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Sample pooling to enhance throughput of brain penetration study

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Abstract

The advent of combinatorial synthesis and high throughput screening in pharmaceutical research has inevitably given rise to a large number of interesting prelead compounds that requires rapid analytical throughput for kinetic characterization. The traditional approach of one-compound-at-a-time bioanalysis has not been able to meet the demand for high productivity of pharmacokinetic screening. This report demonstrates the application of sample pooling in expediting the pharmacokinetic screening, including assessment of brain penetration, of six NK_1 receptor antagonists in rats: CAM 6108 (C1), CAM 6122 (C2), CAM 6178 (C3), CAM 5825 (C4), CAM 6182 (C5), and CAM 6121 (C6). The approach was adopted to avoid complications associated with cocktail dosing where multiple compounds are administered to one animal. The present investigation features individualized dosing (one compound per animal), followed by sample pooling of brain and plasma and bioanalysis via a conventional LC-fluorescence method. Rats were dosed intravenously with each of the six NK_1 receptor antagonists and blood and brain samples were harvested at suitable post-dose time intervals. Plasma or brain homogenate samples from the same time points were pooled into two groups (C1–C3 and C4–C6) for assay. Drug compounds in plasma or brain were extracted by protein precipitation and quantitated using a validated gradient HPLC/fluorescence method, which was made feasible for both groups of compounds with a modification in gradient scheme. Plasma assay precision and accuracy for C1–C6 were $\leq 4.7\%$ and within $\pm 9.8\%$, respectively. Brain homogenate assay accuracy for C1–C6 was within $\pm 7.0\%$. Brain penetration of these compounds was evaluated as the AUC of brain and plasma and their respective brain/plasma AUC ratio. The sample pooling approach helped to quickly identify C1 as the NK_1 receptor antagonist with the greatest extent of brain penetration, followed by C2, C6, C4, C5, and C3 in that order. By employing sample pooling approach, pharmacokinetic parameters and brain penetration of all six compounds were obtained in a fraction of the time required by conventional single compound dosing and analysis.

Keywords: Sample pooling; HPLC assay; Throughput enhancement; Brain penetration; Tachykinin NK1 antagonists; Rats

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1. Introduction

Advancement in combinatorial synthesis and high-throughput screening in pharmaceutical discovery research have resulted in the rapid identification of large number of prelead compounds. In the progression from early discovery to development, pharmacokinetic characterization needed for lead compound selection often becomes a rate-limiting step. Additional expenditure of time and resources in the IND and NDA development phase encountered in the high volume of samples generated in miscellaneous, essential biodisposition studies of lead compounds and their backups. These issues associated with drug discovery and development are, in fact, largely related to bioanalysis.

A throughput-enhanced sample pooling approach has been previously reported to simultaneously investigate the oral absorption profile of six dopamine D_4 receptor antagonists [1]. The present study extends the utility of sample pooling to brain tissue samples, investigating brain penetration and plasma profiles in rats of six NK_1 receptor antagonists in early discovery phase. The rationale of dosing each animal with a single compound followed by sample pooling, instead of cocktail dosing [2,3], was to avoid the complications that might result from the cocktail approach, in which one animal is dosed with multiple compounds.

The throughput enhancement was achieved by pooling individual samples into one combined plasma or brain sample per time point for simultaneous quantitation of drug concentrations. As these six NK_1 receptor antagonists were identified as potential lead candidates by their high receptor binding profiles and were expected to exert their pharmacological effect in the brain, simple comparative data of brain penetration is desirable. In this investigation we reported the development and validation of a traditional gradient HPLC/fluorescence method for brain and plasma assay, and application of the sample pooling approach to enhance the throughput of bioanalysis for brain penetration assessment.

