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Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 359–368
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Brain and plasma exposure profiling in early drug discovery using cassette administration and fast liquid chromatography-tandem mass spectrometry

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Received 9 November 2002; received in revised form 6 June 2003; accepted 2 September 2003

Abstract

A method using reverse phase liquid chromatography-tandem mass spectrometry and cassette administration was developed for in vivo brain and plasma exposure profiling to assist early CNS drug discovery programs. Three to four compounds were grouped in cassettes for dosing and analysis. Compounds in the cassettes were selected to minimize possible analytical interference from each other, as well as from their potential metabolites. In order to improve the confidence of cassette administration, an analogue of the study compounds, with well-established brain penetration data, was included in each cassette as a "biological internal standard". Compounds were administered to rats by intraperitoneal injection and extracted from plasma or brain homogenate by simple protein precipitation. Fast chromatographic separation was achieved by using a short narrow-bore column at a flow rate of 1.0 ml/min with a fast gradient. The brain penetration of the compounds was evaluated by comparing their C_{max} and AUC values in brain and plasma. This approach rapidly provided early brain penetration and plasma exposure information, thus making more of this data available to teams. Comparing the brain exposures to the EC_{50} values (i.e. in vitro potency) of series compounds in the same discovery program provided another dimension of information to select lead compounds for future in vivo assessment. The method described here has been used for providing early brain penetration information in several CNS exploratory and discovery programs.

Keywords: Early in vivo brain and plasma exposure; Fast liquid chromatography–tandem mass spectrometry; Cassette administration; Biological internal standard

1. Introduction

In the last decade, the pharmaceutical industry has developed new technologies, such as combinatorial chemistry, molecular biology and genomics to generate diverse and large compound libraries and identify new targets. High-throughput screening of libraries against multiple disease targets introduced the ability to rapidly identify bio-active molecules. ADME studies and physicochemical property profiles are also conducted early in the drug discovery phase to assist optimization of chemical

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doi:10.1016/S0731-7085(03)00523-5

structures, leading to drug candidates with desirable biopharmaceutical and pharmacokinetic properties. The concentration of a drug and its time course exposure in brain are particularly important for CNS-active compounds, where the ability to penetrate the blood-brain barrier is a prerequisite. The brain penetration of a compound can be estimated by simultaneously measuring its plasma and brain concentrations, following administration. In the past, this information only became available in the later stages of drug discovery, due to the time and manpower required. Conducting in vivo brain exposure studies in the early stages of drug discovery provides useful information to guide structure optimization, to select optimal conditions for in vivo bio-activity assays, to correlate exposure and in vivo bio-activity, and to help rank compounds for CNS exploratory and discovery programs.

Liquid chromatography combined with tandem mass spectrometry (LC–MS/MS) has been widely used for quantitation of organic molecules in biological matrices in drug discovery [1,2]. Due to the sensitivity and selectivity provided by LC–MS/MS, a method for quantitative analysis can be rapidly developed. Often, a generic method can be used for compounds in the same structure class. Recently, there has been great attention to employ new techniques, such as fast LC–MS separation [1,3], on-line SPE [4], and parallel LC–MS analysis [5] for high-throughput analysis. Post-dose sample pooling is another approach to reduce the analysis time. A recent report by Korfmacher, et al. described a 'rapid rat' pharmacokinetic screening model [6]. In their study, plasma samples from individual animals were pooled across time points to provide a smaller number of test samples to be assayed for each test compound. Atherton, et al. also reported the application of sample pooling to increase throughput of brain penetration profiles in rats [7]. The advantages of using the post-dose sample pooling approach are reduction in analysis time, and avoidance of drug–drug interaction. However, this approach does not reduce the number of animals and the time of animal work required for screening. An alternative way to increase throughput is cassette dosing, also referred to as N-in-1 dosing [2,8,9]. In this approach, in vivo exposure and pharmacokinetic parameters of a number of compounds can be simultaneously determined. The methodology reduces: the number of animals, the time for animal work, sample preparation, and analysis. Although the advantages and disadvantages of the approach have been vigorously debated [10], cassette dosing has been widely used for pharmacokinetic screening in the pharmaceutical industry. This methodology, if applied with proper recognition of the pitfalls, can provide useful data to help accelerate lead candidate evaluation [11]. Few studies have been reported using cassette dosing methodology to obtain brain exposure profiles. Frick et al. [8] and Tamvakopoulos et al. [12] discussed the validity of cassette dosing for determining brain concentrations in mice and rats, respectively. Their findings showed that the concentration–time profiles of the specific classes of compounds for cassette dosing were similar to those observed for individual dosing.

