic.*

Table 1.
Display of the cutoff values of the in silico *predictors below or above which the variant is considered non-functional.*

Loss of function (LoF) mutations (frameshift, start lost, stop gained consequences) were identified by filtering for the "HIGH" impact prediction from VEP software.

2.3 PGx analysis

For the 526 samples analyzed with eWES, additional variant calling was performed. The BAM files from the mapping were utilized for PGx variant calling encompassing all positions (n = 1080) listed in the "pharmcat_positions_2.15.3.vcf" file, irrespective of whether changes were observed, by using the "--emit-all-active-sites" argument in GATK. The resultant VCF files that have information for all targeted sites (genotyped as 0/0, 0/1 or 1/1) were merged with bcftools and analyzed with Docker version of PharmCat (v2.15.3). The software is specifically designed for variant identification in predefined genes with documented associations to drug metabolism and corresponding dosing guidelines (by CPIC or DPWG). Currently, it analyzes and reports haplotypes and diplotypes (genotypes) directly from VCF documents for 18 genes: *ABCG2*, *CACNA1S*, *CFTR*, *CYP2B6*, *CYP2C19*, *CYP2C9*, *CYP3A4*, *CYP3A5*, *CYP4F2*, *DPYD*, *G6PD*, *IFNL3*, *NUDT15*, *RYR1*, *SLCO1B1*, *TPMT*, *UGT1A1,* and *VKORC1*. It should be noted that PharmCat reports diplotypes based on the highest functional impact score assigned during the analysis process. If the phase is unknown, as in our case, additional possible genotypes cannot be excluded.

For the *CYP2D6* gene, PharmCat analysis is not enabled by default since haplotypes and diplotypes calling is not recommended directly from VCF file due to the large influence of SV and CNV on phenotype prediction. Additional obstacle for proper identification of correct diplotype are high genetic variability, high similarity with the pseudogene, and existence of alternative haplotypes in Ref. genome. However, we have used the possibility to call *CYP2D6* star alleles that are defined based on SNPs and/or INDELs using PharmCat research mode option, which enables the identification of the *CYP2D6* star alleles that have significant functional consequences. This was made possible since these non-functional alleles are usually associated with one deleterious variant, and they are part of the haplotype of the defined star allele. Definitions for associated deleterious variants (core variants) for *CYP2D6* non-functional star alleles were obtained from published recommendations for clinical testing [24]. Therefore,

for the *CYP2D6* gene we are reporting frequencies only for associated deleterious variants in Tier 1 and Tier2 sets of star alleles defined by Association of Molecular Pathology (AMP) Minimum Sets of Alleles for PGx Testing guideline (https://www.pharmgkb.org/ampAllelesToTest). For other genes, we are reporting diplotypes/haplotypes as identified by PharmCat. Additionally, we have used information for CNV calls made by sequencing provider to identify only whole *CYP2D6* gene deletions (5* allele) that were confirmed with manual inspection of the BAM files and manual confirmation for variant homozygosity in the deleted region.

In order to characterize specific pharmacogenetic profile in our population, we have compared diplotype and haplotype frequencies with those previously summarized and reported for European population by CPIC [25], accessed from https://www.pharmgkb.org/page/pgxGeneRef. For the *CYP2D6, CYP2C19*, *CYP2C9,* and *CYP3A5* genes, we have additionally made a comparison of our findings for associated deleterious variant frequencies with previously reported studies on smaller cohorts from our population [26–29].

3. Results

3.1 Phenotype distribution

Phenotypes for 13 pharmacogenes were assigned based on diplotype interpretation, as detailed in **Table 2**. However, for five genes—*CACNA1S*, *CYP2D6*, *CYP4F2*,

Gene	Phenotype	n	% by gene
ABCG2	Normal function	453	86.1%
	Decreased function	67	12.7%
	Poor function	6	1.1%
CFTR	ivacaftor non-responsive in CF patients	521	99.0%
	ivacaftor responsive in CF patients	5	1.0%
CYP2B6	Normal metabolizer	246	46.8%
	Intermediate metabolizer	167	31.7%
	Indeterminate	49	9.3%
	Poor metabolizer	35	6.7%
	Rapid metabolizer	24	4.6%
	Intermediate metabolizer OR indeterminate	2	0.4%
	No result	2	0.4%
	Ultrarapid metabolizer	1	0.2%
CYP2C19	Normal metabolizer	218	41.4%
	Intermediate metabolizer	150	28.5%
	Rapid metabolizer	127	24.1%
	Ultrarapid metabolizer	19	3.6%
	Poor metabolizer	11	2.1%
	No result	1	0.2%

Gene	Phenotype	n	% by gene
CYP2C9	Normal metabolizer	316	60.1%
	Intermediate metabolizer	191	36.3%
	Poor metabolizer	18	3.4%
	No result	1	0.2%
CYP3A5	Poor metabolizer	439	83.5%
	Intermediate metabolizer	81	15.4%
	Normal metabolizer	6	1.1%
DPYD	Normal metabolizer	509	96.8%
	Intermediate metabolizer	17	3.2%
G6PD	Normal	517	98.3%
	Indeterminate	4	0.8%
	Deficient	3	0.6%
	Variable	2	0.4%
NUDT15	Normal metabolizer	515	97.9%
	Intermediate metabolizer	8	1.5%
	Indeterminate	2	0.4%
	Poor metabolizer	1	0.2%
RYR1	Uncertain susceptibility	525	99.8%
	Malignant hyperthermia susceptibility	1	0.2%
SLCO1B1	Normal function	315	59.9%
	Decreased function	136	25.9%
	Increased function	30	5.7%
	Poor function	29	5.5%
	Possible decreased function OR decreased function	9	1.7%
	Decreased function OR possible decreased function OR indeterminate	6	1.1%
	Indeterminate OR indeterminate	1	0.2%
TPMT	Normal metabolizer	507	96.4%
	Intermediate metabolizer	19	3.6%
UGT1A1	Intermediate metabolizer	223	42.4%
	Normal metabolizer	207	39.4%
	Poor metabolizer	64	12.2%
	Indeterminate	31	5.9%
	No result	1	0.2%

Table 2.
Distribution of pharmacogenetic phenotypes in the 526 studied subjects, shown summarized by gene.

IFNL3, and *VKORC1*—phenotypes could not be determined due to either the lack of established diplotype-to-phenotype mappings or analytical limitations.

CYP2D6 analysis using PharmCAT (research mode) presented particular challenges, yielding a high rate of unresolved classifications: 38% of samples had no result, and 29% were classified as undetermined. This underscores the complexity of *CYP2D6* genotyping, where ambiguous allele combinations often complicate

diplotype assignments. Therefore, phenotype, genotype, and haplotype results were not presented for the *CYP2D6* gene.

Among all 526 individuals, every participant carried at least one non-normal metabolizer phenotype (defined as decreased, intermediate, poor, rapid, and ultrarapid metabolizer statuses) for 13 pharmacogenes, with the number of those phenotypes per subject ranging from one to seven.

3.2 Comparison of diplotype and haplotype frequencies with the European population

Frequency comparisons between the population of North Macedonia and the European populations revealed some notable differences in both diplotype and haplotype distributions. This comparison focused on genes for which frequency data was available

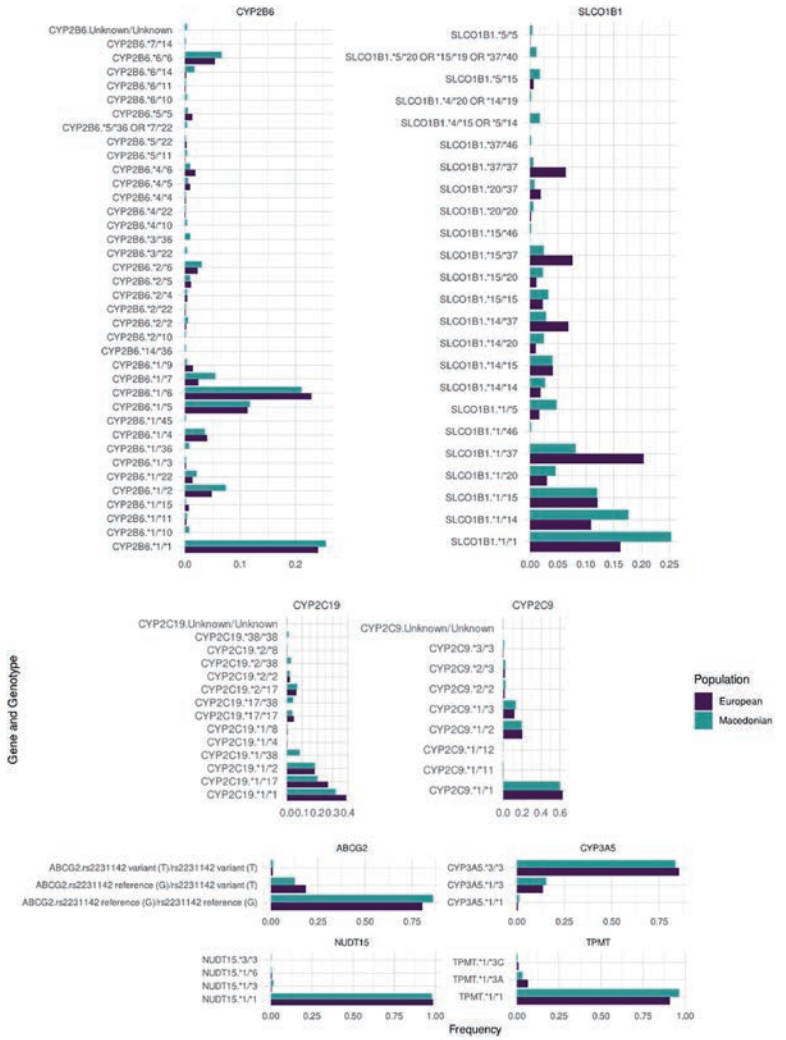


Figure 1.
Comparison of diplotype distributions for eight pharmacogenes between the Macedonian and European populations.

from CPIC, and hence, diplotype frequencies were compared for eight genes (**Figure 1**), while haplotype frequencies were compared for fifteen genes (**Figures 2** and **3**).