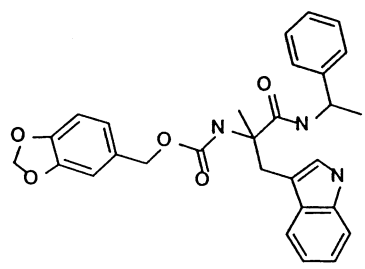
2. Materials and methods

2.1. Chemicals, reagents, and instrumentation

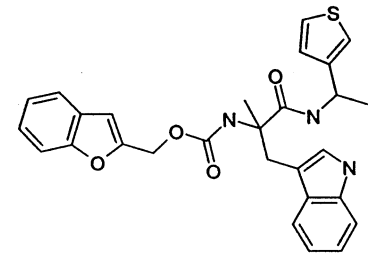
The six NK_1 receptor antagonists (Fig. 1) were obtained from the Parke-Davis Neuroscience Research Center (Cambridge, UK): CAM 5825, [2-(1H-indol-3-yl)-methyl-1-(1-ethiophen-3-yl-ethyl-carbamoyl)-ethyl]-carbamic acid benzofuran-2-yl-methyl ester, [R-(R*,R*)] and [R-(R*,S*)] (MW 502); CAM 6108, 2-(1H-Indol-3-yl)-methyl-1-(1-phenyl-ethylcarbamoyl)-ethyl]-carbamic acid benzo[1,3]dioxol-5-ylmethyl ester, [R-(R*,S*)] (MW 500); CAM 6121, 2-(1H-indol-3-yl)-methoxymethyl-1-(1-phenyl-ethylcarbamoyl)-ethyl]-carbamic acid benzofuran-2-ylmethyl ester, [R-(R*,S*)] (MW 510); CAM 6122, 2-(1H-Indol-3-yl)-methyl-1-methoxymethyl-1-(1-phenyl-ethylcarbamoyl)-ethyl]-carbamic acid benzofuran-2-ylmethyl ester (MW 529), [S-(R*,R*)]; CAM 6178, 2-[(Benzofuran-2-ylmethyl)amino]-3-(1H-indol-3-yl)-2-methyl-N-(1-phenyl-ethyl)-propioamide, [R-(R*,S*)] (MW 453); and CAM 6182, [1-[1-(3-Dimethylamino-phenyl)-ethylcarbamoyl)-2-(1H-indol-3-yl)-1-methyl-ethyl)-carbamic acid benzofuran-3-ylmethyl ester (MW 539). Acetonitrile, water and ammonium acetate were obtained from Mallinckrodt (Paris, KY). Absolute ethanol was obtained from Aaper Alcohol and Chemical (Shelbyville, KY) and PEG 400 was from Union Carbide (Danbury, CT). Heparinized rat plasma was obtained from Pel-Freez Biologicals (Rogers, AR). The HPLC system was composed of a Perkin-Elmer series 410 LC Pump, a Perkin–Elmer ISS 200 autosampler, and a Perkin–Elmer LS 40 Fluoresence Detector (Norwalk, CT). Data were collected with a Spectra Physics SP 4400 integrator interfaced to a ChromNet/2 data acquisition system (San Jose, CA). Sample evaporation was performed with a Turbovap LV evaporator from Zymark Corp. (Hopkinton, MA).

2.2. Standard and quality control preparation

Stock solutions for each of CAM 6108 (C1), CAM 6122 (C2), CAM 6178 (C3), CAM 5825 (C4), CAM 6182 (C5), and CAM 6121 (C6) were made in acetonitrile at 1 mg ml^{-1} and diluted



CAM 6108 (C1)



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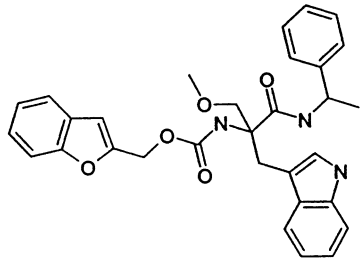

