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In vitro plasma protein binding determination of flunarizine using equilibrium dialysis and liquid chromatography–tandem mass spectrometry

Zhongping John Lin[a,*], David Musiano[a], Anna Abbot[b,1], Linyee Shum[a]

[a] *Avantix Laboratories Inc., 57 Read's Way, New Castle, DE 19720, USA*
[b] *Johnson and Johnson Pharmaceutical Research and Development, L.L.C. Spring House, Route 202, P.O. Box 300, Raritan, NJ 8869, USA*

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Abstract

A highly sensitive method was developed and validated for determining the free fraction of flunarizine in human plasma. Equilibrium dialysis was used for the separation of free (unbound) drug and liquid chromatography/tandem mass spectrometry (LC–MS/MS) was used for quantitation. Post-dialysis plasma or buffer samples of 0.2 mL were extracted using a liquid–liquid extraction procedure and analyzed using a high performance liquid chromatography electrospray tandem mass spectrometer system. The compounds were eluted isocratically on a Supelco Supelcosil ABZ + Plus column, ionized using a positive ion atmospheric pressure electrospray ionization source, and analyzed using multiple reaction monitoring. The ion transitions monitored were *m*/*z* 405 → 203 for flunarizine and *m*/*z* 409 → 207 for flunarizine-d4 (internal standard, IS). The chromatographic run time was 3.5 min per injection, with retention times of 2.1 min for both flunarizine and IS. The calibration curve for flunarizine was linear over the concentration range of 0.25–2000 ng/mL ($r^2 > 0.9989$) in the combined matrix of human plasma and isotonic sodium phosphate buffer (1:1, v/v) with the lower limit of quantitation of 0.25 ng/mL. The inter-assay coefficient of variability (CV) for the quality control samples was less than 13.5%, and the inter-assay percent nominal was greater than 98.2%. In vitro protein binding of flunarizine was determined at concentrations of 5, 10 and 100 μg/mL using the validated method. Flunarizine was extensively bound to plasma protein with a 0.083 ± 0.005% overall percent free drug in plasma and a CV value less than 7.8%. This validated method will be used for the ex vivo assessment of flunarizine protein binding in human plasma from a drug–drug interaction clinical study.

Keywords: Flunarizine; Protein binding; Equilibrium dialysis; LC–MS/MS

1. Introduction

Flunarizine is one of the piperazine derivatives with antihistamine properties and calcium channel blocking activity and has been widely used to treat cerebral and peripheral vascular insufficiency [1]. Topiramate is a chemical compound classified as a sulfamatesubstituted monosaccharide with antiepileptic activity and recently has been demonstrated to be effective for treating the partial onset of seizures in adults and children, Lennox-gastaut sydrome, and generalized tonic–clonic seizures [2]. The accurate determination of unbound (free) fraction of drug in plasma is essential in the therapeutic monitoring of drugs because only the unbound drug is available for distribution, elimination, and pharmacodynamic interaction with receptors [3,4]. This is especially important for highly protein bound drugs (such as flunarizine) where fluctuations in the free fraction can have an impact on the interpretation of total drug measurements. Unbound drug determinations may be important for clinical studies where the protein concentration may vary or drug displacement may occur. Methodologies for the determination of unbound drug concentrations in plasma include equilibrium dialysis [5],

* Corresponding author. Tel.: +1 302 322 9900; fax: +1 302 322 9904.
E-mail address: linj@avantixlabs.com (Z.J. Lin).
[1] Tel: 1 908 704 4727; fax: 1 908 218 0973.

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ultrafiltration [6], unltracentrifugation [7], calorimetry [8], and capillary electrophoriesis (CE) [9]. Extensive reviews on these methodologies can be found in the literature [10–12]. The in vitro protein binding has been reported to be greater than 90% for flunarizine [13], however, to date, no published reports have dealt with the quantitation of the free fraction of flunarizine. In order to evaluate the potential drug–drug protein binding interaction of flunarizine with topiramate, a highly sensitive and selective method was needed. Therefore, this paper describes a method employing equilibrium dialysis for the separation of unbound drug from plasma and LC–MS/MS method for the quantitation of unbound and total flunarizine in human plasma.

