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Research Article

QUANTIFICATION OF FLUNARIZINE IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

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ABSTRACT

A rapid and simple liquid chromatography-tandem mass spectrometric (LC–MS/MS) assay was developed and validated for quantification of flunarizine in human plasma. Samples were prepared by solid phase extraction procedure and cinnarizine was used as internal standard (IS). The analytes were eluted from a C_{18} (50mm×4.6 mm) analytical column using a isocratic mobile phase system consisting of acetonitrile and ammonium formate (85:15 v/v) with a total analysis time of 2.8 min. Analytes were detected by MS/MS using electrospray ionisation in the positive mode with multiple reactions monitoring (MRM) of the precursor ion/product ion transitions 405.2/203.1 for flunarizine and 369.1/167.1 for cinnarizine. Matrix effects were negligible. Standard curve for flunarizine was linear (r^2 ≥0.9976) over the range of 0.2–50 ng/mL, with 0.2 ng/mL representing the lower limit of quantification. Relative standard deviations were <4.2% for intra- and inter-day precision and the accuracy was within ±7.4%.

Keywords: Flunarizine; Human Plasma; Liquid chromatography-tandem mass Spectrometry.

INTRODUCTION

Flunarizine is a selective calcium channel blocker with calmodulin binding properties and histamine H_1 blocking activity. It is effective in the prophylaxis of migraine, occlusive peripheral vascular disease, vertigo of central and peripheral origin, and as an adjuvant in the therapy of epilepsy. Flunarizine, 1-[bis(4-fluorophenyl)methyl]-4-[(2E)-3-phenylprop-2-en-1-yl] piperazine, is a piperazine derivative[1,2]. It prevents cellular calcium overload by reducing excessive transmembrane calcium influx. Flunarizine does not interfere with normal cellular calcium homeostasis. The effects of flunarizine in the prophylaxis of migraine are most pronounced with regards to the reduction of the frequency of attacks. The severity of migraine attacks improves to a lesser extent, while little or no effect is seen on the duration of migraine episodes[3].

Flunarizine is well absorbed with peak plasma levels at 2 to 4 hours after oral administration in healthy volunteers. Plasma concentrations increase gradually during chronic administration of 10 mg daily, reaching a steady state level after 5 to 6 weeks of drug administration. Flunarizine is 99.1% bound; 90% is bound to plasma proteins and 9% distributed to blood cells, leaving less than 1% present as free drug in the plasma. Flunarizine is metabolized principally through N-oxidation and aromatic hydroxylation. During a 48 hour period after a single 30 mg dose, minimal urinary (<0.2%) and fecal (<6%) excretion of flunarizine and/or its metabolites was found.

This indicates that the drug and its metabolites are excreted very slowly over a prolonged period of time. Flunarizine has a long elimination half - life of about 19 days. The Cmax reported 30.5 ng/mL after oral administration of single dose 5 mg flunarizine tablets[3].

Various reported analytical methodologies for determination of unbound drugs in plasma were, include calorimetry[4], ultrafiltration[5], ultracentrifugation[6], equilibrium dialysis[7], capillary electrophoresis (CE)[8] and methods to determine flunarizine like HPLC methods with detailed literature[9], spectrophotometric techniques[10-11], gas chromatographic methods[12-14] and liquid chromatography tandem mass spectrometric methods[15-17]. Recently rapid chromatographic method was reported[18] for determination of flunarizine and its related impurities in dosage forms with runtime of 12 min. The current analytical work presents a method that reduces the analytical runtime to 2.8 min and no method was reported with sensitivity of 0.2 ng/mL. The achieved sensitivity was required for determination of plasma concentrations of flunarizine in human plasma at very low levels in elimination phase of pharmacokinetic and therapeutic drug monitoring studies.