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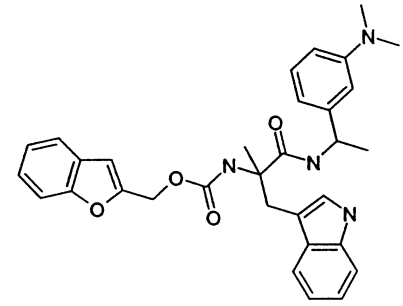
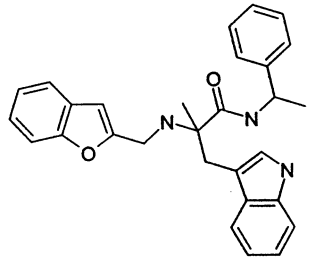
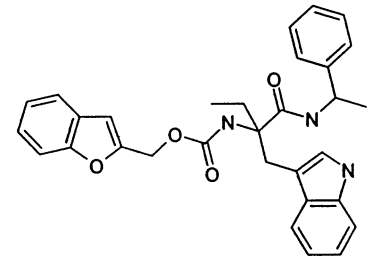


CAM 6182 (C5)



CAM 6178 (C3)



CAM 6121 (C6)

Fig. 1. Chemical structures of CAM 6108 (C1), CAM 6122 (C2), CAM 6178 (C3), CAM 5825 (C4), CAM 6182 (C5), and CAM 6121 (C6).

with water:acetonitrile (90:10) to prepare a 50 μg ml^{-1} stock. These stock solutions were further diluted with water:acetonitrile (90:10) to prepare working standards containing C1, C2 and C3 as group I, or C4, C5 and C6 as group II in a concentration range of 10–4000 ng ml^{-1}. The grouping was made based on their chromatographic property. For rat plasma quality control the stock solutions were diluted to prepare three different concentrations in a range of 25–2000 ng ml^{-1}. Quality control samples were subdivided into 500-μl aliquots, stored frozen, and used for