This article describes the development and application of a method using liquid chromatography-tandem mass spectrometry and intraperitoneal cassette administration to determine brain and plasma exposure during early drug discovery in CNS programs. In this method, rapid LC–MS/MS analysis has been achieved using a short narrow-bore column and a fast mass spectrometric scan. In order to monitor the possible drug–drug interactions, a "biological internal standard" was included in each cassette as a positive control. This control has a well-established brain penetration profile when analyzed by traditional individual analyses. The method described here has been used as a screening tool to provide rapid brain exposure information for several CNS exploratory and discovery programs.

2. Experimental

2.1. Reagents and chemicals

LC grade water, acetonitrile and methanol were obtained from EM Science (Gibbstown, NJ, USA). Ammonium acetate, methylcellulose and formic acid were purchased from Sigma. Dimethylamine was obtained from Aldrich. Tween 80 were from J.T Baker (Phillipsburg, NJ, USA). All analytes were obtained from Wyeth Research (Princeton, NJ, USA).

2.2. Animal dosing

Male Sprague–Dawley rats (Charles River, Willmington, MA, USA), weighting 200–300 g, were fasted overnight prior to dosing. Compounds were dissolved in water–methylcellulose–Tween 80 (97.5:0.5:2, v/v/v) at a concentration of 0.6 mg/ml (3.0 mg/5 ml each compound) and sonicated until a uniform homogenous solution was obtained. The dose was administered intraperitoneally at 3 mg/kg per compound (5 ml/kg) for cassette administration. Three or four compounds were grouped in a cassette according to their structures, molecular weights and LC–MS conditions. A "biological internal standard" was included in each cassette as a positive control for cassette administration. Two animals were used at each time point. Total 12 animals were used for six time points in a cassette analysis. The rat blood and brain were collected at 5, 10, 15, 30, 60 and 120 min. Vehicle-injected animals were sacrificed 30 min post-dosing. Animals were anesthetized with isoflurane prior to sacrifice. Blood (3 ml) was collected into 6 ml heparinized Vacutainer tubes by cardiac puncture. Plasma was separated by centrifugation and frozen at −80 °C until analysis. Following blood collection, the animals were perfused transcardially with 50 ml of cold PBS to remove blood from the brain. The brain was immediately harvested, weighed, polytron homogenized in 5 ml of cold distilled water, and frozen at −80 °C until analysis.

2.3. Sample preparation procedure

Stock solutions of standards were prepared by diluting a 1 mg/ml DMSO solution of each compound with water–acetonitrile (50:50, v/v). The resulting stock solutions of standards contained a mixture of the compounds in a cassette. An internal standard solution (structure analogue of the analytes) was prepared in a concentration of 5 μg/ml in water–acetonitrile (50:50, v/v). Stock solutions of QCs were prepared by separately weighing the solid compounds and dissolving in solvents as described for the standards. Standards and QC samples were prepared by spiking control plasma and brain homogenate aliquots (vehicle-injected) with 20 μl of internal standard and 20 μl of the corresponding stock standard solutions, which contained a mixture of the study compounds, followed by vortexing. The brain and plasma samples were prepared by spiking 20 μl of internal standard solution and 20 μl of water–acetonitrile (50:50, v/v) into plasma and brain homogenate aliquots (200 μl and 400 mg, respectively), followed by vortexing. Protein precipitation was performed by adding 1 or 3 ml of cold acetonitrile for plasma and brain aliquots, respectively, followed by vortexing and centrifugation at 3000 rpm and 5 °C for 10 min. Then, the supernatant was transferred into glass tubes and dried down under N_2 using a Zymark TurboVap evaporator at 40 °C. The residuals were finally reconstituted into 100 μl of water–acetonitrile (50:50, v/v) for LC–MS/MS analysis. Eight standards and six QCs at three different concentration levels for brain and plasma samples in the concentration range 1–2000 ng/g and 1–2000 ng/ml, respectively, were used for each cassette study.