The *CYP2B6* gene shows the widest range of diplotypes in both populations, indicating substantial genetic variability. The most prominent differences were observed in the *SLCO1B1* gene. In the European population, increased heterozygosity was observed for the *37 allele (normal function), whereas in the Macedonian cohort, heterozygosity was more common for the *1 allele (normal function). Moreover, the *1/*1 and*1/*14 diplotypes (normal function) were more frequent in the Macedonian population, while the *1/*37, *14/*37, and especially *37/*37 diplotypes (normal function) were more prevalent in the European population. For diplotypes with decreased function, *15/*37 was more frequent in the European population while *1/*5 was more frequent in the Macedonian population (**Figure 1**).

In addition to *SLCO1B1*, significant differences in haplotype frequencies were observed for *CYP2C19*, *CYP4F2*, *RYR1*, and *UGT1A1* (**Figures 2** and **3**).

For the *CYP2C19* gene the main differences were for the frequency of the *CYP2C19**38 allele which is not reported in the CPIC frequency table. This allele exhibits normal function and is characterized by its identity with the reference sequence, in contrast to the *CYP2C19**1 (also normal function), which features the c.991A > C variant.

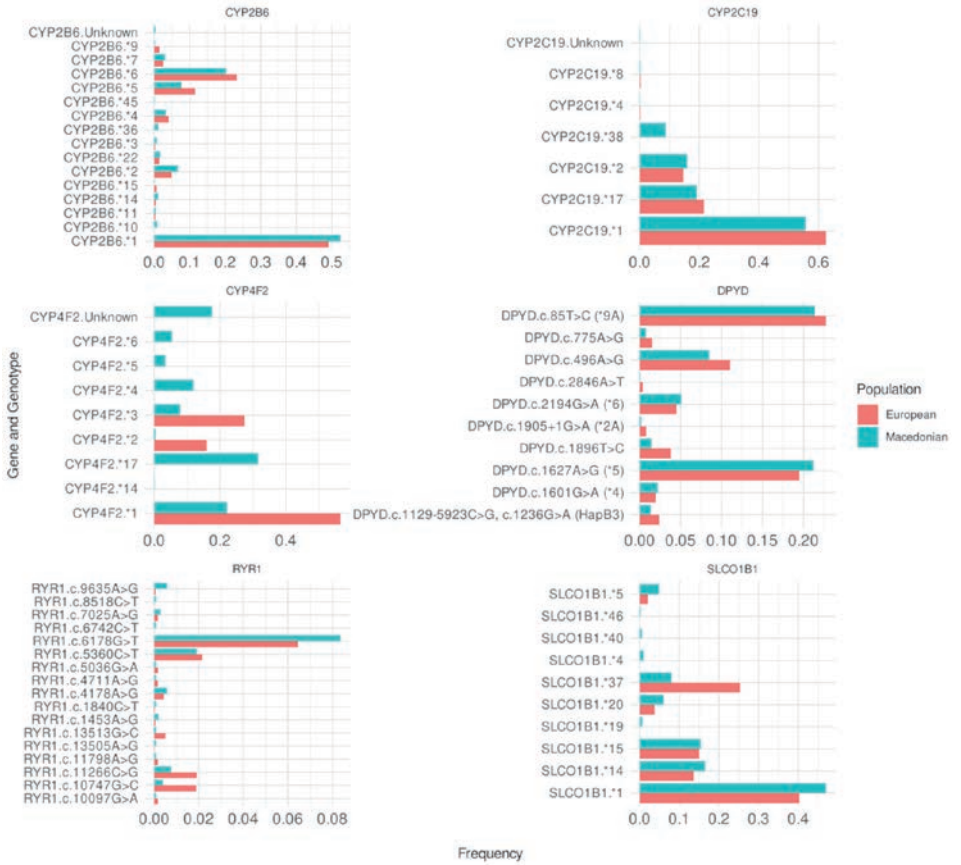


Figure 2.
*Comparison of haplotype distributions for six pharmacogenes (*CYP2B6, CYP2C19, CYP4F2, DPYD, RYR1, *and* SLCO1B1*) between the Macedonian and European populations.*



Figure 3.
*Comparison of haplotype distributions for nine pharmacogenes (*CYP2C9, UGT1A1, ABCG2, CFTR, CYP3A5, NUDT15, TPMT, VKORC1, *and* G6PD*) between the Macedonian and European populations.*

For the *CYP4F2* gene, CPIC frequencies are available only for alleles *1, *2, and *3, while in our population, we identified four additional alleles along with a large proportion of unknown haplotypes.

Concerning the *RYR1* gene, there is clear evidence for a sub-population specific allele frequency distribution. The higher frequency of the *RYR1*:c.6178G > T variant in our cohort is primarily due to the significantly higher frequency in the Romani sub-population (13.5%) compared to the Macedonians (8.4%) and Albanians (7.9%) from our cohort and to the frequencies reported in gnomAD exomes 2.1 for European (non-Finnish) population (7%).

The differences in the frequencies of the *UGT1A1* variants are primarily due to the differences in the methodology for the analysis of this gene. Namely, the *UGT1A1**80 + *28 haplotype reflects the presence of the *28 and *80 together on the same gene copy, while CPIC allele frequencies are mainly based on literature references which does not include the information on the *80 allele, and hence, *28 is typically reported without considering the *80 and vice versa.

3.3 Analysis of AMP's minimum sets of alleles for PGx testing

A total of 78 pharmacogenetic alleles are currently recommended for clinical testing by the AMP, comprising 36 Tier 1 alleles and 42 Tier 2 (optional testing) alleles.

Of these, 27 alleles were identified across 8 of the 10 evaluable genes in our cohort, since no variants were detected in *VKORC1* and *CYP3A4* genes (**Figure 4**). The majority of these alleles (n = 20) were Tier 1, while 7 belonged to Tier 2. All 526 individuals carried at least one of the 27 alleles, with the number per person ranging from one to nine (**Figure 5A**). Even if the *CYP3A5**3, which was observed in 91% of samples, is excluded from analysis, 481 subjects (91.4%) still carried at least one of the remaining 19 Tier 1 alleles, with one to six alleles per person (**Figure 5B**). We have not observed any notable difference in allele frequencies among different ethnic groups.

One of the objectives of our study was to assess whether any star alleles, not previously targeted by conventional genotyping methods in our country, were emerging. When comparing with previous studies for our population for *CYP2D6*