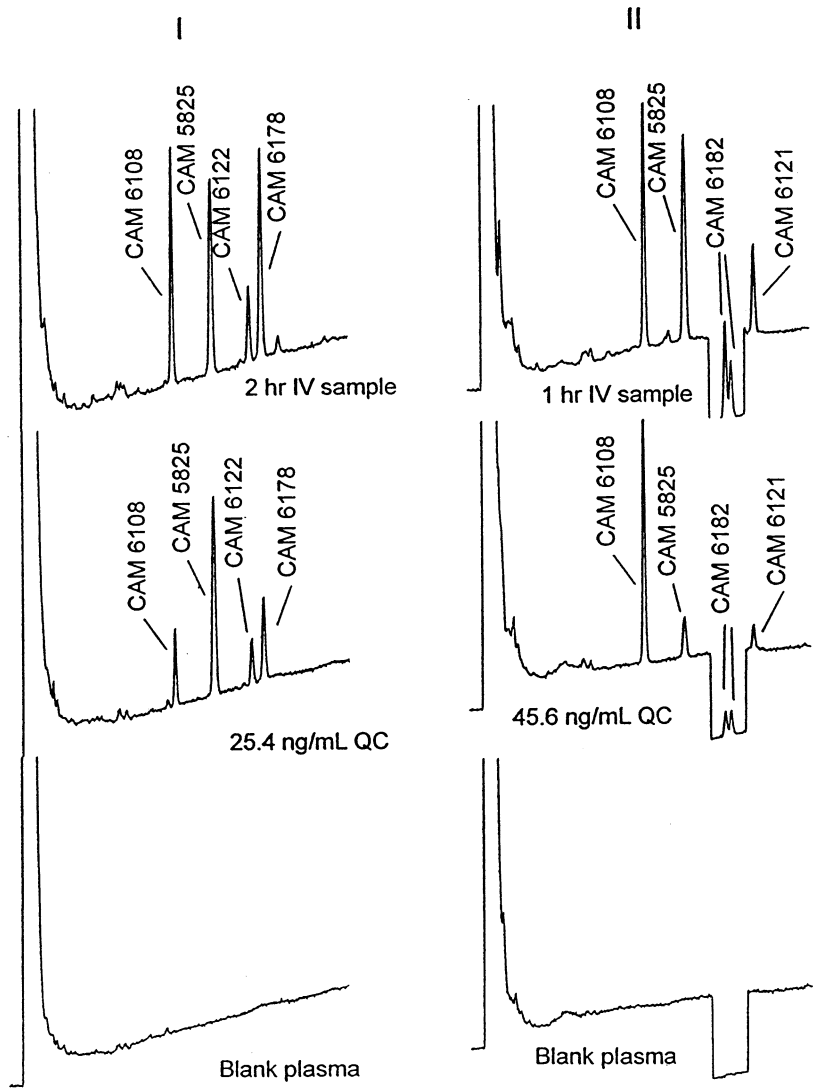


Fig. 2. HPLC chromatograms of pooled plasma samples for CAM 6108 (C1), CAM 6122 (C2) and CAM 6178 (C3) in group I, and CAM 5825 (C4), CAM 6182 (C5) and CAM 6121 (C6) in group II for blank plasma, quality control, and in vivo sample after intravenous injection.

up to one month. Internal standards, C4 for group I and C1 for group II, was prepared by diluting a 100 μg ml^{-1} stock in acetonitrile to 500 ng ml^{-1} in water:acetonitrile (90:10). QC's were made and validated for the plasma assay only.

2.3. Sample pooling and sample pretreatment

Drug compounds in plasma samples were extracted by acetonitrile precipitation. In 12×75 mm disposable glass tubes, 300 μl of control plasma (for standard curve or blank samples),

