LC/MS/MS in drug development: targeting the brain

Pengfei Song, Bernd Meibohm, and Charles R. Yates
The University of Tennessee Health Science Center, Memphis, TN, USA

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INTRODUCTION

Success of drug development generally relies on selecting one or two most drug-like molecules from a large number of new chemical entities (NCEs) generated by combinatorial chemistry and subsequently filtered using *in silico* predictions. Drug-like properties include reliable bioactivity, favorable physical-chemical properties, and acceptable drug metabolism and pharmacokinetic (DMPK) profiles to enable “good” efficacy, availability, persistence, safety, and practicality in human (1). The use of highly sensitive and selective liquid chromatography tandem mass spectrometry (LC/MS/MS) assays during early *in vitro* and *in vivo* testing facilitates selection and advancement of one or two “best” compounds. Characterization of central nervous system (CNS) pharmacokinetics (PK), despite typically being performed during secondary or tertiary screening, is critical for the successful development of drugs targeting the CNS (e.g., anticonvulsants, antidepressants, anesthetics, antibacterials, and anticancer agents). The blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) represent the main anatomical and biochemical (structural or functional) barriers between the peripheral circulation and the CNS. Thus, a key issue during early stage CNS-targeted drug development is lead compound penetrability of the BBB and/or BCSFB. This review will provide an overview of the application of LC/MS/MS in the nonclinical development of CNS drugs.

BRAIN ARCHITECTURE

The brain is a heterogeneous multicompartiment organ, which is physically separated from the blood by the BBB and BCSFB. The BBB and BCSFB limit the transcellular and paracellular movement of xenobiotics from the blood into the CNS by employing numerous passive and active defense mechanisms including tight junctions, drug metabolizing enzymes, and efflux transporters (2–4).

Anatomical Barriers That Limit Drug Uptake

The BBB separates the systemic circulation and the brain parenchyma (e.g., astrocytes, microglia) and is formed by the tight apposition of polarized endothelial cells lining blood vessels in the brain. Tight junctions, limited endothelial pinocytosis, and lack of transendothelial fenestrae on the adjacent endothelial cells enable the BBB to restrict the paracellular permeability of hydrophilic drugs. However, highly lipophilic xenobiotics may penetrate the BBB via transendothelial diffusion.

The BCSFB is formed through tight junctions between polarized epithelial cells lining the choroid plexus, the arachnoid membrane, and circumventricular organs. These specialized epithelial cells, as compared to the endothelial cells forming the BBB, are more permeable and contain numerous fenestrations. As a result, the BCSFB is considered more “leaky” than the BBB.

Drug metabolizing enzymes capable of xenobiotic inactivation have been described in the small microvessels of the brain and particularly the choroid plexus (4) such as phase I enzymes [cytochrome P-450-dependent monooxygenase (5), NADPH-cytochrome P-450 reductase (6), CYP1A1 (7), monoamine oxidase

(MAO) (8), class III alcohol dehydrogenase (ADH), epoxide hydrolases, glutathione peroxidase (5)], and all three classes of phase II enzymes (UGT1A6, SULT1A1, GST) (4). In fact, the choroid plexus has been described as the “small liver in the brain,” because it contains significant oxidative drug metabolizing capacity as well as liver-type drug transporters (4,9).

Active Transport Mechanisms

A variety of ion channels and pumps organized in a polarized fashion facilitate active influx and efflux of xenobiotics at the level of the BBB and the BCSFB. Consequently, active transport processes may alter drug distribution, leading to concentration gradients between the CSF, brain extracellular fluid (ECF), and brain tissue.

The influx transport systems mostly function in the direction of influx from blood to brain at the BBB, including transporters for amino acids (System L), glucose (GLUT-1) (10), monocarboxylic acids (MCT1), organic cations (OCT1, OCT2, OCTN1), and organic anion transporters (Oatp 1 and Oatp2) (2). Transport activity for nucleosides and peptides has also been documented (11). Through appropriate chemical modification, any of the endogenous transport systems could, in theory, be used to transport a pharmacologic agent or pro-drug into the CNS. Utilization of these systems to facilitate CNS delivery remains a challenge in drug discovery and development (12,13).