2. Experimental

2.1. Chemicals and reagents

Flunarizine hydrochloride (>99% purity) and flunarizine-d4 hydrochloride as the internal standard (IS, >98% purity) were provided by Johnson and Johnson Pharmaceutical Research and Development, L.L.C. (Raritan, NJ, USA). The chemical structures of flunarizine and flunarizine-d4 are shown in Fig. 1. Deionized water was produced from an ultra high quality polishing system unit (UHQ-PS) (High Wycombe, Bucks, UK). Hexane, acetonitrile, methanol, sodium hydroxide and hydrochloric acid were provided by Fisher Scientific (St. Louis, MO, USA). Ammonium acetate, disodiumhydrogen phosphate heptahydrate, potassium dihydrogen phosphate, and sodium chloride were provided by Aldrich (Milwaukee, WI, USA), and trifluoroacetic acid (TFA) was provided by Burdick and Jackson (Muskegon, MI, USA). Blank human sodium heparinized plasma was obtained from Bioreclamation Inc. (Hicksville, NY, USA) and was stored frozen at −20 °C. Pooled human plasma from six different lots were used in this study. All mobile phase solvents were HPLC grade and all reagents were analytical reagent grade.

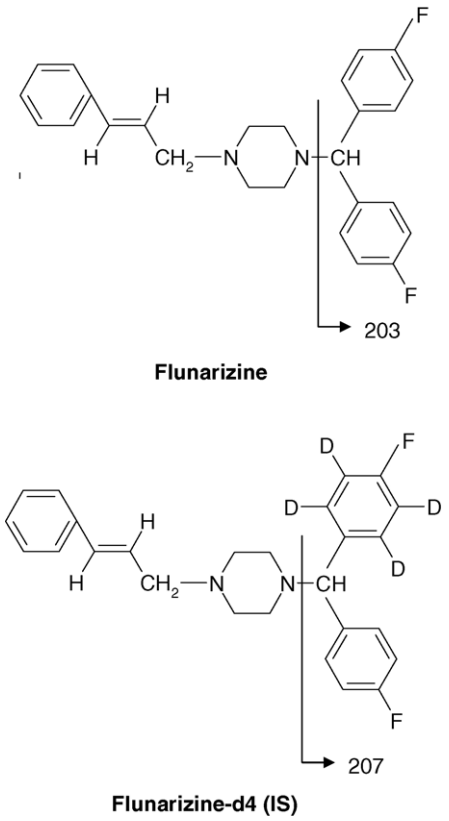

Fig. 1. Chemical structures of flunarizine and flunarizine-d4 (IS).

2.2. Equilibrium dialysis

Equilibrium dialysis was performed in cells made from Teflon with compartment volumes of 1 mL. A Multi-equilibrium dialyser with variable speed drive unit (Amika Corp., Columbia, MD, USA) was used to rotate the cells during dialysis. Dialysis membranes with very high permeability and a molecular weight cut-off of 10,000 Da were used (Harvard Apparatus, Holliston, MA, USA). Plasma samples were titrated to pH 7.4 ± 0.05 using either 1.0N hydrochloric acid or 0.1N sodium hydroxide. A 0.8 mL aliquot of each plasma sample was dialyzed against 0.8 mL of isotonic sodium phosphate buffer, pH 7.4, in a temperature controlled water bath at a nominal temperature of 37 ± 2 °C and rotated at a minimum of 22 rpm for 2.5 h. After dialysis was complete, the resulting plasma and buffer dialysates were promptly recovered from the Teflon cells and analyzed by LC–MS/MS after sample preparation.

2.3. Calibration standards and quality control samples

Standards and quality control samples (QCs) were made from two separate stock solutions (1 mg/mL in methanol expressed as free base). Working calibration standards at concentrations of 0.25, 0.5, 1, 5, 20, 50, 200, 1000 and 2000 ng/mL were prepared daily in the combined matrix of blank human plasma and pH 7.4 isotonic sodium phosphate buffer (1:1, v/v). Seven levels of QC samples, 0.25, 0.5, 1, 20, 200, 1000 and 2000 ng/mL, were prepared daily in the combined matrix for the determination of inter-assay accuracy and precision. Working standards and QCs were prepared by spiking the corresponding standard or QC acetonitrile solutions into the sample tubes, evaporating the acetonitrile under nitrogen and dissolving the drugs with 1:1 (v/v) blank human plasma/isotonic sodium phosphate buffer (pH 7.4). QCs exceeding the upper limit of quantitation (ULOQ) were prepared at 5 and 100 μg/mL in human plasma for the determination of dilution linearity.