To determine the recovery of analytes from brain homogenate, the analyte brain homogenate and the control brain homogenate samples were prepared using the same sample clean up procedure as discussed above. The analyte brain samples were prepared by spiking 400 mg of brain homogenate with 20 μl of stock solution of compound mixture, followed by adding 3 ml of cold acetonitrile, vortexing and centrifugation. The supernatant was dried down under N_2 and reconstituted into 100 μl of water–acetonitrile (50:50, v/v). The control brain samples were prepared by aliquoting 400 mg of brain homogenate, followed by protein precipitation and centrifugation. The supernatant was dried down under N_2 and reconstituted into 80 μl of water–acetonitrile (50:50, v/v). Twenty microliters of stock solution of compound mixture were then spiked into the reconstituted solution to make final volume of 100 μl.

2.4. Chromatography

A Waters 2790 liquid chromatograph (Waters, Milford, MA, USA) was used for all LC analysis. Chromatographic separation was carried out using cartridge columns (2 mm i.d., 20 mm length, and 3 or 5 μM particle site) with a 0.5 μM prefilter frit (Keystone Scientific Inc., Bellefonte, PA, USA) at an oven temperature of 50 °C. The columns used were Xterra MS C18 (Waters, Milford, MA, USA), Aquasil C18 and BDS Hypersil C8 (Keystone Scientific Inc., Bellefonte, PA USA). The mobile phase consisted of

solvent A: 10 μM ammonium acetate (or 0.1% dimethylamine, or 0.1% formic acid, v/v) in water–acetonitrile (95:5, v/v) and solvent B: 10 μM ammonium acetate (or 0.1% dimethylamine, or 0.1% formic acid, v/v) in water–acetonitrile (5:95, v/v). The column and mobile phase were selected for optimal electrospray ionization efficiency, and for optimal chromatographic separation and peak shape. A generic fast linear gradient started from 5% of B for 0.5 min, ramped to 95% of B in 3 min, held at 95% B for 0.1 min, then ramped back to 5% of B in 0.4 min, and finally held at 5% of B for 1 min. The flow rate was 1 ml/min and the effluent was diverted from the mass spectrometer for the first 0.5 min. The 1 ml/min effluent from the LC column was split before the MS and ∼0.3 ml/min effluent was directed into the electrospray interface of the mass spectrometer. The chromatographic conditions for study compounds were tested in a cassette mode.

2.5. Mass spectrometry

On-line LC–MS/MS analyses were performed using a Micromass Quatro Ultima tandem quadrupole mass spectrometer (Micromass, Beverly, MA, USA) operated in negative or positive electrospray ionization mode with the source temperature at 120 °C. The split LC elute was sprayed into the mass spectrometer at a desolvation temperature of 300 °C and at a spray voltage of +3.5 or −2.5 kV for positive and negative ionization, respectively. Nitrogen was used as both desolvation (1000 h^{-1}) and nebuliser gas (fully open). The pressure of the argon collision gas was set at 5 psi and adjusted to an analyzer pressure of 2.0–3.0 × 10^{-4} mbar. The mass spectrometer was optimized prior to the analysis by post column infusion of 10 ng/μl of analytes with a LC flow at 300 μl/min flow rate. Multiple reaction monitoring (MRM) conditions for each compound were then developed. The MRM analyses were performed by passing protonated ($[M+H]^+$) or deprotonated ($[M-H]^-$) molecular ions through the first quadrupole (Q1) and collision dissociating the molecular ions in the second quadrupole (collision cell—Q2). A selected product ion, based on intensity and structure characteristics, was isolated by the third quadrupole (Q3) and detected with the photomultiplier set at 650. The Dwell time for each MRM transition was set at 0.13 s. This approach provided a sensitive and selective analysis that is unique for individual analytes. The concentrations of analytes in plasma and brain were determined by their area ratios to that of the internal standard using a weighting linear fit. Compound exposure following administration was determined by calculating the AUC (area under the curve of the mean concentrations from 0 to 120 min) using Win-Nonlin professional version 3.1 (Pharsight Corporation, Cary, NC, USA).

3. Results and discussion

In the study, intraperitoneal administration was chosen to reduce first pass metabolism of compounds and to simplify the operation. In the study, the brain penetration of the compounds was evaluated by comparing their AUC values in brain and plasma. Therefore, cassette dosing is amenable to all types of administration, such as IV, oral, SC, etc.