Although influx transporters may be exploited to facilitate brain uptake, most attention during CNS drug development has focused on efflux transporters [e.g., P-glycoprotein (P-gp) and multidrug resistance protein family (MRP1–9)], because of their potential to limit brain uptake. P-gp, a 170-kDa phosphorylated and glycosylated plasma membrane protein, is the translated product of gene *ABCB1* in humans and the *mdr1a/1b* genes in rodents. P-gp is expressed on the subapical surface of the epithelium of the choroid plexus (BCSFB) (14,15) as well as the luminal surface of the endothelium of capillary microvessels (BBB) (16–18). The ability of P-gp to significantly limit the brain uptake of numerous drugs, including dexamethasone, digoxin, cyclosporin (19), ondansetron, and loperamide (20), has been demonstrated using P-gp knockout (*mdr1a/1b* $-/-$) mice.

The multidrug resistance-associated proteins (MRPs) belong to the ABC protein superfamily. There are at least nine human MRP isoforms expressed in the BBB, BCSFB, or brain tissue. However, the expression and location of these MRPs in the brain is far from being understood completely and has so far primarily only been verified by *in vitro* models (2). MRPs are probenecid-sensitive, multi-specific organic anion transporters that accept glucuronide, glutathione- and sulfo-conjugates as substrates and thus complement the drug metabolizing processes by extruding the conjugated metabolites formed within the cell (4). Active transport out of the brain by MRP family transporters in the BBB and BCSFB appears to be the predominant mechanism limiting access of nucleoside reverse transcriptase inhibitors to the brain (21). In summary, with the interplay of BBB and BCSFB, drug metabolizing enzymes and efflux transporters, the entry of the xenobiotics into the CNS is extremely limited.

LC/MS/MS PLATFORM

General Considerations in Brain Sample Analysis

The revolutionary expansion of LC/MS/MS resulted in the sudden disappearance of analytical techniques traditionally used in drug development including immunoradiometric assay (IRA), gas chromatography (GC), high-performance liquid chromatography (HPLC)-UV, or fluorescence (22). The sensitivity, selectivity, and wide ranging applicability of mass spectrometry has led to the selection of the triple-quadrupole LC/MS/MS as the primary bioanalytical tool in clinical bioanalysis and absorption, distribution, metabolism, and excretion (ADME) studies related to drug discovery and lead optimization. With regard to sensitivity, the triple quadrupole features a linear dynamic range covering four to five orders of magnitude, which permits detection of low picogram levels of analyte (on column). Superior selectivity is accomplished through the use of multiple-reaction monitoring (MRM), which permits precursor ion isolation in the first quadrupole of the instrument, fragmentation of this ion in the second quadrupole (collision chamber), and then isolation of a selected product ion in the third quadrupole. Moreover, MRM increases signal-to-noise ratio, which also leads to greater sensitivity.

There are a number of reasons why studies designed to characterize CNS drug disposition require high sensitivity as provided by LC/MS/MS techniques. First, the drug concentration in the CSF or brain interstitial fluid (ISF) generally represents unbound drug concentrations, since only the unbound drug can penetrate the BBB and BCSFB. Thus, highly protein bound drugs, in general, achieve much lower brain concentrations relative to total plasma concentration (ISF:plasma or CSF:plasma). Second, sample volume is limited whether one directly samples the CSF via cannulation or the CSF/ISF using microdialysis. Third, intrinsically low analyte recovery rates associated with microdialysis further reduce the concentration of recovered analyte. Lastly, buffers for brain tissue extraction, medium for *in vitro* screening, CSF, and ISF microdialysate all contain high salt concentrations as well as endogenous compounds, which may lead to matrix effects (e.g., loss in signal due to ion suppression and/or adduct formation). Numerous strategies to matrix effects (23) followed by sample cleanup have been proposed to improve analysis (24–26).