MATERIALS AND METHODS

Materials and Reagents

The flunarizine and cinnarizine (IS) reference standards were obtained from Clearsynth Labs (Mumbai, India). Gradient grade methanol, acetonitrile (ACN), analytical grade ammonium formate, ammonia solution and formic acid (GR grade) were purchased from Merck specialties (Mumbai, India). The solid phase extraction cartridges strata-X-C were purchased from phenomenex Ltd (Mumbai, India). Ultrapure type-1 water from Milli-Q system (Millipore, Bedford, MA, USA) was used for aqueous preparations in the study. Polypropylene RIA vials (ABDOS, New Delhi, India) and volumetric flasks (type A) were obtained from Tarsons products Pvt. Ltd (New Delhi, India).

Mass spectrometric and chromatographic conditions

The LC–MS/MS system with a Shimadzu LC-20AD HPLC system (Shimadzu Corporation, Japan) interfaced with a 3200-triple quadrupole turbo ion spray mass spectrometer (Applied Biosystems, Canada) operated with analyst TM software version 1.4.2 was used for instrument control and data acquisition. Positive mode electrospray ionisation was used, the ionspray voltage was 5000V and the source temperature was 450 ◦C. Curtain gas, nebuliser gas and collision gas were set to flow rates of 25, 45 and 10 arbitary units. Data acquisition was performed via multiple reaction monitoring (MRM) with a dwell time of 200 ms. Collision energy (CE), declustering potential (DP) were optimised for the individual analytes; flunarizine 405.2/203.1 (CE 25 V, DP 16 V) and cinnarizine (IS) 369.1/167.1 (CE 25 V, DP 18 V). Calibration curves were constructed by plotting analyte/IS peak area ratios versus analyte concentrations using the analyst quantification software. Analysis of linearity was performed by $1/x^2$-weighted linear regression.

A Kintex EVO C_{18} (50mm×4.6 mm i.d., 5 µm) column was used with a C8 guard column 4mm×3.0mm (Phenomenex, Torrance, CA, USA). The column was heated to 35 ◦C whilst the auto-sampler was cooled to 5 ◦C. Mobile phase consisting of 10 mM ammonium formate buffer, pH adjusted to 3.5 with formic acid solution and acetonitrile in the ratio of 15:85 v/v. The run time was 2.8 min. Between each run the injection needle was washed before and after injection with 0.3 mL of acetonitrile:water (90:10 v/v) solution.

Calibration standards and quality control samples

Stock solutions of flunarizine and IS were prepared in methanol at 1mg/mL concentration. Standard solutions were prepared in water: methanol (50:50 v/v) solution and spiked into plasma to produce calibration standards of 0.2, 0.4, 1, 4, 10, 25, 40, 50 ng/mL of flunarizine. Quality control (QC) samples were prepared from separately weighed stock solutions and spiked into plasma to get the concentrations of 0.2 ng/mL (LLOQQC-Lower limit of quantification quality control), 0.6 ng/mL (LQC-Low quality control), 21 ng/mL (MQC-Medium quality control), 38 ng/mL (HQC-High quality control) and 100 ng/mL (DQC-Dilution quality control) of flunarizine. An IS solution of 200 ng/mL cinnarizine was prepared in methanol. All solutions were stored in refrigerator at 2-8◦C and spiked plasma samples were stored at −70 ◦C.

Sample preparation

As per method validation plan, the required spiked samples were retrieved from the freezer made them to thaw to room temperature. To a 5ml polypropylene tube containing 50 µl of IS solution, 300 µl of spiked plasma sample and 300 µl of 2% formic acid solution was added, then contents in tube were mixed for 30 seconds on a vortexer. The sample was loaded on to strata-X-C SPE (solid phase extraction) ion exchange cartridge

(30 mg/1cc) which is preconditioned with l ml of 100% methanol followed by equilibration with 1ml of 0.2% formic acid solution. After loading the sample, the cartridge was washed with 1ml of 0.2% formic acid solution, followed by 1ml of methanol-water (10:90 v/v) solution containing 1% formic acid. The cartridge was allowed to dry for 3 minutes. Then cartridge was eluted with 1 ml of methanol: ammonia (98:2 v/v), then the solution was evaporated to dryness and reconstituted with 0.3 ml of mobile phase.