3.1. Recovery of analytes from brain homogenate

A simple protein precipitation method was used for both brain and plasma sample preparation. The acetontrile protein precipitation method has been commonly used for plasma sample clean up. The recovery of analytes from brain homogenate after protein precipitation using acetonitrile was determined by comparison of area ratios of an analyte peak in analyte brain homogenate samples to that in control brain homogenate samples. The control brain and analyte brain homogenate samples were prepared by using the same sample clean up procedure as discussed in the experimental section. However, for the analyte brain homogenate samples, 20 μl of stock solution of compound mixture were spiked before the protein precipitation step. For the control brain homogenate

Table 1
Recoveries (%) of four compounds in a cassette from brain homogenate by acetonitrile protein precipitation

Compound	57 ng/g	740 ng/g
A	85	93
B	84	79
C	90	85
D	87	79

samples, 20 μl of stock solution of compound mixture were spiked after the reconstitution step. This approach allowed minimization of the matrix effect during the ionization process, which might cause false positive or negative results. The recoveries of four compounds in a cassette at two different concentrations are shown in Table 1. The recovery of these four compounds at two different concentrations, 57 and 740 ng/g, was in a range of 79–93%. This simple protein precipitation method was employed generically in our laboratory to assess structures from various CNS discovery projects. Recoveries of better than 80% were achieved for most compounds in these studies.

3.2. LC–MS/MS analysis

Multiple reaction monitoring provided high sensitivity for quantitation. Generally, the MRM transition

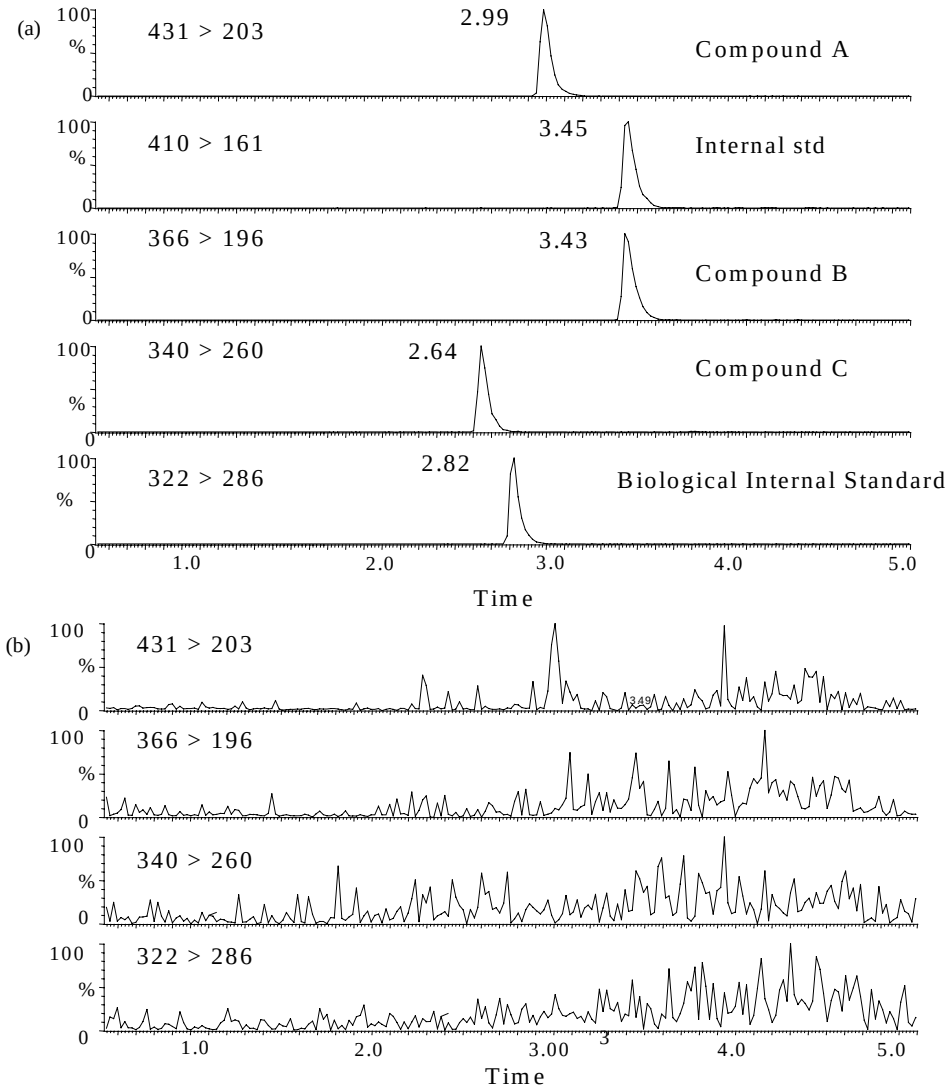

Fig. 1. Multiple reaction monitoring chromatograms of (a) brain homogenate extract spiked with three study compounds, a "biological internal standard" and a quantitation internal standard in a cassette and (b) vehicle brain homogenate extract.

was chosen based on a fragmentation from a molecular ion to a characteristic product ion. In some cases, the molecular ion did not dissociate or mainly dissociated by loss of a non-characteristic neutral fragment (e.g., H_2O). If there was no interference from the biological matrix and potential metabolites, a transition from molecular ion to molecular ion or from molecular ion to a non-structure specific product ion could be used for these types of compounds. The selectivity of tandem mass spectrometry using MRM mode allowed simultaneous determination of a number of compounds without chromatographic separation.