Cassette Dosing/Cassette Analysis

To take advantage of the high sensitivity and selectivity of LC/MS/MS, methods such as cassette dosing (N-in-one dosing) and cassette analysis by sample pooling strategies have been developed to analyze several potential lead compounds simultaneously in a cassette, thereby speeding up the lead selection and optimization. Streamlining early stage *in vitro*, *in vivo*, and *ex vivo* drug development by cassette dosing is made possible because (i) far fewer samples are analyzed in cassette analysis by sample pooling, thus minimizing the number of animals required and (ii) moving directly into *in vivo* testing avoids the issue of potential lack of correlation between *in vitro* and *in vivo* results (27–31).

Although frequently used to accelerate the examination of compounds *in vivo*, cassette dosing remains controversial, as it has a number of possible scientific limitations. First, certain compounds should not be mixed in the cassette including those having: (i) the potential to produce common daughter ions (i.e., isobars); (ii) solubility incompatibility; and (iii) different ionization modes (negative versus positive). Second, interpretation of the results may be problematic by unintended drug-drug interactions at the level of CYP450 isoenzymes and/or transporter proteins, like P-gp. An alternative approach is postdose pooling of samples (32). This method combines samples only when they are ready to be assayed, thereby

avoiding the problem of drug-drug interactions. This method is most valuable when generation of samples is relatively easy, as is the case for some *in vitro* screens where there is minimal contamination and crosstalk between analytes.

IN VITRO/IN VIVO CHARACTERIZATION OF CNS LEAD COMPOUNDS

In order to achieve good CNS availability and persistence after systemic administration, it is essential that a drug possesses an acceptable ADME profile in the systemic circulation. Industry and academia rely heavily on LC/MS/MS techniques to help generate ADME profiles (1,22,33). In addition, LC/MS/MS has been used extensively to characterize the CNS PK of drugs targeting the brain (e.g., plasma protein binding, *in vitro* permeability, and *in vivo* penetrability of the BBB/BCSFB after systemic drug administration).

Plasma Protein Binding

Plasma protein binding is considered of limited clinical relevance for drugs with peripheral targets (34). However, it is critical for drugs targeting the CNS, since it is generally accepted that passage of endogenous solutes and drugs across the BBB is restricted to the unbound drug fraction (35). For this reason, equilibration or partitioning of solutes between blood and brain or between blood and CSF is often expressed on the basis of unbound plasma concentration. Plasma protein binding, therefore, is a key determinant of a drug's CNS availability. Numerous methods exist for determining the extent of plasma protein binding of drugs. Equilibrium dialysis and ultrafiltration are the most routinely utilized methods. Both of these methods have been proven to be experimentally sound and to yield adequate protein binding data (36).

Recently, LC/MS/MS was used to develop a semiautomatic, high-throughput method involving a cocktail ultrafiltration method in a 96-well filtrate assembly to determine protein binding for 32 compounds in one single experiment, instead of 1–2 compounds using conventional methods (37).

BBB/BCSFB Permeability

A compound's BBB permeability is almost always of interest, either as a property to screen for a CNS indication or to counter-screen when CNS penetration is undesirable. During the past years, a number of *in vitro* cell culture models have been developed for the purpose of predicting the passage of drug candidates across the BBB and the BCSFB (32). A cell-based model offers the potential to account for transcellular and paracellular drug diffusional processes, metabolism, and active transport processes, as well as nondefined interactions between a drug and cellular material that may impact a membrane's overall permeability profile (38). Most *in vitro* models are designed to identify compounds that are P-gp substrates, since P-gp is the best characterized efflux transporter found in the BBB and BCSFB. For example, the colorectal carcinoma-derived cell line (Caco-2) (39–41) and the Madin Darby canine kidney (MDCK)-derived cell line (42) have been widely used to screen for P-gp substrates. These systems offer advantages common to *in vitro* models including (i) relatively high-throughput and the clean matrices favored by analytical chemists and (ii) the ability to isolate a single process or group of processes, so that it can be studied free from interferences caused by other factors (32).