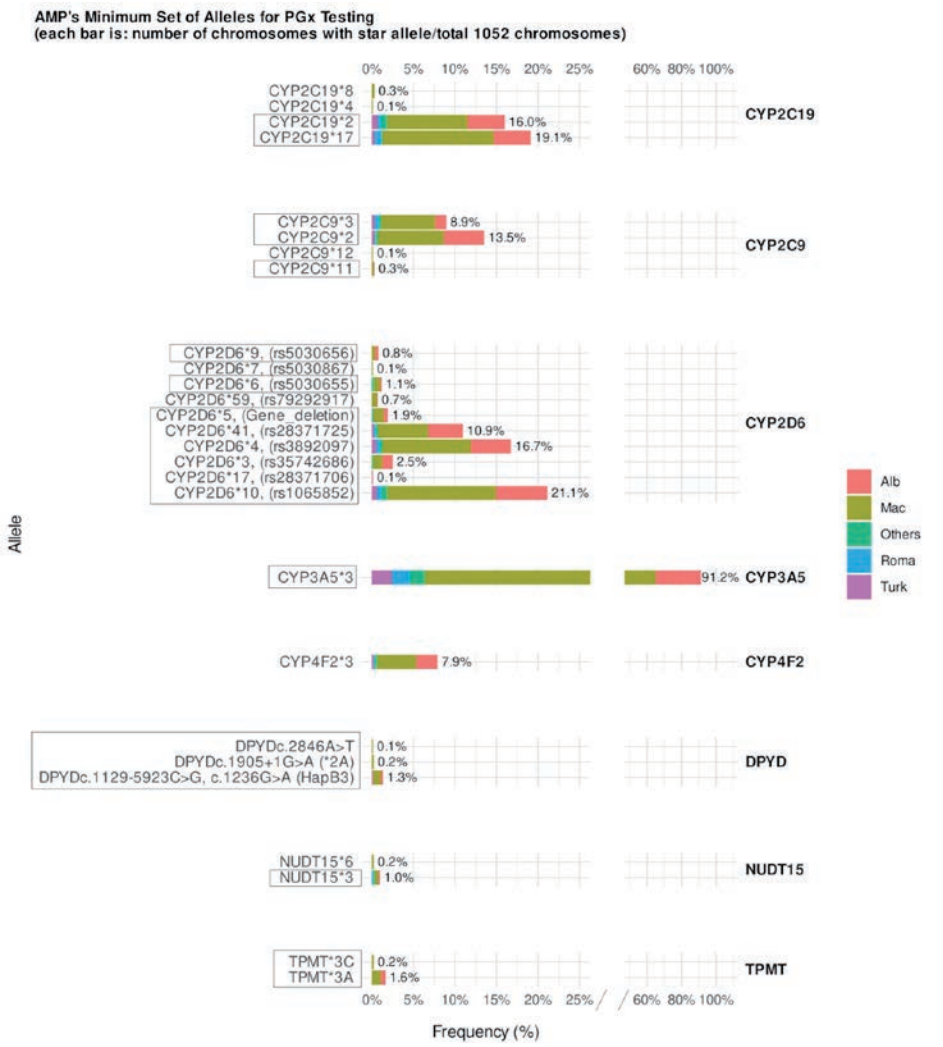


Figure 4.
Overview of the 27 AMP-recommended alleles detected in the population of North Macedonia across 8 pharmacogenes, categorized by the ethnicity of the subjects. For the CYP2D6 *gene, only frequencies for core variants (rs numbers in parenthesis) are presented. Tier 1 alleles are marked with a square. Alb—Albanian, Mac—Macedonian, Roma—Romani, and Turk—Turkish ethnic background.*

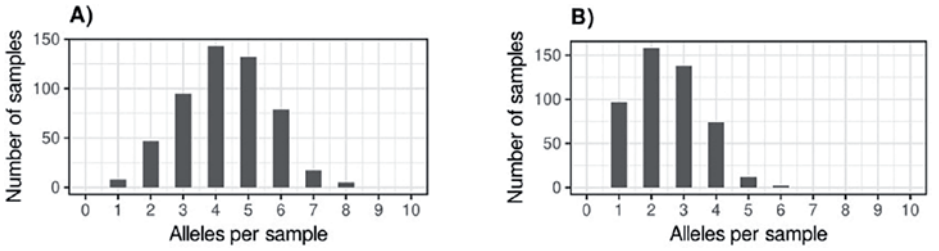


Figure 5.
Distribution of AMP-recommended alleles per individual. (A) Number of AMP-recommended alleles per individual (all identified 27 AMP-recommended alleles included); (B) number of Tier 1 AMP-recommended alleles per individual, excluding CYP3A5**3 (19 alleles).*

gene [26, 27], we have not observed significant difference for identified alleles from the AMP's minimum sets. Two *CYP2D6* alleles, *59 (found in 7 individuals) and *7 (found in 1 individual), were newly discovered alleles not targeted by previous studies, although they are Tier 2 alleles. Likewise, for the *CYP2C9*, *CYP2C19*, and *CYP3A5* genes, our results showed no significant divergence from allele frequencies reported in previous studies [27–29] and our study. We did identify an additional four alleles from the recommended AMP sets, albeit at low frequencies: *CYP2C9**11 (in three samples), *CYP2C9**12 (in one sample), *CYP2C19**8 (in three samples), and *CYP2C19**4 (in one sample). These findings imply that existing genotyping protocols used in our country, such as qPCR-based assays, could be relatively easily broadened to encompass the complete spectrum of clinically relevant AMP-recommended alleles.

3.4 Identification of novel non-functional variants

To identify novel non-functional variants, we analyzed 19 pharmacogenes in a cohort of 1415 individuals, which were previously assessed for known variants using PharmCat. Notably, *NUDT15*, which was not included in the clinical exome panel, was analyzed in the 1125 subjects with whole-exome sequencing data.

Across the examined genes, we identified a total of 1269 distinct variants within exons and flanking intronic regions. To prioritize putative non-functional variants, we applied a filtering strategy based on *in silico* prediction tools (**Table 1**), classifying variants into three categories: HIGH, MODERATE, and SPLICE. The HIGH-impact variants were predicted using the VEP annotator, while SPLICE variants were identified using SpliceAI. For MODERATE impact variants (missense variants), we considered only those predicted as pathogenic by a majority of the *in silico* tools (≥4 out of seven algorithms).

Following this filtering process, a set of 163 variants was retained, which comprised 35 variants previously targeted by PharmCat and 128 novel variants. No variants in *NUDT15* met our filtering criteria.

The comparison of the allele counts of novel versus known (PharmCAT-targeted) variants for each gene is shown in **Figure 6**. It should be noted that due to the absence of phasing information we were unable to determine the cis or trans configuration of alleles of multiple variants. Interestingly, for 8 genes (*ABCG2*, *CACNA1S*, *CYP3A4*, *CYP3A5*, *CYP4F2*, *IFNL3*, *UGT1A1,* and *VKORC1*), only novel putative non-functional variants were identified, whereas for three genes (*CYP2C19*, *CYP2C9,* and *CYP2D6*), only known variants met our filtering criteria.

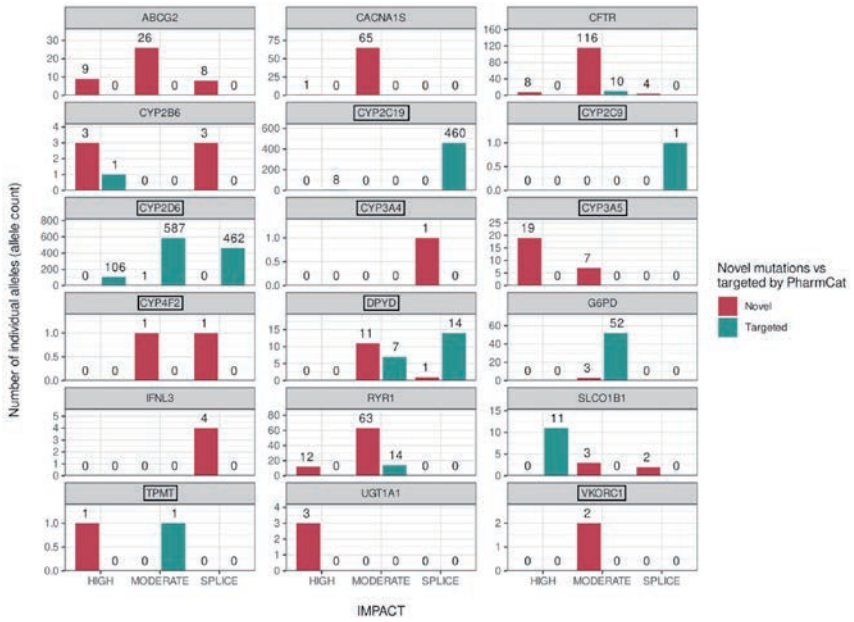


Figure 6.
Comparison of the allele counts and predicted impact of the novel and known putative non-functional variants identified in this study across the 19 analyzed pharmacogenes. Note that NUDT15 *is not represented as no variants met the filtering criteria for this gene. Variants are grouped based on their predicted functional impact: HIGH, MODERATE, and SPLICE. The y-axis represents the number of alleles for given variant category for a given gene (indicated above each panel). AMP-recommended genes are marked with a square.*

Our analysis identified 17 novel putative non-functional variants across 7 of the 10 genes included in the AMP's minimum sets of alleles for PGx testing (**Table 3**). These novel variants were observed on a total of 45 individual allele occurrences within our cohort. In contrast, we detected 16 known PharmCAT-targeted variants in 5 of the 10 genes included in the AMP's minimum sets of alleles for PGx testing (data not shown), with these variants observed a total of 1414 times within the cohort, indicating 1414 individual allele occurrences (**Figure 6**, **Table 3**). Notably, for one of the AMP recommendations gene, the *DPYD* gene, we found comparable number of alleles for known and novel non-functional variants. We have observed 21 alleles of 3 known AMP-recommended variants and 12 alleles in 8 novel variants (**Figure 6**, **Table 3**). Similarly, for *CYP3A5* also, we identified three novel variants with a combined allele count of 23 (**Table 3**), in parallel with the *CYP3A5**3 star allele, which was already identified at a high frequency in our cohort (**Figure 4**). Conversely, for *CYP3A4* and *VKORC1*, two other AMP-recommended genes, we detected no alleles corresponding to the known star alleles listed by AMP (**Figure 1**). However, we did identify novel potentially non-functional variants in both *CYP3A4* (c.866-1G > T) and *VKORC1* (c.118C > T) (**Table 3**), indicating the presence of uncaptured genetic variation.