The smaller column dimension (20 mm × 2.0 mm i.d.) used in conjunction with the fast gradient, starting from 5% of B for 0.5 min in the generic method, resulted in early elution of polar matrix interferences, which were diverted from the mass spectrometer for the first 0.5 min. This approach rendered minimal ion suppression typically observed during electrospray ionization. In quantitative analysis of a cassette of compounds, chromatographic separation might also be needed for reducing the possible ion suppression and minimizing the interference. Use of a short narrow-bore column (20 mm length) packed with 3 μM porous particles at a flow rate of 1 ml/min provided fast gradient separation with good chromatographic resolution and sensitivity. Reducing the column i.d. from conventional 4.6 mm to 2 mm resulted in a five-fold gain in sensitivity due to less dilution [13].

Volatile mobile phases were selected based on the mode of ionization as well as the ionization efficiency. The sensitivity of analyses was improved by splitting the 1 ml/min LC effluent, so that ∼0.3 ml/min effluent was directed into the electrospray interface of the mass spectrometer. The robustness of the analysis was achieved by diverting the first 0.5 min of the effluent from the mass spectrometer.

Fig. 1(a) shows multiple reaction monitoring chromatograms of brain homogenate extract spiked with three study compounds, a “biological internal standard” and a quantitation internal standard from

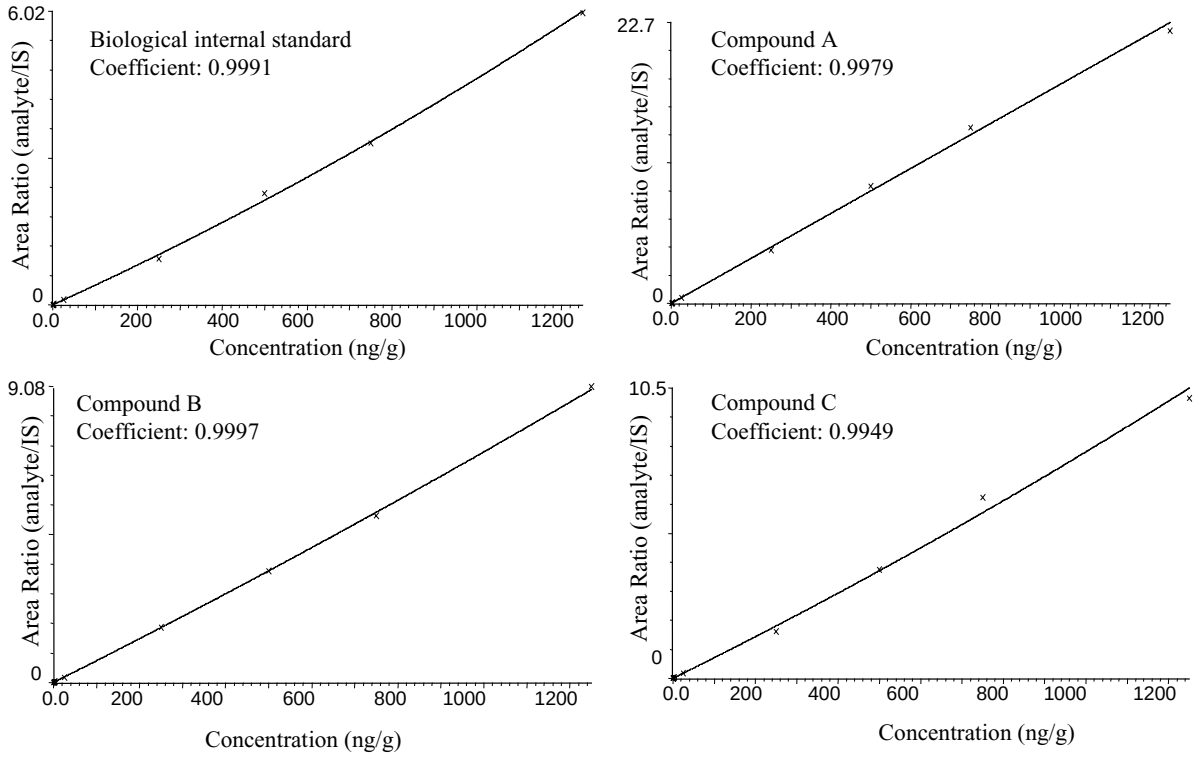

Fig. 2. Calibration curves for three study compounds and a “biological internal standard” compound spiked in brain homogenate in a cassette.