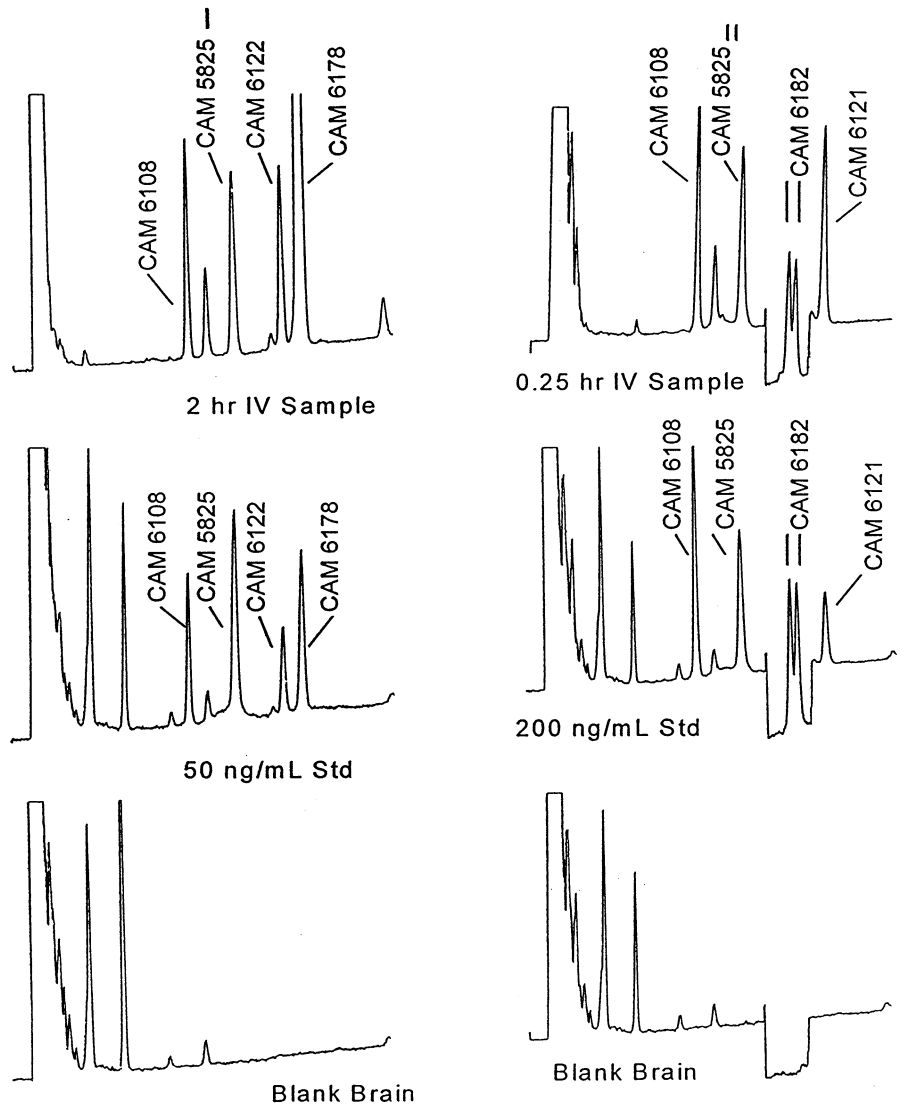


Fig. 3. HPLC chromatograms of pooled brain samples for CAM 6108 (C1), CAM 6122 (C2) and CAM 6178 (C3) in group I, and CAM 5825 (C4), CAM 6182 (C5) and CAM 6121 (C6) in group II: blank plasma, quality control, and in vivo sample after intravenous injection.

quality control samples or unknown samples (100 μl × 3) were mixed with 100 μl working standard (group I or II) or water:acetonitrile (90:10), 100 μl of internal standard (C4 for group I or C1 for group II) and 500 μl of acetonitrile. The precipitated plasma mixtures were vortexted, centrifuged, and supernatants removed for evaporation to dryness in the Turbo-Vap at 50°C under a gentle stream of nitrogen. Samples were reconstituted in 200 μl of mobile phase and 150 μl of the resulting samples was injected into the HPLC. Drug compounds in the whole brain samples were extracted by homogenation followed by acetonitrile precipitation. To 20-ml glass