2.4. LC–MS/MS methods

LC–MS/MS analyses were performed using a Hewlett Packard 1100 system (Wilmington, DE, USA) coupled to a

Micromass Quattro LC triple-quadrupole mass spectrometer (Manchester, UK). The mass spectrometer was operated using an electrospray atmospheric pressure ionization source in positive ion mode (ESI^+) with multi-reaction monitoring (MRM). The analytical column was a Supelco Supelcosil ABZ + Plus, 50 mm × 2.1 mm, 3 μm (Bellefonte, PA, USA). The mobile phase consisted of methanol:water (80:20) with 10 mM ammonium acetate and 0.02% TFA. The sample injection volume was 5 μL and the total run time was 3.5 min using an isocratic flow rate of 0.2 mL/min. System carry-over was determined by injecting the highest calibration standard followed by mobile phase. No carry-over was observed.

Sensitivity of MRM was optimized by infusing a two-in-one solution of 50 ng/mL flunarizine and flunarizine-d4 in the mobile phase. The capillary voltage was maintained at 3.8 kV. The cone and the extractor voltages were set to 15 and 3 V, respectively. The desolvation and ion source temperatures were 400 and 120 °C, respectively. Ions were activated at a collision energy of 20 eV and at an indicated argon pressure of 2.8×10^{-3} Torr. Both quadrupoles were maintained at unit resolution in order to assay all analytes, and the transitions (precursor to daughter) monitored were *m*/*z* 405 → 203 for flunarizine and *m*/*z* 409 → 207 for IS. The dwell time for each transition was 200 ms and the interchannel delay was 20 ms. MRM data was acquired and the chromatograms were integrated using MassLynx^{TM} NT, Version 3.2 software. A weighted (1/*x*) linear regression was used to generate the calibration curve from standards and to calculate the sample concentrations.

2.5. Sample preparation

For calibration standards and QCs, 200 μL of standard or QC acetonitrile solutions and 100 μL of IS (100 ng/mL in acetonitrile) were added into the sample tubes, and then evaporated to dryness under nitrogen followed by adding 400 μL of 1:1 (v/v) blank human plasma/isotonic sodium phosphate buffer (pH 7.4). For post-dialysis plasma samples, 100 μL of IS was added into the sample tubes, and then evaporated to dryness under nitrogen. Aliquots of 200 μL plasma sample and 200 μL blank isotonic sodium phosphate buffer were then added. For post-dialysis buffer sample, 100 μL of IS was added into the sample tubes, and then evaporated to dryness under nitrogen. Aliquots of 200 μL buffer sample and 200 μL blank plasma were then added. All samples, standards and QCs were acidified by addition of 1 mL of 0.1 M aqueous ammonium acetate adjusted the pH to 4 with acetic acid, and were extracted into 4 mL of hexane. The extraction tubes were shaken at high speed for 20 min followed by centrifugation at 4000 rpm for 20 min. The organic phase was transferred to clean glass tubes and evaporated to dryness in a 45 °C water bath under a nitrogen stream. The samples were dissolved in 200 μL of mobile phase, vortexed for 1 min, and transferred into glass inserts of autosampler vials. All samples were then centrifuged for 5 min at 4000 rpm and a 5 μL aliquot of each sample was injected onto the LC–MS/MS system.

2.6. Validation of the LC–MS/MS method

The method was validated for accuracy, precision, sensitivity, specificity, calibration linear range, and reproducibility according to the FDA guideline for bioanalytical methods validation for human studies [14]. The standard curve range was 0.25–2000 ng/mL using nine calibration standards and five replicates of QC samples at each concentration level in three separate batch runs. Each batch run also contained additional samples such as stability samples for processing and storage.