a typical LC–MS/MS analysis. The chromatographic separation was achieved by using an Aquisal C18 column (2 mm × 20 mm, 5 μM) with a fast LC gradient. The peak widths at half peak heights of all components were less than 6 s, providing good chromatographic resolution and sensitivity. No interference was observed in control brain homogenate samples (see Fig. 1(b)).

The standard curves for three analytes and a "biological internal standard" in brain extracts are shown in Fig. 2. Good linearity in a concentration range of 1–1220 ng/g was obtained for all compounds with deviation within (±) 20%. For early discovery projects, lower acceptance criteria were used for quantitation with measurement deviation (defined as a ratio of ((measured concentration) − (actual concentration))/(actual concentration)) within (±) 30% and linear coefficients within 0.96, being acceptable.

3.3. Cassette administration

One major challenge facing in vivo brain exposure is the time and manpower needed to carry out these experiments. The cassette administration approach allows for reduction in the time involved with drug administration, blood and brain sample collection, plasma preparation, brain perfusion and homogenization, and LC–MS/MS analysis. A common concern for cassette administration is the possible drug–drug interaction due to inhibition of enzymes and transporter proteins, as well as competition for plasma protein binding, which may lead to errors in the measured pharmacokinetic parameters. Reports showed that using the smallest detectable doses and limiting compounds in a cassette to 4 or 5 could minimize the potential for errors [10,11]. Thus, the dose level of 3 mg/kg was used in these studies. Including a "biological internal standard" (also called a benchmark compound) can also safeguard the accuracy of the results. In order to improve the confidence of cassette administration, an analogue of the study compounds, selected based on its well-established brain penetration data, was included in each cassette as a "biological internal standard" in our studies. Fig. 3(a) and (b) show the brain and plasma concentration–time course profiles of a "biological internal standard" from four cassette studies. The time course brain and plasma