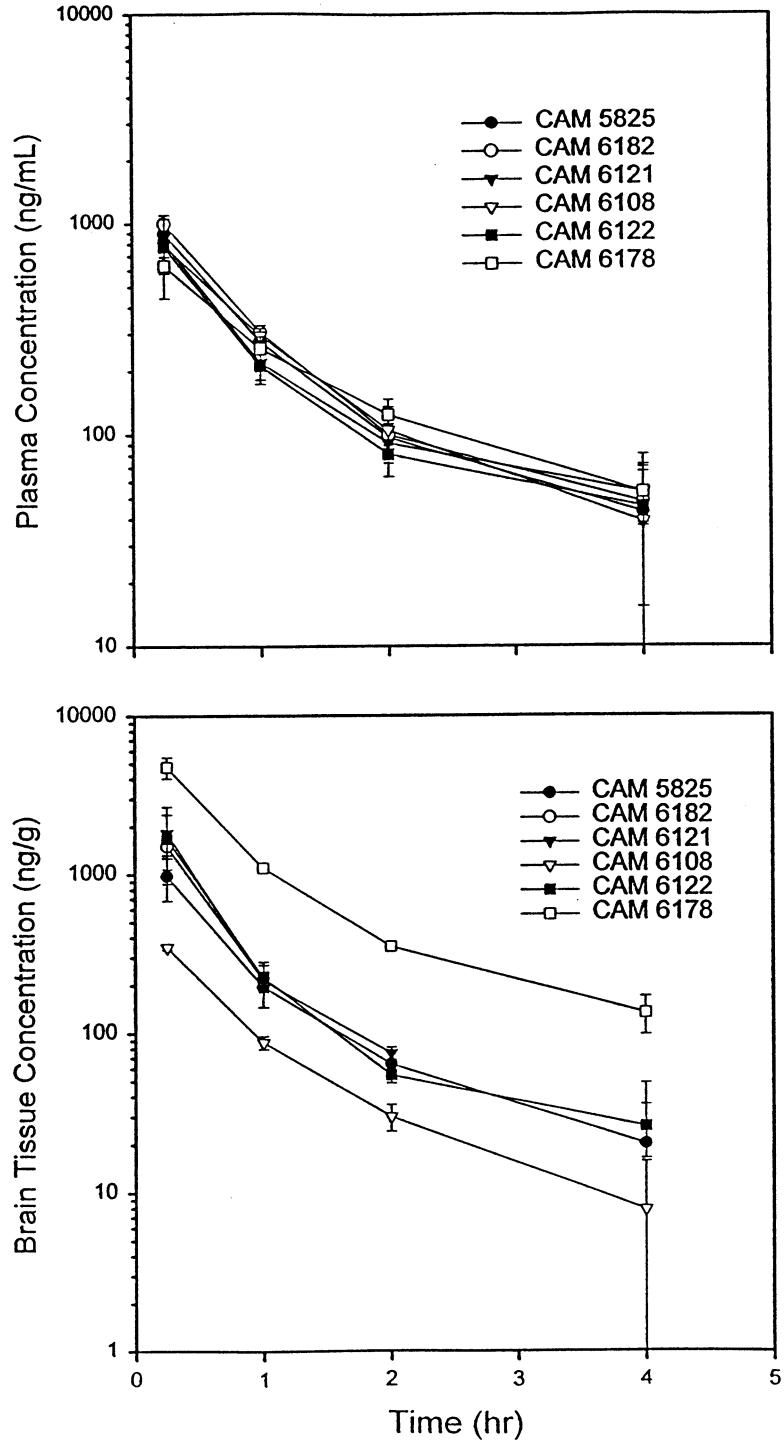


Fig. 4. Plasma and brain profiles of six NK_1 receptor antagonists in rats receiving an intravenous dose of 5 mg kg^{-1} CAM 6108 (C1), CAM 6122 (C2), CAM 6178 (C3), CAM 5825 (C4), CAM 6182 (C5) or CAM 6121 (C6).

Table 1
Intra-assay and inter-assay for the group I compounds (C1–C3) in plasma quality controls and brain standards at three different concentrations: observed concentration (precision, %RSD) (accuracy, %RE)

Concentration (ng ml^{-1})	Plasma intra-assay ($n = 3$)	Plasma inter-assay ($n = 8$)	Brain inter-assay ($n = 2$)
C1			
25	25.4 (3.1%) (1.6%)	24.7 (3.9%) (−1.2%)	25.0 (1.1%) (0.0%)
50	106 (1.9%) (6.0%)	101 (4.7%) (1.0%)	102 (1.4%) (2.0%)
500	528 (0.9%) (5.7%)	518 (2.8%) (3.6%)	512 (0.7%) (2.3%)
C2			
50	46.4 (3.9%) (−7.3%)	47.6 (3.8%) (−4.96%)	48.5 (5.7%) (−3.1%)
200	192 (3.4%) (−4.2%)	189 (5.5%) (−5.6%)	199 (0.7%) (−0.5%)
1000	992 (0.8%) (−0.8%)	986 (2.0%) (−1.4%)	1026 (2.2%) (2.6%)
C3			
25	47.1 (2.0%) (−5.8%)	45.3 (5.0%) (−9.4%)	49.4 (0.9%) (−1.2%)
200	187 (1.3%) (−6.7%)	183 (3.6%) (−8.4%)	199 (0.7%) (−0.5%)
1000	937 (0.9%) (−6.3%)	943 (3.2%) (−5.7%)	1022 (0.6%) (2.2%)

scintillation vials containing pre-weighed control brains or brains harvested from brain penetration study, a 3-fold volume of acetonitrile was added, assuming that 1 g of brain is equal to 1 ml volume. The brain samples were homogenated by a Polytron PT 10/35 homogenizer. In 12 × 75 mm disposable glass tubes, 300 μl of pooled group I (C1–C3) or group II (C4–C6) homogenized brain or 300 μl of control homogenized brain, 100μl of group I (or group II) working standard or water:acetonitrile (90:10), and 250 μl of acetonitrile were mixed. The precipitated brain mixtures were vortexed, centrifuged, evaporated, and reconstituted in a manner similar to the plasma sample mixtures. A total of 150 μl of the reconstituted sample was chromatographed on the HPLC system described below.