4. Discussion

Pharmacogenetics is crucial for advancing personalized medicine since the integration of the PGx information into clinical decision-making holds the potential to

Gene_Variant	dbNSP	IMPACT	Consequence	Pathogenicity prediction by	AC	AF	gnomADe 2.1 AF
CYP2D6:c.1258 T > G	.	M	missense	CADD, BayesDel, MetaRNN, ESM1b, AlphaMissense, PHACTboost, Mutformer	1	0.000353	0
CYP3A4:c.866-1G > T	rs1413275858	S	splice_ acceptor	SplaceAI	1	0.000353	0
CYP3A5:c.1064A > G	rs149888520	M	missense	CADD, BayesDel, MetaRNN, ESM1b, AlphaMissense, Mutformer	1	0.000353	0.000271
CYP3A5:c.247dup	rs753330469	H	frameshift	VEP	1	0.000353	0.000155
CYP3A5:c.245 T > G	rs56244447	M	missense	MetaRNN, ESM1B, AlphaMissense, Mutformer	6	0.00212	0.000159
CYP3A5:c.92dup	rs28383469	H	frameshift	VEP	18	0.00636	0.006923
CYP4F2:c.1169G > A	rs372871763	M	missense	CADD, MetaRNN, ESM1b, AlphaMissense, PHACTboost	1	0.000353	2.78E-05
CYP4F2:c.344-1G > A	rs763450661	S	splice_ acceptor	SplaceAI	1	0.000353	1.2E-05
DPYD:c.2060A > C	rs755692084	M	missense	CADD, BayesDel, MetaRNN, ESM1b, Mutformer	3	0.00106	1.2E-05
DPYD:c.623G > T	rs376073289	M	missense	CADD, BayesDel, MetaRNN, ESM1b, AlphaMissense, PHACTboost, Mutformer	1	0.000353	1.99E-05
DPYD:c.1229G > A	rs199646142	M	missense	CADD, BayesDel, MetaRNN, Mutformer	1	0.000353	0.000179
DPYD:c.2071G > T	rs202212118	M	missense	CADD, MetaRNN, ESM1b, AlphaMissense, PHACTboost, Mutformer	1	0.000353	0.000156
DPYD:c.2137A > G	rs773407491	M	missense	CADD, MetaRNN, ESM1b, AlphaMissense, PHACTboost, Mutformer	1	0.000353	7.98E-06
DPYD:c.2329G > T	rs672601276	M	missense	CADD, BayesDel, MetaRNN, ESM1b, Mutformer	2	0.000707	2.79E-05
DPYD:c.2330C > A	rs374825099	M	missense	CADD, BayesDel, MetaRNN, ESM1b, Mutformer	2	0.000707	2.79E-05

Gene_Variant	dbNSP	IMPACT	Consequence	Pathogenicity prediction by	AC	AF	gnomADe 2.1 AF
DPYD:c.2059-2122 T > G	rs747189276	S	intron	SplaceAI	1	0.000353	1.2E-05
TPMT:c.676C > T	rs761990479	H	stop_gained	VEP	1	0.000353	2.79E-05
VKORC1:c.118C > T	.	M	missense	CADD, BayesDel, MetaRNN, ESM1b, AlphaMissense, PHACTboost, Mutformer	2	0.000707	0

AC: allele count; AF: allele frequency in the study population; H: HIGH impact; M: MODERATE impact; S: SPLICE impact; gnomADe_AF: allele frequency in the gnomAD v3.1.2 exome dataset (non-Finnish European population).

Table 3.
Seventeen novel putative non-functional variants identified in seven of the ten genes included in the AMP recommended minimum set of alleles for pharmacogenetic (PGx) testing. Allele frequency (AF) is calculated as allele count (AC) divided by allele number (n = 2830).

significantly improve treatment outcomes by enabling personalized drug selection and dosing regimens. However, the successful clinical translation of PGx relies heavily on accurate, population-specific knowledge of pharmacogenetic allele frequencies. Marked differences exist in the distribution of clinically actionable variants across geographically and ethnically diverse populations. While targeted pharmacogenetic testing has become increasingly common, comprehensive approaches are essential to fully characterize the pharmacogenetic landscape, particularly in understudied populations, like those in the Balkan Peninsula, including N. Macedonia. Whole exome sequencing (WES) offers a powerful and unbiased method to identify both known and novel pharmacogenetic variants across the entire protein-coding genome. We aimed to provide the first comprehensive analysis of pharmacogenetic (PGx) allele and phenotype distribution in a sizable cohort representative of the population of N. Macedonia, leveraging the power of whole exome sequencing, particularly extended WES.

When analyzing phenotype distribution, we have found that every single participant in our cohort carried at least one variant metabolizer allele across 13 pharmacogenes where phenotypes could be assigned based on allele combinations. This is in line with studies from large research cohorts, which also have demonstrated that vast majority of individuals (over 99%) harbor at least one divergent phenotype [11, 30]. The detailed comparison of diplotype and haplotype frequencies of our population with aggregated European populations revealed significant differences for several genes, (*SLCO1B1*, *CYP2C19*, *RYR1,* and *UGT1A1*). Such inter-population variability in the frequency of pharmacogenetic variants is well-documented [12] and emphasizes the importance of characterizing PGx landscapes in specific ethnic or geographic groups to inform local clinical practice and guideline implementation.

Our analysis also revealed the presence of 27 out of 78 clinically relevant AMP-recommended alleles within our cohort, many of which were observed at high frequency. A substantial majority of our cohort (91.4%, excluding *CYP3A5*3 which is observed in nearly all subjects) carried at least one Tier 1 AMP-recommended allele. Comparing our findings for key genes (*CYP2D6*, *CYP2C9*, *CYP2C19*, and *CYP3A5*) with previous, smaller-scale genotyping studies in North Macedonia [26–29], we observed largely consistent frequencies for the most common alleles. However, our WES-based approach allowed for the identification of additional, lower-frequency AMP alleles (e.g., *CYP2C9**11, *12; *CYP2C19**8, *4) and specific *CYP2D6* variants (*59, *7) not typically included in standard genotyping panels used locally. This suggests that existing targeted genotyping protocols used in our country, such as qPCR-based assays, could be relatively easily broadened to encompass the complete spectrum of clinically relevant AMP-recommended alleles that our study has revealed, building upon the existing coverage of the most common variants.

One significant advantage of employing sequencing-based methods like WES is the potential to identify novel genetic variants within pharmacogenes, extending beyond predefined targets. Our analysis on the larger cohort (1415 subjects) revealed 128 novel variants predicted to have putative non-functional impacts (HIGH, MODERATE, or SPLICE) across 19 pharmacogenes. While the allele frequency of these novel variants was generally lower compared to the known, targeted variants detected in the cohort, their presence, particularly within AMP-recommended genes, is noteworthy. We identified 17 novel putative non-functional variants in 7 of the 10 AMP genes analyzed, indicating that relevant genetic variation is not limited to currently recognized star alleles.

The identification of novel putative non-functional variants in genes like *DPYD*, *CYP3A4*, and *VKORC1*, which are crucial determinants of response to widely used

drugs (e.g., fluoropyrimidines for *DPYD*, many substrates for *CYP3A4*, warfarin for *VKORC1*), highlights the potential limitations of relying solely on panels targeting established variants. For *DPYD*, we found a comparable number of unique novel variants (8) as targeted ones (3), even if the total allele counts differed (12 novel vs. 21 known), suggesting a potentially broader landscape of functional alterations beyond the most common HaPB3 allele. It was already suggested that comprehensive assessment of the *DPYD* sequence could lead to substantial advancements in the pre-treatment identification of high-risk patients [31]. More strikingly, for *CYP3A4* and *VKORC1*, where no known AMP-recommended star alleles were detected in our cohort, we identified novel putative non-functional variants (*CYP3A4*:c.866-1G > T and *VKORC1*:c.118C > T). This finding underscores that relying only on current AMP lists might miss potentially clinically relevant variations in specific populations or individuals. Functional characterization of these novel variants is essential to ascertain their true impact on drug metabolism or response and to inform future updates of PGx guidelines and testing panels.

Despite its advantages in variant discovery, standard short-read WES faces limitations in fully resolving the complex genetic architecture of certain PGx genes, most notably *CYP2D6*. The high rate of unresolved or indeterminate classifications for *CYP2D6* with PharmCAT from WES VCFs is a well-known challenge [13], primarily due to the gene's pseudogene homology, structural variations (CNVs), and the difficulty of determining variant phase with short reads. These factors complicate accurate star allele assignment and phenotype prediction. While we utilized WES-based CNV calls to identify *CYP2D6* deletions (e.g., *5), future steps should involve validating these structural variant calls using orthogonal methods like MLPA to ensure accuracy and enable reliable reporting of deletions and duplications, which are critical for *CYP2D6* phenotype prediction. The optimal approach for complex genes like *CYP2D6*, and for unambiguously determining the phase of multiple variants, will require the integration of long-read sequencing data, which can span large or repetitive regions and improve haplotype resolution.