Stability of flunarizine in plasma and combined matrix (plasma/buffer, 1:1, v/v) was investigated at room temperature for approximately 4 h and at 4 °C for approximately 9 days. Post-preparative stability was also determined. The extraction recovery of flunarizine was calculated by comparing the peak areas of extracted plasma standards to the peak areas of post-extraction plasma blanks spiked at corresponding concentrations. The overall absolute recovery from human plasma was determined by comparing the peak areas of extracted plasma standards to those prepared in mobile phase. The method specificity was evaluated by screening six lots of blank sodium heparinized human plasma.

3. Results and discussion

3.1. Method optimization of equilibrium dialysis

One of the common problems for the determination of free faction of drugs using equilibrium dialysis is non-specific drug adsorption to sample containers, especially hydrophobic and highly protein bound drugs. A comparison test showed that no drug loss occurred in plasma samples stored in glass containers while there was significant drug loss in buffer samples. This was possibly due to the drug extensive protein binding and the lack of available drug for adsorption in plasma. Silianization of containers and use of plastic vials was tried to protect flunarizine from adsorption in buffer samples, but this process was not as efficient as adding blank plasma into containers prior to draining the post-dialysis buffer samples from dialysis cells after equilibrium dialysis. Therefore, all subsequent work was carried out by adding pre-weighed blank plasma into the buffer sample containers in order to prevent adsorption of flunarizine to sample containers. In addition, extra caution was needed during the sample preparation and sample handling, especially at low concentrations.

3.2. LC–MS/MS method

The standards were prepared in the combined matrix of human plasma and isotonic sodium phosphate buffer (1:1, v/v) in order to quantify the free drug concentrations in buffer

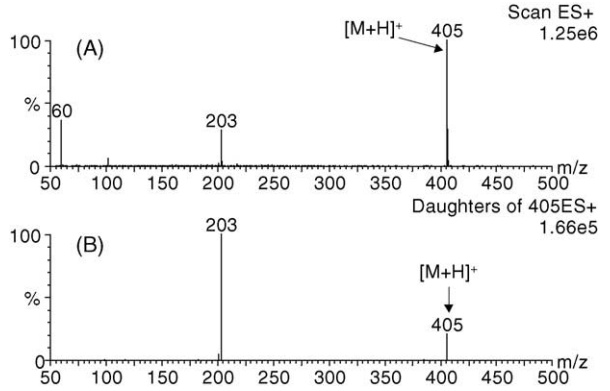

Fig. 2. Q1 scan spectrum (A) of flunarizine and product ion scan spectrum (B) of *m*/*z* 405.

dialysate and the total drug concentrations in plasma dialysate using the same calibration curve. The electrospray ionization gave the optimum sensitivity for flunarizine in positive ion mode. The Q1 mass spectra of flunarizine and IS showed protonated molecular ions $[M + H]^+$ at *m*/*z* 405 and 409, respectively. The product ion scan spectra of *m*/*z* 405 for flunarizine and *m*/*z* 409 for IS showed high abundance fragment ions at *m*/*z* 203 and 207, respectively. The fragmentation patterns are shown in Fig. 1. The ion transitions of *m*/*z* 405 → 203 for flunrizine (Fig. 2) and *m*/*z* 409 → 207 for IS were chosen for multiple reaction monitoring.

3.3. Specificity, sensitivity and calibration linear range

Blank human plasma samples from six different subjects were extracted and analyzed for flunarizine as a true blank (double blank without spiking analyte and IS), or spiked only with IS, or with flunarizine as a single blank. There were no endogenous peaks that interfered with the quantitation of flunarizine or IS. There was no interference from IS contributing to the flunarizine *m*/*z* channel or from flunarizine contributing to the IS *m*/*z* channel. The ratio of signal to noise from an extracted lower limit of quantitaion (LLOQ) sample (0.25 ng/mL) was at least 40 for flunarizine. There was no significant lot-to-lot variation in matrix effect and no carry-over observed from the ULOQ to the blank sample. The calibration curve was linear over the concentration range of 0.25–2000 ng/mL using the linear regression with a weighting factor of the reciprocal of the concentration (1/*x*) for flunarizine. The coefficients of determination (r^2) were 0.9989 or better. Representative chromatograms of blank spiked with flunarizine at LLOQ and IS, an extracted post-dialysis buffer sample and an extracted post-dialysis plasma sample, generated from a plasma sample spiked with flunarizine at 1 μg/mL, are shown in Figs. 3–5.