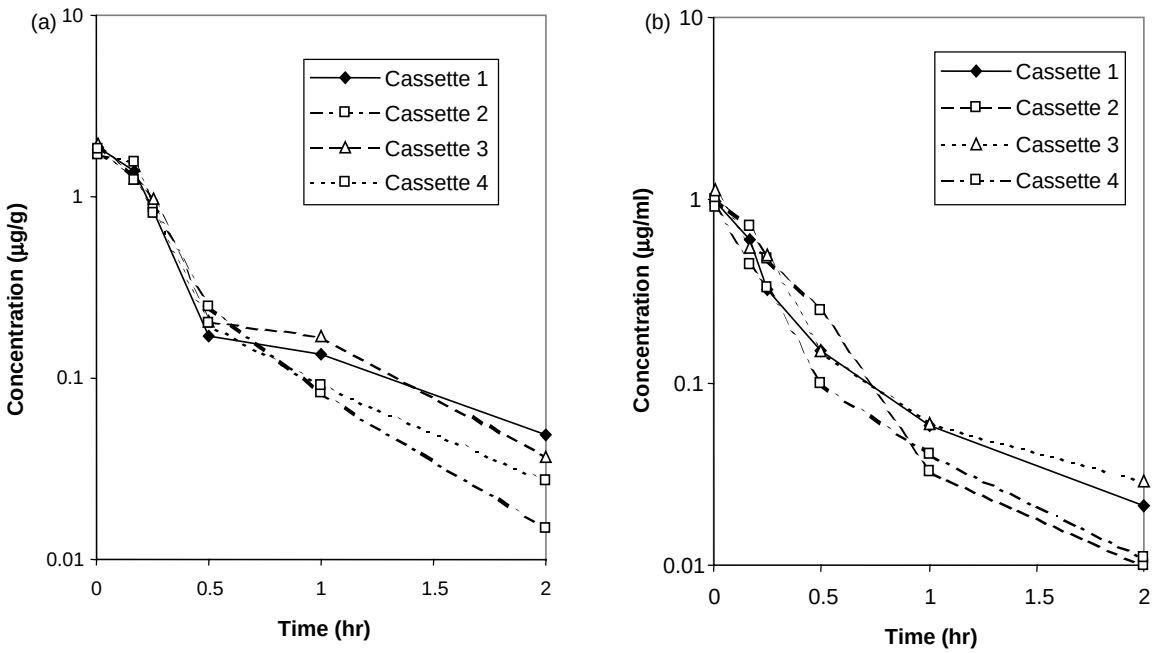

Fig. 3. The in vivo exposure of a "biological internal standard" compound in (a) brain and (b) plasma from four cassette studies after IP cassette administration in male rats at 3 mg/kg per compound.

Table 2
The time course brain and plasma exposure of a "biological internal standard" compound in four cassette studies after IP cassette administration in male rats at 3 mg/kg per compound

Cassette	AUC brain (h ng/g)	AUC plasma (h ng/ml)	AUC brain/AUC plasma
1	652	320	2.0
2	643	375	1.7
3	698	361	1.9
4	595	259	2.3

exposures of the biological internal standard from four cassette studies following a cassette IP dose are summarized in Table 2. Each cassette had different compounds from the same structure class. The brain and plasma profiles of the "biological internal standard" in these four cassettes were in good agreement, suggesting there was no observed drug–drug interaction in these studies.

Another common concern for cassette dosing is the possible analytical interference due to in vivo metabolites, leading to 'false positive results'. In our study, the compounds were grouped in ways that eliminated the possible interference from potential metabolites. Thus, a compound with the same molecular weight as a potential metabolite of a study compound should not be included in the same cassette (e.g., two compounds with molecular weight differences of 14, 16 or 32 amu should not be included in the same cassette). In addition, the good chromatographic separation used for these studies further minimized the interference.

3.4. Applications

This method has been used as a screening tool to provide early in vivo brain penetration information. Fig. 4 shows the time course brain and plasma exposures of 15 compounds in a CNS discovery program after IP cassette administration in male rats at 3 mg/kg per each compound. The absolute brain exposure was dependent on the degree of brain penetration as well as the level of plasma exposure. The degree of brain penetrations of the study compounds was evaluated by comparing their C_{max} and AUC values in brain and plasma (Fig. 5). The study allowed rapid assessment of compounds with poor brain penetration in CNS exploratory programs as well as the prioritization of lead compounds by correlating the in vivo brain exposure to the EC_{50} values (i.e. in vitro potency). Fig. 6 shows the correlation of the in vivo brain exposure with the in vitro potency of 15 compounds in a CNS program for compound ranking. This approach allows rapid selection of lead compounds for future in vivo assessment. Correlating the in vivo brain exposure with data

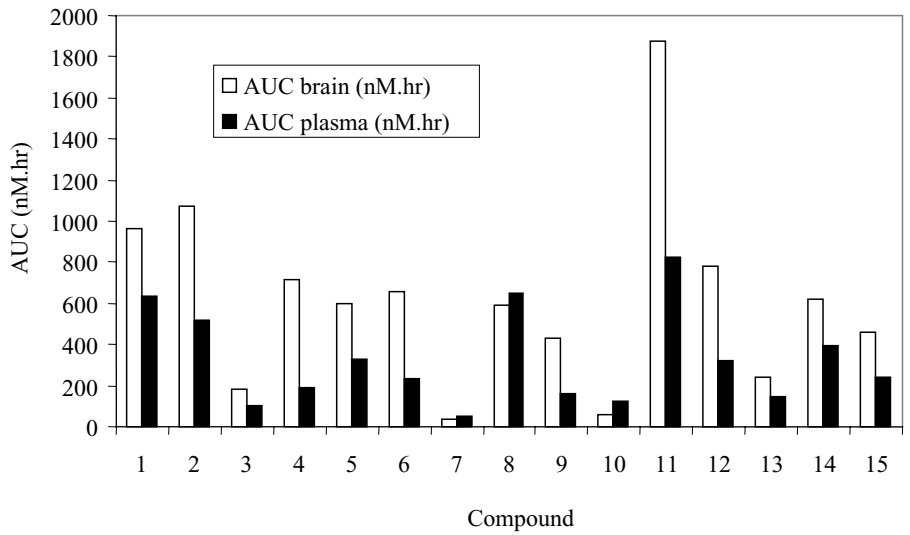

Fig. 4. Brain and Plasma exposures of 15 compounds in a CNS discovery program after IP cassette administration in male rats at 3 mg/kg per compound.