Studies employing WES or whole-genome sequencing for PGx profiling have increasingly demonstrated their value in providing a more comprehensive genetic picture compared to targeted genotyping [10, 11, 13]. These approaches enable the simultaneous assessment of numerous PGx genes and the discovery of novel variants. However, translating this wealth of sequencing data into actionable clinical recommendations requires robust bioinformatics tools and updated guidelines. Our findings suggest a need to refine current clinical PGx testing strategies. Firstly, methodologies should evolve to better handle complex genes like *CYP2D6* and provide phasing information where relevant. Secondly, expert consortia and regulatory bodies should establish clear criteria and pathways for reporting novel, potentially non-functional variants identified through sequencing, especially those with *in silico* predictions of impact comparable to or stronger than established PGx variants, as observed for some of our novel findings in AMP-recommended genes.

5. Conclusion

In conclusion, this study provides a comprehensive pharmacogenetic landscape of the population in our country, confirming the high prevalence of non-normal metabolizer phenotypes and highlighting significant inter-population variability. While previous targeted genotyping has captured the most common PGx alleles,

WES revealed additional known low-frequency AMP alleles and, importantly, novel putative non-functional variants within key PGx genes. These findings underscore the utility of comprehensive sequencing approaches for population-specific PGx characterization and variant discovery. However, moving forward, clinical PGx testing requires advancements in recommended sequencing methodologies to accurately capture complex variants and structural variations, as well as updated guidelines for the systematic evaluation and reporting of novel, potentially clinically relevant genetic variations.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 6

The State of the Art: Pharmacogenomics, Multi-Omics, and Translational Barriers in Antipsychotic Therapy

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Abstract

Pharmacogenomics is a crucial area in neuropsychopharmacology, offering a scientific foundation for precision medicine in treating psychiatric disorders, particularly schizophrenia spectrum disorders (SSD). Patients with SSD exhibit significant variability in their response to antipsychotic drugs, ranging from lack of efficacy to severe adverse drug reactions. This variability is largely due to genetic differences affecting drug metabolism (e.g., CYP450 enzymes such as CYP2D6 and CYP1A2) and drug targets (e.g., dopamine D2 and serotonin 2A receptors). For instance, an individual's metabolizer status can dramatically alter drug concentrations, influencing both therapeutic effectiveness and the risk of side effects, such as weight gain and extrapyramidal symptoms. Recent advancements including multi-gene panels and multi-omics approaches (genomics, epigenomics, and metabolomics) are deepening our understanding of these complex drug responses. While promising case studies and clinical trial data highlight the predictive power of pharmacogenetics, its routine clinical integration faces substantial challenges. These include the need for standardized protocols, more robust real-world evidence, and improved omics literacy among healthcare providers. Overcoming these hurdles through collaborative efforts and technological advancements is essential to fully achieve the potential of pharmacogenomics in personalizing antipsychotic treatment, ultimately leading to improved patient outcomes and more effective mental healthcare.

Keywords: pharmacogenomics, schizophrenia, antipsychotics, drug metabolism, precision medicine

1. Introduction

In recent years, pharmacogenomics, with its core component of pharmacogenetics, has become a central focus in neuropsychopharmacology research, providing a scientific

basis for developing precision medicine in the treatment of psychiatric disorders [1–4]. Specifically, this has become a critical area of exploration for schizophrenia spectrum disorders (SSD), which are severe mental disorders that affect around 1% of the global population [5]. The variability in patients' responses to antipsychotic drugs, ranging from lack of therapeutic effect to severe adverse drug reactions (ADRs), remains a significant clinical challenge [6]. Genetic variations affecting drug metabolism, transport and target engagement are now recognized as key contributors to these differences, and their identification may provide clinicians with powerful tools to optimize treatment regimens across diverse populations [7].

In addition to the aforementioned factors, a patient's response to a specific pharmacotherapy is a multifactorial phenomenon and is often complex. It is influenced by a diverse array of factors, such as pharmacokinetics, age, gender, ethnicity, and concomitant drug usage [8]. Even after considering these factors, accurately assessing the probability of ADRs and predicting drug efficacy remains a challenge. The differences in patient response to a drug can, at least partially, be attributed to genetic variations that affect drug metabolism and distribution, or to variations in the target proteins responsible for the drug's mechanism of action [9, 10]. In 1956, a hereditary glucose-6-phosphate dehydrogenase deficiency was linked to the hemolytic reaction caused by the antimalarial drug primaquine, suggesting for the first time the impact of genetic factors on drug side effects [9]. Furthermore, it has been well established that individual variations in drug serum concentrations may arise from genetically determined variations within the cytochrome P450 (CYP450) family [11].

Building on early discoveries, advances in sequencing technologies, such as next-generation sequencing (NGS), have significantly expanded the identification of genetic variants, including rare polymorphisms in drug-metabolizing enzymes and therapeutic targets [12]. However, translating this growing body of genetic data into clinically actionable guidance for antipsychotic treatment remains a major challenge [12, 13].

2. Pharmacogenetic mechanisms in antipsychotic treatment

Prescribing the optimal antipsychotic treatment for schizophrenia is very important, as it is well-established that individual patients have different responses to the available antipsychotic drugs.

2.1 Genetic variations affecting drug metabolism

The CYP450 family of enzymes is primarily responsible for metabolizing most antipsychotic drugs [14, 15]. Genetic variations, or polymorphisms, in the genes that encode these enzymes are a major contributor to the inter-individual variability in drug metabolism [15]. These polymorphisms can lead to a wide spectrum of metabolic phenotypes, classifying individuals into distinct categories: poor metabolizers (PMs), intermediate metabolizers (IMs), extensive/normal metabolizers (EMs/NMs), and ultra-rapid metabolizers (UMs) [15]. Each phenotype is associated with a different level of enzyme activity, which directly influences a patient's capacity to metabolize a drug and, consequently, affects drug efficacy and bioavailability. The most clinically relevant cytochrome (CYP) enzymes for antipsychotics metabolism include CYP2D6, CYP1A2, CYP2C19, and CYP3A4 [16].

Among the clinically relevant CYP enzymes, CYP2D6 is particularly significant in the metabolism of antipsychotic drugs and exhibits the largest degree of genetic

polymorphism, with more than 80 genetic variations identified [14]. Genetic variation has a significant impact on an individual's drug metabolism and response to a wide range of medications, and the *CYP2D6* gene is highly polymorphic, with over 135 distinct alleles described. The combination of these alleles in an individual can be used to calculate an activity score, which allows for the prediction of their metabolizer type [17]. The distribution of these phenotypes varies significantly across ethnicities; for example, roughly 5–10% of the European population is classified as PMs and 1–2% as UMs [14]. This metabolic variability has critical clinical implications, as metabolizer status highly influences required drug dosage. For example, carriers of low-activity *CYP2D6* alleles can have up to 80% higher serum concentrations of risperidone compared to those with the wild-type allele [14]. This impact was highlighted in a prospective study of adolescents with schizophrenia, where extensive metabolizers (EMs) showed a significant decrease in positive and negative syndrome scale (PANSS) scores, indicating improved effectiveness and fewer adverse reactions compared to IMs or PMs [18]. However, studies investigating the direct association between CYP2D6 metabolizer status and long-term clinical outcomes have yielded mixed results, with some research indicating a link between higher activity scores and increased symptom severity [19]. This is also reflected in clinical guidelines. The FDA recommends that in patients who are known CYP2D6 poor metabolizers (PMs), the usual dose of aripiprazole should be reduced by half to prevent toxicity [20].

Following CYP2D6, CYP1A2 emerges as another pivotal enzyme in the metabolic processing of numerous widely prescribed antipsychotics [18, 21]. Constitutively expressed in the human liver, it accounts for approximately 13% of the total hepatic CYP content [17]. CYP1A2 catalyzes the metabolism of roughly 9% of commonly used drugs, including the oxidation of drugs, such as theophylline, olanzapine, and propranolol [22]. Notably, it is the main CYP enzyme involved in the metabolism and clearance of clozapine [22, 23]. Previous studies have also identified a substantial 40- to 130-fold inter-individual difference in CYP1A2 activity and a 40-fold difference in protein expression among individuals, with 35–75% of these differences attributed to genetic factors [22, 23].