3.4. Precision, accuracy and dilution linearity

Table 1 shows the flunarizine validation data on accuracy and precision for each standard concentration. The inter-

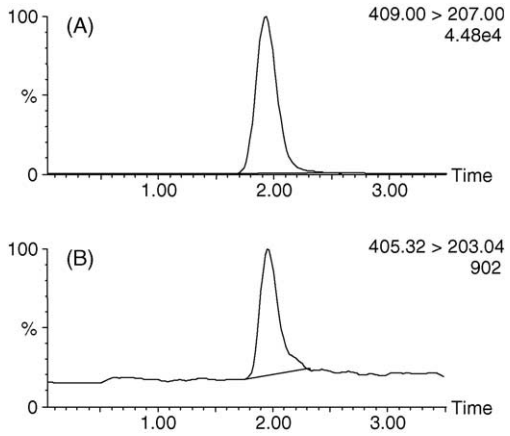

Fig. 3. Chromatograms of an extracted combined blank matrix (1:1 human plasma/buffer) spiked with flunarizine at LLOQ (0.25 ng/mL). (A) IS channel: *m*/*z* 409 → 207; (B) flunarizine channel: *m*/*z* 405 → 203.

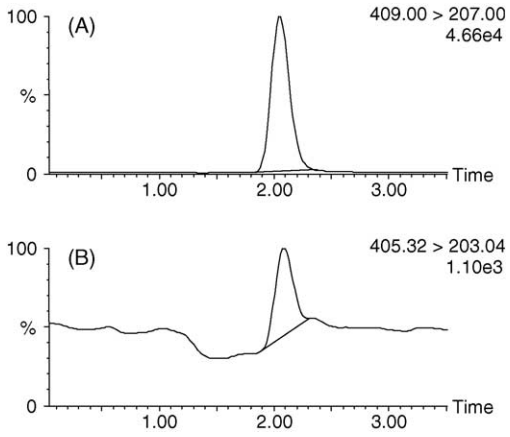

Fig. 4. Chromatograms of an extracted post-dialysis buffer sample generated by dialyzing plasma sample spiked with flunarizine at 1 μg/mL. (A) IS channel: *m*/*z* 409 → 207; (B) flunarizine channel: *m*/*z* 405 → 203.

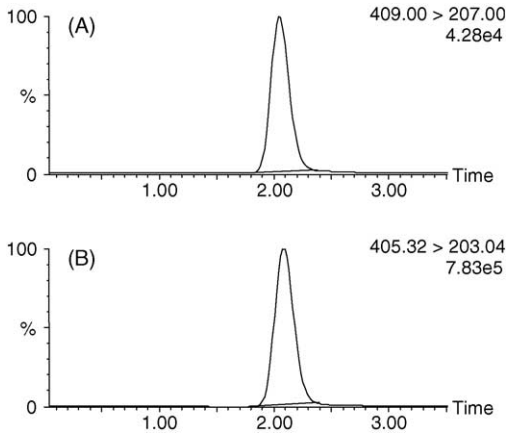

Fig. 5. Chromatograms of an extracted post-dialysis plasma sample spiked with flunarizine at 1 μg/mL. (A) IS channel: *m*/*z* 409 → 207; (B) flunarizine channel: *m*/*z* 405 → 203.

Table 1
Precision and accuracy of calibration standards ($N = 6$)