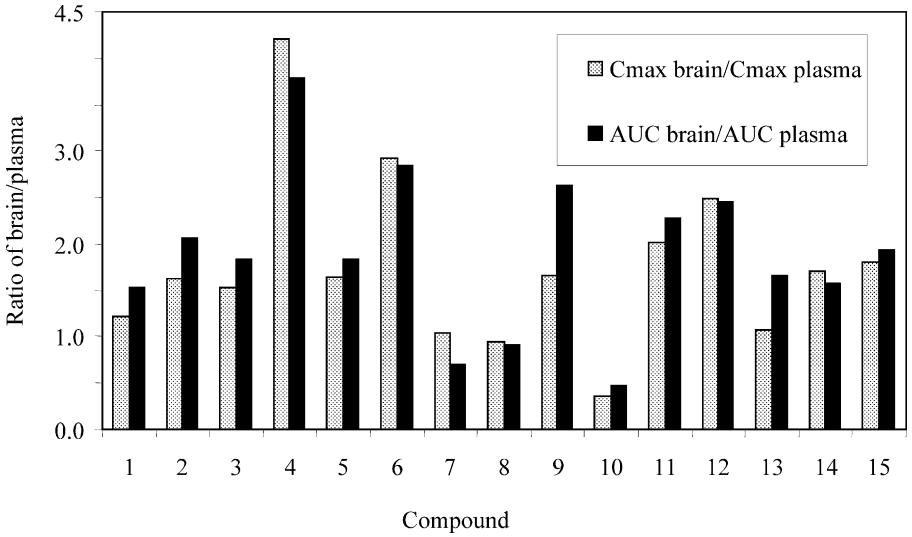

Fig. 5. The ratios of brain exposure to plasma exposure of 15 compounds in a CNS discovery program after IP cassette administration in male rats at 3 mg/kg each compound.

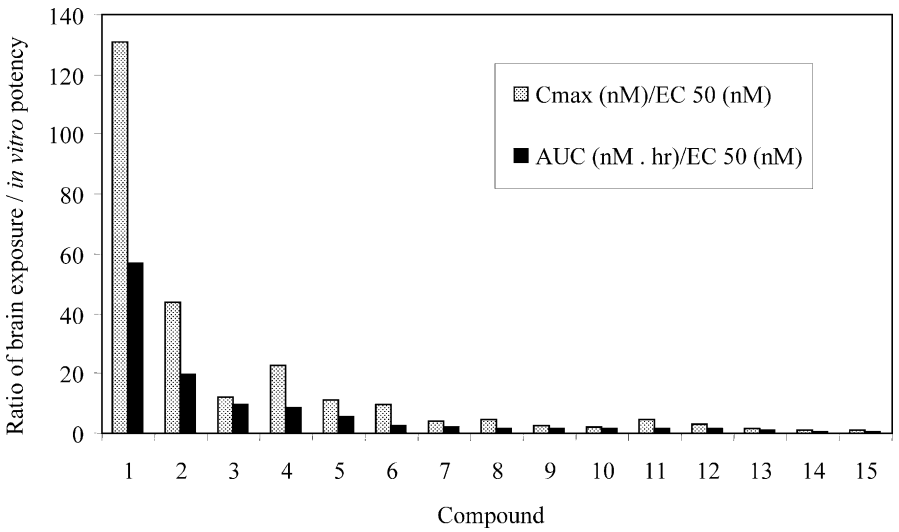

Fig. 6. Correlation in vivo brain exposure with the in vitro potency of 15 compounds in a CNS discovery program to select lead compounds for future in vivo assessment.

from in vitro high throughput assays, such as passive diffusion BBB assay [14] and Pgp assay using Caco-2 allowed understanding of the brain penetration mechanism of the compounds for structure optimization. The brain exposure information was also used for selecting the optimal administration route, dosing vehicle, dosing level as well as the treatment window for in vivo bio-activity assays in CNS exploratory programs. This approach efficiently reduced the time and manpower required for in vivo activity studies.

4. Conclusion

The method combines: fast LC chromatography, generic sample extraction, sensitive and selective

MRM tandem mass spectrometry, intraperitoneal dosing, and cassette dosing analysis. The results are reliable for drug discovery research. The approach speeds up data production by ∼10-fold and sacrifices one-third of the animals.

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