The predictive power of these models is somewhat controversial, since they are not brain endothelial cells. In order to address this limitation, *in vitro* cell permeability models must possess certain characteristics believed to be critical for recapitulation of the BBB. For example, the model system must display a restrictive

paracellular pathway, a physiologically realistic cell architecture, functional expression of transporter mechanisms such as the P-gp and MRP, and ease of culture to facilitate high-throughput screening with reproducibility and validated by internal controls of low (e.g., sucrose) and high (e.g., diazepam or propranolol) brain-penetrating solutes. It is important to recognize that, for appropriate predictions of brain penetration, the quantitative relationships between in vitro and in vivo transporter function must be established. To this end, the primary bovine (or porcine) brain microvessel endothelial cell (BMEC) monolayer has been applied in high-throughput drug discovery screening for ranking lead compounds. Primary or low passage brain capillary endothelial cells provide the closest phenotypic resemblance to in vivo brain endothelial cells, and the cultured monolayers derived from them generate a restrictive paracellular barrier to solute permeability. A major disadvantage is that BMECs are not an immortalized cell line. Therefore, they must be primary-cultured from cow or porcine brain after tedious and time-consuming isolation, which inherently results in intra- and inter-batch variability (38).

BBB/BCSFB Penetrability

In vitro systems are now widely used during drug discovery to screen/counter-screen compounds by ranking their likelihood of CNS penetration. However, given the complexity of the heterogeneously multicompartmental brain, it is unlikely that in vitro BBB models alone can provide accurate, quantitative predictions of in vivo CNS availability. Therefore, CNS availability of promising drug candidates is evaluated in vivo during the later stages of preclinical drug development. The CNS availability after systemic drug administration may be determined by sampling brain tissue (i.e., brain homogenate), CSF, or brain extracellular fluid/ ISF concentrations at various times after systemic administration of a compound(s).

Brain Tissue Homogenate Sampling

Analyzed with LC/MS/MS, a brain homogenate was used by Tamvakopoulos et al. (31) to determine brain penetration of a group of compounds following cassette dosing. Verification of applicability with individual compound administration suggested no major differences (ranking order) in brain or plasma concentrations between mixture dosing and single compound administration (31). Frick et al. (32) showed for 89 triazines that concentrations of compounds at one hour post-dose in the brains of mice following cassette dosing ($n = 9$) were correlated with those observed after dosing with discrete compounds. These data illustrate that combination of cassette dosing and LC/MS/MS detection of drug concentration in brain tissue homogenates can dramatically increase the throughput for CNS lead optimization. However, many limitations may lead to misinterpretation of drug penetration to the brain, such as the questionable relevance of drug concentration in the heterogeneous brain compared to the target sites, the nonspecific binding to or sequestration of a candidate compound in the brain tissue, and the general lack of correction for the contribution of brain blood to the drug concentration in brain homogenate.

In Vivo Brain Sampling

Implantation of a catheter for direct sampling of the CSF has been used to overcome technical limitations associated with measuring brain homogenate concentrations as a marker of CNS availability. For example, serial CSF sampling can easily be performed through catheters inserted into the cisterna magna, thus enabling measurement of detailed drug concentration versus time curves in the CSF. The choroid plexi of the lateral, third, and fourth ventricles are responsible for CSF production (approximately

0.4 mL/min in humans and 2.1 μ L/min in rats). The CSF leaves the CNS by reabsorption back into blood via the arachnoid villi. In large animals, such as the dog or nonhuman primates, repeated studies in the same chronically catheterized animal make crossover studies possible to compare a series of candidate compounds in early stages of development (35).