While *CYP1A2* exhibits genetic polymorphisms with up to 40 polymorphic variants reported, its metabolic function is also profoundly and uniquely shaped by environmental factors, a phenomenon known as phenoconversion [21–23]. Among these, smoking stands out as a critical influencer, with polycyclic aromatic hydrocarbons (PAHs) in tobacco smoke powerfully inducing CYP1A2 expression through the aryl hydrocarbon receptor (AHR) pathway [21]. Such induction boosts enzyme activity so significantly that smokers metabolize CYP1A2 substrates considerably faster than non-smokers. This accelerated metabolism can result in lower serum drug concentrations and, consequently, a diminished therapeutic effect [21]. Examples of variants include the *CYP1A2**1C allele, associated with decreased enzyme activity, and the *CYP1A2**1F allele, which is the most frequent and highly characterized, linked to increased enzyme activity and heightened inducibility by smoking [22]. High frequencies of this variant have been reported worldwide, including 60% of the Japanese population. Clinical examples of its impact include decreased blood levels of olanzapine in schizophrenic patients carrying the *CYP1A2* -163C > A polymorphism [22]. Furthermore, this variant contributes to an UM phenotype in smokers, yet a normal metabolizer phenotype in non-smokers [21]. This marked alteration in a metabolic capacity poses a significant clinical challenge upon smoking cessation, as it can lead to a rapid increase in drug levels, thereby escalating the risk of adverse drug reactions [21].

2.2 Genetic factors influencing drug targets

While pharmacogenetic variability stemming from drug-metabolizing enzymes significantly influences drug pharmacokinetics, an equally critical aspect lies in genetic factors influencing drug targets, which shape how drugs exert their therapeutic and adverse effects within the body. These targets, often receptors and transporters, determine the direct mechanisms underlying antipsychotics actions. Among the most crucial are variations in the dopamine D2 receptor (DRD2), a primary site of action for nearly all antipsychotics [24]. Investigations into DRD2 gene polymorphisms have revealed their influence on individual treatment outcomes. For instance, the deletion allele of the –141C insertion/deletion polymorphism in the promoter region is thought to reduce D2R2 density and activity and has been associated with poorer antipsychotic response [25]. Similarly, the A1 allele of the TaqIA polymorphism was hypothesized to influence treatment response by leading to reduced gene expression. However, findings across studies have been inconsistent, and a recent meta-analysis did not detect any association [25]. Other DRD2 polymorphisms, such as TaqIB or A-241G, have also been associated with clinical outcomes, but these findings require further investigation [26, 27]. Concurrently, the serotonin 2A receptor (HTR2A) gene, particularly the T102C (rs6313) polymorphism, has been suggested to influence the efficacy of antipsychotic drugs. However, the results of different studies have been inconsistent [28]. A meta-analysis confirmed that this inconsistency is largely influenced by ethnicity [28]. Specifically, a subgroup analysis revealed that in the Caucasian population, antipsychotic drugs were more effective in patients with the T allele. Conversely, East Asian patients with the TC genotype had better treatment outcomes, while in those with the CC genotype, the treatment was less effective [28]. Furthermore, the TC genotype was a good predictor of the efficacy of clozapine treatment [28]. Providing a specific clinical example of this pharmacodynamic variability, a prospective study in North Indian patients found that the presence of DRD2 Taq 1 D2D2 (rs180498) and 5-HT2A C516T (rs6305) CT genotypes was more likely to be associated with non-response to risperidone treatment [29]. Together, these genetic differences in drug targets provide essential insights into the diverse pharmacodynamic responses observed among patients receiving antipsychotic treatment, complementing the pharmacokinetic variations.

2.3 Genetic impact on pharmacokinetics and pharmacodynamics

The genetic variations in drug-metabolizing enzymes and drug targets, as discussed above, collectively contribute to a detailed understanding of how pharmacogenetics influences a drug's entire journey through the body, from uptake to elimination. Specifically, polymorphisms in the genes for CYP enzymes, such as CYP2D6 and CYP1A2, directly influence drug metabolism and excretion, which in turn causes significant individual variability in drug levels [22]. For instance, UMs metabolize drugs more quickly, resulting in lower therapeutic levels, while PMs accumulate drugs, increasing the risk of toxicity [14]. Furthermore, genetic variations in drug transport molecules, such as P-glycoprotein (encoded by the *ABCB1* or *MDR1* gene), also play a crucial role in affecting drug absorption and distribution [30, 31]. Ultimately, understanding how genetic differences alter the absorption, distribution, metabolism, and excretion (ADME) of drugs is essential for predicting a patient's full response and advancing toward truly personalized psychiatric care. Concurrently, genetic variations in drug target sites, such as the DRD2 and HTR2A receptors, alter drug

pharmacodynamics by affecting binding affinity, receptor density, or downstream signaling [24]. These variations can modify a drug's therapeutic effect and side effect profile even when its concentration is within the normal range (**Table 1**).

Enzyme (gene)	Primary role/function	Example Drug substrates (general)	Notes on clinical relevance
CYP2D6	Metabolism of ~25% of all drugs, particularly many antidepressants, antipsychotics, and opioids.	Antidepressants: Amitriptyline, nortriptyline, fluoxetine, paroxetine, venlafaxine, duloxetine Antipsychotics: Aripiprazole, risperidone, haloperidol, olanzapine Opioids: Codeine, tramadol, oxycodone (prodrug activation) Beta-blockers: Metoprolol, propranolol	Highly polymorphic. Genetic variation can lead to absent, decreased, normal, or increased enzyme activity, significantly impacting drug levels and risk of side effects or lack of efficacy.
CYP2C19	Metabolism of proton pump inhibitors (PPIs), some antidepressants, and antiplatelet drugs, such as clopidogrel (prodrug activation).	Antidepressants: Citalopram, escitalopram, imipramine PPIs: Omeprazole, lansoprazole, pantoprazole Antiplatelets: Clopidogrel Anticonvulsants: Phenytoin	Highly polymorphic. Genetic variants are associated with altered drug metabolism, impacting drug efficacy (e.g., clopidogrel) or toxicity (e.g., PPIs).
CYP2C9	Metabolism of NSAIDs, oral anticoagulants (e.g., warfarin), and some antidiabetic drugs.	NSAIDs: Celecoxib, ibuprofen, naproxen Anticoagulants: Warfarin Antidiabetics: Glipizide, tolbutamide Anticonvulsants: Phenytoin	Genetic variants significantly affect drug clearance, leading to increased risk of bleeding with warfarin or reduced efficacy/increased toxicity with NSAIDs.
CYP1A2	Metabolism of caffeine, clozapine, olanzapine, and some antidepressants. Influenced by smoking.	Antipsychotics: Clozapine, olanzapine Antidepressants: Duloxetine, imipramine Other: Caffeine, theophylline	Polymorphisms and induction (e.g., by smoking) can alter drug levels, impacting efficacy and side effect risk (e.g., clozapine).
UGT1A1	Glucuronidation of bilirubin and various drugs, including irinotecan.	Chemotherapy: Irinotecan (active metabolite SN-38) Other: Atazanavir, raltegravir (minor substrates)	Polymorphisms (e.g., *28 allele) are associated with reduced enzyme activity, increasing the risk of severe toxicity (e.g., neutropenia with irinotecan).
TPMT	Metabolism of thiopurine drugs (e.g., azathioprine, mercaptopurine, 6-mercapto-purine).	Thiopurines: Azathioprine, mercaptopurine, thioguanine	Genetic variants can lead to very low or absent enzyme activity, causing severe myelosuppression if standard thiopurine doses are given. Pre-emptive testing is standard.

Enzyme (gene)	Primary role/function	Example Drug substrates (general)	Notes on clinical relevance
DPYD	Metabolism of fluoropyrimidine chemotherapy drugs (e.g., 5-fluorouracil, capecitabine).	Fluoropyrimidines: 5-Fluorouracil (5-FU), Capecitabine, Tegafur	Genetic variants can lead to reduced or absent enzyme activity, significantly increasing the risk of severe, life-threatening toxicities (e.g., myelosuppression, neurotoxicity) with standard doses. Pre-emptive testing is recommended.
NAT2	Metabolism (acetylation) of various drugs and xenobiotics.	Antibiotics: Isoniazid, sulfasalazine Anti-arrhythmics: Procainamide anti-hypertensives: Hydralazine	Polymorphisms result in "slow" or "rapid" acetylator phenotypes, affecting drug accumulation and toxicity (slow acetylators) or therapeutic failure (rapid acetylators).
SLCO1B1	Encodes OATP1B1, a transporter involved in hepatic uptake of statins.	Statins: Simvastatin, atorvastatin, pravastatin, rosuvastatin	Polymorphisms (e.g., *5 allele) can reduce transporter function, leading to increased systemic statin concentrations and a higher risk of myopathy.

This table outlines major drug-metabolizing enzymes relevant to pharmacogenomics, along with examples of their common drug substrates, including antipsychotics. The list of substrates is not exhaustive. Abbreviations: PPIs, proton pump inhibitors; NSAIDs, non-steroidal anti-inflammatory drugs. Information primarily derived from the Pharmacogenomics Knowledgebase (PharmGKB) [32] and Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines [33].

Table 1.
Key pharmacogenomic enzymes and their drug substrates.