2.4. Chromatographic conditions and data acquisition

Two assays with internal standards were developed and validated for the simultaneous determination of C1–C3 or C4–C6 in rat plasma. Standard curve linearity assessment of C1–C3 and C4–C6 in rat brain homogenates was also accomplished. Preliminary HPLC/fluorescence profiling indicated a necessity of placing C1–C3 into group I using C4 as the internal standard, and C4–C6 into group II using C1 as the internal standard for optimal compound separation. Plasma and brain extracts from group I and II were chromatographed by gradient elution on a Zorbax Rx-C_8 column (4.6 mm × 25 cm, 5 μm) from MAC-MOD Analytical (Chaddds Ford, PA), which was protected by a RP-18 Newguard cartridge (3.2 × 15 mm, 7 μm) from Applied Biosystems (San Jose, CA). The gradient system consisted of two mobiles phases: 20 mM ammonium acetate buffer (pH 5):acetonitrile at a ratio of 55:45 for mobile phase A and of 25:75 for mobile phase B. For analytes in brain extracts, the ammonium acetate/acetonitrile ratio in mobile phase A was 50:50 for a faster elution. The flow rate was 1.2 ml min^{-1} for all cases. The separation gradient was operated from 100%A to 50%A/50%B in 40 min for plasma or 30 min for brain extracts. Between injections the column was washed and re-equilibrated with 100% mobile phase A for 10 min.

All analytes were detected by fluorescence at excitation 280 nm and emission 350 nm, except for analyte V which was detected at excitation 256 nm and emision 356 nm between 27.5 and 32.5 min for plasma extract or between 19.0 and 22.8 min for brain extracts. The change in fluoresence setting for analyte V was to achieve maximum peak response. Chromatographic responses of calibration standards were integrated and peak area ratios calculated using weighted (1/concentration^2) linear regression. For compound V which exists as a

Table 2
Intra-assay and inter-assay for the group II compounds (C4–C6) in plasma quality controls and brain standards at three concentrations: observed concentration (precision, %RSD) (accuracy, %RE)

Concentration (ng ml^{-1})	Plasma intra-assay ($n=3$)	Plasma inter-assay ($n=8$)	Brain inter-assay ($n=2$)
C4			
50	45.6 (3.0%) (−8.9%)	48.4 (7.7%) (−3.3%)	51.2 (6.4%) (2.4%)
200	203 (2.3%) (1.7%)	204 (2.0%) (2.2%)	203 (2.8%) (1.5%)
1000	1027 (2.0%) (2.7%)	1038 (1.9%) (3.9%)	970 (0.1%) (−3.1%)
C5			
100	95.7 (1.1%) (−1.6%)	100 (5.9%) (0.3%)	101 (2.9%) (0.9%)
400	405 (1.9%) (1.3%)	401 (2.5%) (0.3.%)	394 (5.4%) (−1.5%)
2000	2037 (1.9%) (1.8%)	2010 (2.6%) (0.5%)	2006 (1.5%) (0.3%)
C6			
50	47.8 (4.7%) (−4.5%)	48.3 (8.7%) (−2.6%)	50.5 (4.5%) (1.0%)
200	187 (0.6%) (−6.3%)	193 (3.3%) (−3.6%)	203 (1.7%) (1.3%)
1000	902 (2.0%) (−9.8%)	945 (3.9%) (−5.6%)	969 (1.2%) (−3.1%)

diastereomer with doublet peak, the summated peak area for each sample was used for calculation. Analyte concentrations in quality controls and unknown samples were calculated using the regression parameters of their respective standard curves.

2.5. Animal study in rats

Each compound was administered IV at a dose of 5 mg kg^{-1} in a solution of PEG 400:ethanol: water (2:1:1) via tail vein to fasted male Wistar rats in duplicate. Blood samples were collected by cardiac puncture under anesthesia and whole brain samples were harvested after trans-cardiac perfusion with 0.9% NaCl at 0.25, 1, 2, and 4 h postdose. The throughput enhancement was achieved by pooling individual samples from the same time point into one combined plasma or brain sample for simultaneous quantitation of drug concentrations. For sample pooling, plasma or brain samples at the same time point from rats receiving C1, C2, or C3 were pooled as group I, and those from rats receiving C4, C5, and C6 were pooled as group II for assay.