It is worthwhile to note that the CSF is often regarded as being essentially free of drug binding proteins (albumin and α_1 -acid glycoprotein). In fact however, the CSF contains low levels of plasma-derived proteins that represent approximately 0.5% or less of plasma protein levels (35). Even at these low levels, binding of drugs to the CSF proteins can become significant and may influence the exchange of freely diffusible drugs between the CSF and the brain parenchyma (35). This implies that CSF samples should be treated with acetonitrile or methanol to precipitate CSF protein when necessary. Direct CSF sampling coupled with LC/MS/MS has been widely used to characterize the CSF PK of various drugs (43–48). Despite the advantages of direct CSF sampling over brain tissue homogenate sampling, the fact remains that CSF may only be considered a surrogate marker for ISF concentrations in discrete regions of the brain, the concentration at the site of action.

With the advent of microdialysis coupled to LC/MS/MS, it is now technically feasible to directly measure the unbound concentration of virtually every soluble compound in the brain ISF. Microdialysis facilitates sampling of drug in the ISF via a surgically implanted probe fitted with a semipermeable membrane that permits passage of small unbound substances. The implanted probe is continuously perfused with an artificial physiological solution. The amount/type of drug that passes into the perfusate is determined in part by solubility, molecular weight, and protein binding. In addition, the perfusate is typically an aqueous fluid with high ionic strength, meaning that hydrophilic drugs are more readily detected in the perfusate than hydrophobic drugs. Analytes that are extremely hydrophobic avidly adsorb to the microdialysis sampling tubing, which results in dramatically reduced analyte recovery and sensitivity. Finally, since the semipermeable membrane restricts the passage of large molecular weight species (e.g., the binding protein albumin), drug found in the perfusate represents only the unbound fraction of drug. The combination of these factors dramatically reduces analyte recovery, particularly for highly hydrophobic drugs, thus demanding the sensitivity provided by LC/MS/MS for this kind of studies.

During microdialysis, no fluid is actually removed, so continuous sampling can be performed with minimal disruption of the physiological system. Postsample clean-up prior to injection is usually not required, since the microdialysate is protein-free. However, since the perfusate ionic strength is high, there is a potential for reduced sensitivity due to matrix effects. Acceptable precision (i.e., coefficients of variation of approximately 20% or lower) following microdialysate sampling have been demonstrated (49).

CSF/ISF Pharmacokinetics

In recent years, the ability to directly sample both the ISF and CSF has provided unprecedented insights into the kinetics of drug exchange across the BBB and the BCSFB, as well as drug distribution between the brain ISF and CSF. Shen et al. (35) analyzed the plasma-CSF-brain partitioning data of 104 (10 classes) CNS drugs with CSF-to-plasma (CSF:Pu), brain-to-plasma, and CSF-to-brain concentration ratios. A hyperbolic relationship between the CSF:Pu ratio and the diffusibility index $\log[P_c \times MW^{-1/2}]$ for drugs that cross membranes by passive diffusion was observed. For hydrophilic and/or large molecules, the index is small, and permeability across the BBB and/or BCSFB is rate limiting. As a result, the sink action of CSF leads to a CSF:Pu ratio well below unity. As lipophilicity increases, CSF:Pu ratio is expected to rise towards unity. A plateau approaching

unity is expected for drugs with high lipophilicity and permeability. In addition, only 10 of the 104 drugs showed a CSF:Pu ratio greater than 2, which supports the notion that in vitro plasma protein binding studies can be used for counter-screening of CNS drugs (i.e., drugs that are highly protein bound are less attractive CNS drugs since they are likely to have very low CNS penetrability). Dubey et al. (50) suggested that the drug uptake into the brain that extends beyond the plasma unbound fraction has never been demonstrated at steady-state in the presence of intact cerebral perfusion, and Shen et al. (35) concluded that the free drug rule remains a generally proven governing principle in uptake of drugs from blood into the brain.