3. Current evidence of pharmacogenetics in antipsychotic response

3.1 Studies linking genetic polymorphisms to the efficacy of antipsychotics

Genetic polymorphisms in genes encoding drug targets, particularly dopamine and serotonin receptors, have been extensively studied to identify their association with antipsychotic efficacy [1]. A prominent area of research has focused on variations in the dopamine D2 receptor gene (*DRD2*), a primary target for most antipsychotics. For example, a clinical study found a strong association between the *DRD2* rs1079597 polymorphism and clinical response in patients treated with antipsychotic drugs. Although this association was not significant for all drugs or patient populations, it highlights the complexity of these relationships [34]. Similarly, the serotonin 2A receptor gene (*HTR2A*) has been a key research focus, with the *HTR2A* rs6313 polymorphism being associated with varying degrees of antipsychotic efficacy [28].

Beyond receptor genes, polymorphisms in drug-metabolizing enzymes and other transporters also play a significant role by influencing the concentration of the drug available at its target site [16]. As previously discussed, variants in the genes for cytochrome P450 enzymes, such as CYP2D6 and CYP1A2, can lead to UM or PM phenotypes, directly impacting plasma drug levels and, consequently, clinical efficacy [15, 18, 19]. For instance, one study found that the CYP2D6 metabolizer status significantly affects the plasma concentration of risperidone and its active metabolite, which can influence therapeutic response in adolescents with psychosis [18].

Recent research has also explored the role of the *COMT* gene, which encodes an enzyme catechol-O-methyltransferase, responsible for degrading dopamine, in influencing treatment response [35]. While findings have been mixed, some studies suggest that the *COMT* Val158Met polymorphism may be associated with better treatment outcomes in some patients [35]. Additionally, polymorphisms in the *ABCB1* gene (P-glycoprotein), which affect a drug's ability to cross the blood-brain barrier, have been associated with varying antipsychotic responses in some studies [30, 31].

3.2 Genetic predictors of adverse drug reactions

Beyond predicting therapeutic efficacy, pharmacogenetic research has advanced significantly in identifying genetic variants associated with susceptibility to ADRs. As in the case of efficacy, a patient's pharmacokinetic profile serves as a primary determinant of ADR risk. Individuals classified as PMs for key CYP450 enzymes may exhibit elevated plasma drug concentrations, thereby increasing the likelihood of dose-dependent adverse effects [15, 18, 19].

One of the most clinically significant ADRs of second-generation antipsychotics is rapid weight gain, which can increase the risk of metabolic syndrome, diabetes, and cardiovascular disease. Polymorphisms in the serotonin 2C receptor gene (*HTR2C*) have been consistently linked to this side effect, with certain variants predicting a greater propensity for weight gain, especially with drugs such as olanzapine and clozapine [36]. Other genes related to appetite regulation and metabolism, such as the melanocortin-4 receptor gene (*MC4R*) and the alpha-2A adrenergic receptor gene (*ADRA2A*), have also been associated with antipsychotic-induced weight gain [36, 37].

Extrapyramidal symptoms (EPS), including Parkinsonism, dystonia, and akathisia, are primarily caused by excessive dopamine D2 receptor antagonism, especially with first-generation antipsychotics. Genetic variants in the *DRD2* gene have been extensively studied as predictors of EPS and the more severe, long-term side effect of tardive dyskinesia (TD) [38, 39]. Certain polymorphisms, such as the *DRD2* Taq1A allele, have shown inconsistent but noteworthy associations with the risk of developing EPS and TD in some populations [38]. More recent findings also suggested a role of polymorphisms located in the genes coding for dopamine transporter (DAT) and various serotonin receptors (5-HTR) in the development of haloperidol-induced EPS, such as Parkinsonism and akathisia [40, 41].

Additionally, genetic variants in drug-metabolizing enzymes can contribute to EPS by increasing drug concentrations and, thus, the degree of D2 receptor antagonism [22, 39]. Finally, a recent study demonstrated that genetic variants can also predict the risk of other specific side effects, such as hyperprolactinemia. Particularly, polymorphisms in the *ABCB1* gene, which encodes the P-glycoprotein transporter, were significantly associated with risperidone/paliperidone-induced hyperprolactinemia, suggesting that genetic differences in drug transport across the blood-brain barrier can directly influence side effect profiles (**Table 2**) [31].

3.3 Case examples and clinical trial data

A compelling case study highlights the practical application of pharmacogenetic analysis in managing complex clinical situations [30]. The report details a patient with bipolar disorder who experienced a challenging clinical course, with frequent manic episodes, poor response to multiple drug regimens, and severe side effects, leading to numerous hospital admissions. A pharmacogenetic analysis was conducted

to guide treatment, revealing genetic variations in several key enzymes and transporters, including decreased expression of the MDR1 transport protein/P-glycoprotein, as well as alterations in CYP2B6, CYP2C9, and CYP3A5. Guided by these results,

Enzyme (gene)	Key polymorphisms	Metabolizer/ transporter status	Clinical outcome/recommendation	References
CYP2D6	*3, *4, *5 (no function); *10, *17, *41 (reduced function). Duplications (*1xN, *2xN) (increased function)	PM IM NM UM	PM/IM: Increased drug exposure (e.g., higher antidepressant levels, increased side effects such as orthostatic hypotension with tricyclic antidepressants; reduced activation of prodrugs such as codeine, leading to lack of efficacy). Dose reduction or alternative drugs are often recommended. UM: Decreased drug exposure (e.g., lower antidepressant levels, lack of efficacy; increased activation of prodrugs such as codeine, leading to increased toxicity). Dose increase or alternative drugs are often recommended.	[42]
CYP2C19	*2, *3 (no function); *17 (increased function)	PM IM NM UM	PM/IM: Reduced clopidogrel activation, leading to increased risk of cardiovascular events. Alternative antiplatelet recommended. PM/IM: Increased PPI exposure, potentially leading to increased efficacy for some conditions, but also possible side effects. UM: Decreased PPI exposure, potentially leading to reduced efficacy.	[43, 44]
CYP2C9	*2, *3 (reduced function)	IM PM	IM/PM: Decreased warfarin metabolism, leading to increased bleeding risk. Lower starting doses and careful monitoring are recommended. IM/PM: Increased NSAID exposure, potentially increasing side effect risk.	[45]
UGT1A1	*28 (TA-repeat polymorphism in promoter region)	Decreased enzyme activity	Decreased activity: Increased risk of severe neutropenia and diarrhea with irinotecan. Dose reduction or close monitoring is recommended. Associated with Gilbert's syndrome (benign unconjugated hyperbilirubinemia).	[46]
TPMT	*2, *3A, *3B, *3C (reduced/no function)	PM IM NM	PM: Severely increased risk of myelosuppression with standard thiopurine doses. Significantly reduced doses (e.g., 10%) or alternative therapy is required. IM: Increased risk of myelosuppression. Dose reduction (e.g., 30–50%) or close monitoring is recommended.	[47]
DPYD	*2A (IVS14 + 1G > A), c.1679 T > G (*13), c.2846A > T, c.1236G > A (HapB3)	Reduced/ absent enzyme activity	Reduced/absent activity: High risk of severe, potentially fatal toxicities (e.g., myelosuppression, neurotoxicity, and mucositis) with fluoropyrimidine chemotherapy. Avoidance or substantial dose reduction (e.g., 5-FU) is critical. Preemptive genotyping is recommended.	[48]

Enzyme (gene)	Key polymorphisms	Metabolizer/ transporter status	Clinical outcome/recommendation	References
SLCO1B1	c.521 T > C (*5)	Decreased transporter activity	Decreased activity: Increased systemic concentrations of statins, particularly simvastatin, increasing the risk of statin-induced myopathy. Lower statin dose or alternative statin recommended.	[49]

This table details selected pharmacogenomic polymorphisms, outlining their associated metabolizer or transporter status and specific clinical outcomes or recommendations. Examples of star (*) alleles and single nucleotide polymorphisms (SNPs) are provided. References are included for specific guidelines. Abbreviations: PM, poor metabolizer; IM, intermediate metabolizer; NM, normal metabolizer; UM, ultrarapid metabolizer; 5-FU, 5-fluorouracil; PPIs, proton pump inhibitors; NSAIDs, non-steroidal anti-inflammatory drugs.
*Note on star alleles and polymorphisms: Star (*) alleles denote specific genetic variants or haplotypes, where *1 typically represents the fully functional (wild type) allele, and other numbers (e.g., *2, *3) indicate alleles with reduced, no, or increased enzyme function. SNPs (single nucleotide polymorphisms) are common DNA sequence variations at a single nucleotide.*

Table 2.
Common pharmacogenomic polymorphisms and clinical outcomes.

the patient's complex polypharmacy regimen was modified to select drugs not metabolized by the altered pathways. Following these changes, the patient showed progressive clinical improvement, a reduction in side effects (such as sedation and weight gain), and a return to normal functioning. The case demonstrates how pharmacogenetic analysis can inform treatment decisions, leading to clinical stabilization, improved quality of life, and a reduction in healthcare resource consumption [39].

Beyond individual case reports, recent clinical trial data also demonstrate the predictive power of pharmacogenetics [50]. For instance, a study focusing on aripiprazole-induced weight gain developed a predictive model that combined clinical and genetic information. This research identified specific genetic variants that could predict an individual's susceptibility to significant weight gain, offering a proactive tool for clinicians to mitigate this common side effect [36].