Nominal (ng/mL)	Mean	CV (%)	%Nominal
0.25	0.225	16.0	90.0
0.5	0.467	9.2	93.4
1	1.09	11.9	109.0
5	5.11	3.5	102.2
20	20.2	4.0	101.0
50	49.6	2.2	99.2
200	198	2.0	99.0
1000	1014	2.6	101.4
2000	1989	3.0	99.5

assay coefficient of variation (CV) for the back-calculated calibration standards ranged from 2.0 to 16.0% and the nominal concentrations ranged from 90.0 to 109.0%, respectively. The precision and accuracy data for QCs are summarized in Table 2. Intra-assay CV values were less than 10.6% and the nominal concentrations ranged from 95.8 to 112.8%. Inter-assay CV values were less than 13.5% and the nominal concentrations ranged from 98.2 to 106.8%. The CV and nominal concentration values indicated reproducible LC–MS/MS conditions and that the assay is consistent and reliable. For partial volume analysis, QC samples at 5 and 100 μg/mL were diluted 10- and 100-fold, respectively, with blank plasma prior to extraction. The dilution linearity data showed the CV was 3.2 and 2.2% and the nominal concentrations ranged from 95.4 to 111.3%, respectively. These results support sample dilution up to 100-fold for analysis.

3.5. Recovery and matrix effect

The extraction recovery was determined by comparing the peak areas of extracted standards at 0.5, 20 and 2000 ng/mL in triplicate to the peak areas of post-extraction combined matrix blanks spiked at corresponding concentrations. Extraction recovery from the combined matrix ranged from 95.3 to 106.1% for flunarizine and 93.0 to 102.2% for IS. The overall absolute recovery ranged from 76.7 to 121.1% for flunarizine and 74.2 to 83.4% for IS indicating the matrix effect was less than approximately 20% for both flunarizine and IS.

Table 2
Precision and accuracy of quality control samples

Nominal (ng/mL)	Mean	CV (%)	%Nominal
Intra-assay ($N = 5$)			
0.25	0.282	10.6	112.8
0.5	0.479	4.6	95.8
1	0.994	2.8	99.4
20	20.1	2.0	100.5
200	194	2.6	97.0
1000	1027	1.2	102.7
2000	2048	1.2	102.4
Inter-assay ($N = 15$)			
0.25	0.267	13.5	106.8
0.5	0.491	5.5	98.2
1	1.02	2.9	102.0
20	20.5	3.4	102.5
200	199	3.0	99.5
1000	1027	1.9	102.7
2000	2027	1.5	101.4

Table 3
Precision of flunarizine unbound drug (%Free) determination by equilibrium dialysis and LC–MS/MS

	5 μg/mL	10 μg/mL	100 μg/mL
Mean ($N = 5$)	0.075	0.102	0.071
S.D.	0.004	0.008	0.002
%CV	5.3	7.8	2.8

3.6. Stability of flunarizine

Flunarizine was stable throughout the equilibrium dialysis (37 °C up to 6 h) and sample extraction procedures. Flunarizine showed acceptable 4 h room temperature stability in plasma and in the combined matrix at 0.5, 20 and 2000 ng/mL. Flunarizine was also stable at 4 °C up to 9 days in the combined matrix at concentrations of 0.5, 20 and 2000 ng/mL. Extracted analytes were allowed to stand at an ambient temperature in mobile phase for at least 24 h prior to LC–MS/MS analysis, with no observed effect on quantitation.

3.7. Determination of in vitro protein binding of flunarizine

The equilibrium dialysis time between plasma and buffer was investigated. Plasma spiked with a fixed amount of flunarizine (1 μg/mL) was dialyzed against isotonic sodium phosphate buffer (pH 7.4) for 0.5, 1, 2, 4 and 6 h. Flunarizine crossed the dialysis membrane rapidly and reached a steady state at approximately 2 h. Therefore, a dialysis time of 2.5 h was used for all subsequent equilibrium dialysis experiments. Flunarizine in vitro protein binding was determined by dialyzing plasma spiked with 5, 10 and 100 μg/mL of flunarizine against isotonic sodium phosphate buffer, pH 7.4, followed by LC–MS/MS analysis as described. The free fraction of funarizine and the precision of the determination are summarized in Table 3. The %Free values ranged from 0.071 ± 0.002 to 0.102 ± 0.008% over the concentration range of 5 to 100 μg/mL with an overall %Free drug in plasma of 0.083 ± 0.005% and CV less than 7.8%. The results were calculated without consideration of observed slight volume shifts during dialysis. Flunarizine was bound extensively to plasma protein (>99% bound) and the %Free remained relatively unchanged within the concentration range of 5–100 μg/mL, suggesting that flunarizine has no binding saturation in human plasma.

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