3. Results and discussion

3.1. Chromatographic results

The HPLC chromatograms of the six compounds, group I (C1–C3) and II (C4–C6), spiked in rat plasma or from plasma samples collected after intravenous dosing (5 mg kg^{-1}) are shown in Fig. 2, and those corresponding to brain samples are shown in Fig. 3. They were all well separated without interference from plasma or brain constituents. The profile of chromatographic retention indicated that among the six compounds CAM 6122 (C2) and CAM 6178 (C3) would interfere with CAM 6182 (C5), necessitating that C5 be placed in a separate group. C5 was present as a doublet peak due to its existence as diastereomer, and a switch of fluorescence wavelength setting for maximum peak response (from ex. 280/em. 350 nm to ex. 256/em. 356 nm for a few minutes) resulted in negative baseline deflection.

There was no apparent formation of metabolites which interfered chromatographically with any of the parent compounds. It should be noted that, compared to the mean value of duplicate peak areas at time zero from plasma incubated at room temperature and 37°C, all drug compounds were found to be stable in rat plasma for at least 4 h at both temperatures. No degradation component(s) was detected for any of the six compounds.

3.2. Linearity, precision, and accuracy

The peak area ratios were linearly related to quantifiable plasma and brain concentrations over the range of 10–1000 ng ml^{-1} for C1, 20–2000 ng

Table 3
Mean brain and plasma AUC(0–4) [ng·h ml^{-1}] ($n = 2$) and brain/plasma AUC ratio after an intravenous dose of 5 mg kg^{-1} C1, C2, C3, C4, C5, or C6 in rats

Time (h)	CAM 6108 (C1)	CAM 6122 (C2)	CAM 6178 (C3)	CAM 5825 (C4)	CAM 6182 (C5)	CAM 6121 (C6)
Brain (B)	308	1143	4006	777	836	1138
Plasma (P)	988	894	885	1045	1150	939
B/P	0.31	1.28	4.53	0.74	0.73	1.21

ml^{-1} for C2, C3, C4, and C6, and 40–4000 ng ml^{-1} for C5. The best-fit line was determined by least squares linear regression of the calibration data using 1/concentration^2 weighting. At the limit of quantitation, the chromatographic signal-to-noise ratio was > 10 for all compounds.

Validation for the present HPLC method was evidenced by the excellent intra- and inter-assay precision and accuracy obtained for the quality control samples at three different concentrations (Tables 1 and 2). The intra-assay precision and accuracy for plasma samples were observed on one occasion with three replicates of each plasma quality control; and the inter-assay precision and accuracy for plasma samples were obtained from three occasions with two to three replicates for all compounds. Due to limited resource of control rat brain, only inter-assay was characterized with one experiment on two occasions. Standard, quality control, and unknown samples were randomized just prior to injection. Plasma assay precision (%RSD) and accuracy (%RE) for C1–C6 were within 4.7 and 9.8%, respectively. Brain homogenate assay accuracy for C1–C6 was within 7.0%.

3.3. In vivo application

The sample pooling approach has been applied to brain penetration studies of six compounds in rats. Following IV dosing, all six compounds rapidly penetrated into rat brain as indicated by early maximum brain concentrations occurring at 0.25 h postdose (Fig. 4). Of six compounds, CAM 6178 (C3) was found to have the highest extent of penetration with a brain/plasma AUC(0–4) ratio of 4.5. CAM 6108 (C1) has the lowest brain penetration potential with a brian/plasma AUC(0–4) of 0.31 (Table 3). Note that IV plasma profiles (concentrations over time and AUC) of these six compounds are similar, while the corresponding brain profiles (concentrations over time and AUC) are different. These suggest that they have markedly different tissue distribution capacity, of which CAM 6178 may have the highest tissue (brain receptor) binding potential.

3.4. Summary

An HPLC method based on sample pooling approach was successfully validated with good precision and accuracy for the simultaneous analysis of six compounds (C1–C3 in group I and C4–C6 in group II) in both plasma and rat brain homogenates. The CAM 6178 (C3) has been identified as having the highest extent of brain penetration with a brain/plasma AUC ratio of 4.5. Sample pooling approach dramatically enhances the brain penetration screening. Much longer time would be needed to complete the study if the traditional one-compound-at-a-time bioanalytical measure was used.

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