4. Novel views and advances in pharmacogenetics

4.1 Emerging biomarkers and genetic testing

Although early pharmacogenetic research in psychiatry laid the groundwork by concentrating on essential metabolic enzymes, such as the cytochrome P450 family, the field has since broadened considerably in scope [1, 51, 52]. A major advancement has been the shift from single-gene testing to the use of multi-gene panels [51]. These comprehensive panels simultaneously analyze a broader range of genes involved in both pharmacokinetics and pharmacodynamics. In addition to traditional metabolizer genes, these panels often include variants in drug target genes beyond the dopamine D2 receptor, such as the dopamine D3 receptor gene (*DRD3*) and various serotonin receptor genes, such as *HTR2A* and *HTR2C* [28]. Other critical genes that are now regularly assessed include those encoding drug transporters, such as ABCB1 (P-glycoprotein), and genes encoding enzymes involved in neurotransmitter degradation, such as the *COMT* gene, with specific polymorphisms, such as the Val158Met variant, showing potential association with treatment outcomes [30, 35].

Furthermore, variants within the HLA system, such as *HLA*-B1502*, have been identified as crucial biomarkers for predicting severe adverse drug reactions to certain antipsychotics [22]. This multi-gene approach aims to provide a more holistic genetic profile, offering a richer dataset for anticipating complex drug responses [53].

Beyond genetic panels, cutting-edge research is exploring a more dynamic, multi-omics approach to biomarker discovery [53–56]. This involves integrating genomic data with transcriptomic (RNA expression), proteomic, and metabolomic information to capture a more complete picture of a patient's biological state [57]. For instance, the study of microRNAs (miRNAs), small non-coding RNA molecules that regulate gene expression, is an emerging area showing promise in identifying new biomarkers for antipsychotic efficacy [57, 58]. Furthermore, hypothesis-free, genome-wide approaches such as genome-wide association studies (GWAS) and whole-exome sequencing are being used to discover novel, previously unimplicated genes [59–61]. These studies have uncovered potential links between antipsychotic response and genetic variants in glutamatergic signaling pathways (e.g., *GRID2, GRM7*), suggesting that the genetic influences on treatment outcomes are more complex and distributed across multiple biological systems than initially believed [62, 63]. These emerging biomarkers and testing methods have the potential to move precision psychiatry toward a more robust and comprehensive predictive model [64].

4.2 Integration of multi-omics approaches (genomics, epigenomics, transcriptomics, proteomics, and metabolomics)

While pharmacogenetics has traditionally focused on single genetic variants, it is increasingly recognized that this single-layer approach may not fully capture the complexity of patient variability in antipsychotic treatment outcomes [53, 54, 65]. The integration of multi-omics data, including genomics, epigenomics, transcriptomics, proteomics, and metabolomics, provides a more comprehensive framework to dissect the molecular mechanisms underlying drug efficacy, tolerability, and adverse reactions [53]. This system-level approach moves beyond a static view of the genome to provide a dynamic and holistic picture of a patient's biological state.

For instance, epigenetic modifications such as DNA methylation and histone acetylation can dynamically regulate gene expression in response to environmental and pharmacological stimuli, thereby influencing treatment response independently of DNA sequence [55, 66–68]. Similarly, metabolomic profiling can reveal downstream biochemical signatures of drug metabolism and toxicity that are not evident from genetic data alone [69, 70]. Combining these layers of information enables the identification of multi-dimensional biomarkers with greater predictive power for clinical outcomes than genomics alone [53, 54].

To process these vast and diverse datasets, emerging computational methods and machine learning models are essential for their integration and interpretation, providing a clear path toward more precise and individualized antipsychotic therapy [52, 54]. However, significant challenges remain in standardizing data collection across studies, ensuring reproducibility of findings, and translating these complex, omics-derived biomarkers into routine clinical practice [54, 70].

4.3 Persisting challenges in the clinical implementation of pharmacogenomics

Despite significant scientific progress, the clinical implementation of pharmacogenomics in antipsychotic treatment continues to face multiple challenges [54, 70].

These barriers span from data generation and methodological limitations to clinical translation and broader ethical concerns. A major barrier is the lack of harmonized protocols across laboratories and studies [54, 70, 71]. Differences in sample handling, sequencing technologies, and analytical pipelines lead to inconsistencies, which limit the comparability and reproducibility of findings and slow the accumulation of robust clinical evidence. Furthermore, the integration of multi-omics data requires advanced bioinformatic pipelines and significant computational resources. While machine learning and AI-based methods show promise, they introduce new challenges regarding interpretability, model validation, and generalization across diverse populations [54, 56].

Translating pharmacogenetic associations from research to clinical guidance has also proven difficult [72]. Although a growing number of large-scale, prospective clinical trials demonstrate clear improvements in patient outcomes and cost-effectiveness, this evidence is not yet universally considered sufficient to overcome the gap in broader regulatory endorsement and reimbursement of pharmacogenomics testing in psychiatry [72–74]. Additionally, the utility of pharmacogenomics depends on clinician awareness and competence [72, 75, 76]. Many psychiatrists and other healthcare providers lack sufficient training in genomics and may be hesitant to incorporate genetic test results into prescribing decisions. The growing use of genetic information also raises important ethical, legal, and social implications (ELSI), including concerns about patient privacy, data security, and ensuring equitable access to testing to avoid exacerbating healthcare disparities [76, 77].

Despite these challenges, several initiatives offer promising paths forward. The expansion of large-scale biobanks and population cohorts with linked genomic and clinical data enables a more rigorous evaluation of pharmacogenomics utility in real-world settings [67, 68]. Advances in artificial intelligence and federated machine learning may help address computational bottlenecks and enhance predictive modeling [54, 56, 77, 78]. Furthermore, global efforts to develop standardized guidelines and integrate pharmacogenomics data into electronic health records are helping to bridge the gap between discovery and practice [78]. As evidence accumulates and technological barriers diminish, pharmacogenomics-guided drug prescribing may become a cornerstone of personalized psychiatry.

5. Discussion and future outlook

The field of pharmacogenetics has made remarkable strides, evolving from early discoveries of single-gene metabolism differences to the comprehensive, multi-omics approaches discussed in this review. The evidence presented underscores the significant role of genetic variations, particularly within key CYP450 enzymes and drug target receptors, in explaining inter-individual variability in antipsychotic response, encompassing both efficacy and the risk of adverse drug reactions. The expanding use of multigene panels and the integration of multi-omics data represent critical advances that can lead to a more holistic, system-based approach to precision psychiatry. However, as this review has demonstrated, the path to routine clinical implementation remains complex. The gap between research discovery and clinical practice is marked by persisting challenges, including the lack of standardized protocols, a need for more robust real-world evidence of cost-effectiveness, and insufficient genomic literacy among healthcare providers. As a result, the full potential of pharmacogenomics to guide antipsychotic prescribing has yet to be achieved in everyday clinical settings.

Despite these obstacles, several promising paths are emerging to bridge this translational gap. The expansion of large-scale biobanks and population cohorts, coupled with electronic health records, is critical for generating real-world evidence and validating genetic associations in diverse populations. Moreover, advances in artificial intelligence and machine learning are poised to address computational bottlenecks and enhance predictive modeling, ultimately paving the way for more sophisticated clinical decision support systems.

The integration of pharmacogenomics into clinical practice will require global collaborative efforts to establish robust, standardized guidelines. As technological barriers continue to decrease and costs lessen, pre-emptive pharmacogenomic testing may become a cornerstone of personalized psychiatric care, allowing clinicians to tailor treatment to a patient's genetic makeup. This approach will not only improve patient outcomes by optimizing drug efficacy and minimizing side effects but also ensure a more equitable approach to mental healthcare, announcing a new era for precision medicine in psychiatry.

6. Conclusion

Pharmacogenomics research has progressed significantly, moving from identifying single-gene metabolism differences to employing comprehensive multi-omics approaches. The evidence clearly shows that genetic variations, particularly in CYP450 enzymes and drug target receptors, are crucial in explaining the diverse responses to antipsychotic drugs, affecting both their effectiveness and the likelihood of adverse reactions. The adoption of multi-gene panels and the integration of diverse "omics" data are key advancements pushing us toward a more holistic, system-based approach in precision psychiatry.

However, realizing the full potential of pharmacogenomics in daily clinical practice for antipsychotic drug prescribing still faces considerable obstacles. These include a need for standardized protocols, more robust real-world evidence of cost-effectiveness, and improved omics literacy among healthcare professionals. Despite these challenges, promising avenues are emerging. Expanding large-scale biobanks and population cohorts linked with electronic health records will generate critical real-world evidence, while advances in artificial intelligence and machine learning can enhance predictive modeling. Ultimately, establishing global, standardized guidelines and making pre-emptive pharmacogenomic testing a routine part of care will allow clinicians to truly personalize treatment. This will not only optimize drug efficacy and minimize side effects but also promote a more equitable approach to mental healthcare, marking a new era for precision medicine in psychiatry.

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Conflict of interest

The authors declare no conflict of interest.

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