Moreover, as ISF and CSF drug concentrations reflect the unbound drug fraction in the brain, it is now possible to characterize the CNS drug concentration-effect relationship. For example, pharmacokinetic-pharmacodynamic (PK-PD) models can be derived to explain the lag in the time course of a central pharmacological effect relative to that of drug concentration in the circulation following systemic drug administration (i.e., as revealed by a counterclockwise hysteresis in the serum concentration-effect relationship of CNS drugs) (35).

Wang et al. (47) investigated serum, CSF, and ISF kinetic interrelationship of tiagabine in a freely moving rat model. After intraperitoneal (i.p.) administration of tiagabine, blood, CSF, and ISF samples were collected at timed intervals, and tiagabine concentrations were measured by LC/MS/MS. Results showed that the elimination from CSF was comparable to that of serum, whereas the half-life ($t_{1/2}$) in ISF was three times longer. The authors concluded that tiagabine free protein-unbound concentrations in serum are not reflective of CSF concentrations, and furthermore, the 3-fold slower elimination of tiagabine from the brain explains the relatively long duration of action of tiagabine. The short tiagabine $t_{1/2}$ in serum, which is generally perceived as a disadvantage for the clinical therapeutics of tiagabine, may actually only be of limited relevance (47).

Direct CSF sampling via cannulae implanted in the cisterna magna (35,51–53) offers a practical sampling approach in terms of sensitivity, cost, and throughput in early drug discovery and development (35). CSF concentration is a good indicator of drug availability to the CNS for hydrophilic or large molecular weight compounds with poor-to-moderate permeability (35), although it's controversial (21). There are several excellent reviews regarding the use CSF concentration as a surrogate measure of CNS drug availability as well as the applicability and limitations of CSF sampling for assessing the concentration-effect relationship of drug candidates with varying physicochemical properties during drug discovery and development (21,35,54).

CONCLUDING REMARKS

Historically, CNS drug development has relied upon relatively imprecise methods to assess CNS availability (e.g., measuring drug concentration in brain tissue homogenates or using surrogate markers such as plasma drug concentration). However, recent advances in the field of mass spectrometry (e.g., increased sensitivity and selectivity), have enabled the sampling of discrete brain compartments previously considered inaccessible for various technical reasons. Cassette dosing followed by determination of unbound drug concentrations at the intended site of action in the brain by LC/MS/MS, either with or without the use of microdialysis, allows drug development scientists to increase the speed and efficiency of throughput screening of lead compounds targeting the CNS. In addition, pharmaceutical scientists can now directly compare in vivo data with data obtained from in vitro BBB permeability models, with the intended goal of refining existing models and/or developing models that more accurately recapitulate the whole animal.

Despite revolutionizing small molecule drug discovery, cassette dosing followed by determination of drug concentration by LC/MS/MS represents only the latest tool to facilitate the use of mass spectrometry in drug discovery. Future innovations involve subpharmacological/subtherapeutic dosing following by drug concentration determination by ultrasensitive "big-physics" instruments: accelerator mass spectrometry (AMS) (55) and positron emission tomography (PET) (56,57). In theory, the use of next-generation mass spectrometers with ultrasensitivity [i.e., zeptogram (10^{-21} g)] permits cassette microdosing, where a human microdose is defined as one-hundredth of the proposed pharmacological dose derived from animal and in vitro models (58). Microdosing is proposed to be added to the drug development scientist's armamentarium within the next 10 years and is expected to further accelerate the drug development process by allowing one to generate in vivo human disposition data for lead compounds at an early development stage (56,57).

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address correspondence to:

Charles R. Yates
The University of Tennessee, Memphis
Department of Pharmaceutical Sciences
874 Union Avenue
Crowe Building Rm 5P
Memphis, TN 38163, USA
e-mail: cyates@utmem.edu