Method validation

A complete method validation of flunarizine in human plasma was carried out as per US Food and Drug Administration (USFDA)[19], Agencia Nacional de Vigilancia Sanitaria (ANVISA)[20] and European medical agency (EMA)[21] bioanalytical method validation guidelines. The experimental parameters evaluated were carryover test, selectivity, specificity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability. A carryover experiment was performed to verify the carryover of analyte and IS, which influence the method performance in consecutive analytical runs. The carryover experiment was performed by injecting the following sequence of samples: blank plasma sample→ULOQ sample→blank plasma sample→LLOQ sample to check for any interference at the retention times of analyte and IS owing to carryover. Selectivity was assessed by comparing the chromatograms of six different human blank plasma lots along with each two lipemic and hemolyzed plasma containing K_2 EDTA (Ethylene diamine tetra acetic acid) as anticoagulant. Potential interference from the concomitant drugs like acetaminophen, ibuprofen, metamizole, ketorolac and ondansetron were evaluated. Sensitivity was determined by analyzing six replicates of extracted LLOQ samples. The effect of matrix on quantitation was checked with six different lots of K_2 EDTA plasma along with each two lots of hemolyzed and lipemic plasma. Three replicates of each LQC and HQC were prepared from ten different lots of plasma (60 QC samples in total) and analysed against the calibration curve. The linearity of the method was determined by analysis of standard plots constructed with a 8-nonzero standards for three different days. In addition to nonzero standards, blank plasma samples were also analyzed to confirm the absence of direct interference.

A set of spiked calibration standards (n = 6 for 0.2, 0.4, 1, 4, 10, 25, 40, 50 ng/mL) and QC samples (n = 36 for 0.2, 0.6, 21, 38 and 100 ng/mL) were prepared and analysed on three different days to evaluate precision and accuracy. This includes the lowest concentration calibration standard (0.2 ng/mL), which represented the lower limit of quantification (LLOQ) of the assay.

Relative extraction recoveries of flunarizine and IS were determined by comparing the obtained peak areas of the LQC, MQC, HQC samples (n = 6) to the peak areas of blank plasma extracts spiked with flunarizine and IS at concentrations corresponding to 100% recovery. Matrix effects from the plasma extracts were assessed by comparing the peak areas of the spiked blank plasma extracts to the peak areas from flunarizine and IS spiked in mobile phase, which represented 100% intensity (i.e. no matrix effect).

Stock solution stability was determined by comparing peak areas of flunarizine stock solutions stored at 2-8 ◦C for 20 days with those of freshly prepared ones. Long term stability in plasma was determined by analysing LQC, HQC samples stored at −70 ◦C for 41 days (n=6) against freshly prepared calibration curve. For a combination of short term and freeze-thaw stability, QC samples (n = 6 at LQC, HQC level) were subjected to three freeze-thaw cycles (thaw at room temperature, freeze at −70 ◦C for 12–24 h) followed by bench top storage at room temperature for 14 h. The results were then evaluated by determining the QC concentrations against calibration curve. Processed sample stability (dry extract) was evaluated by determining the concentrations of a set of QC samples (n = 6 at 2 levels) in refrigerator at 2-8 ◦C for 51 h and also storing the samples in autos ampler for 42 h at 5 ◦C.

RESULTS AND DISCUSSION

Method development

Since the assay was planned to use for analysing several hundred clinical samples, solid phase extraction was considered preferable for sample preparation as it is a simple, reliable technique compared to other extraction procedures such as protein precipitation or liquid–liquid extraction[22] which produce ‘dirty ‘extracts, a potential issue of co-elution with matrix components that can lead to changes in the ionisation processes in the electrospray source of the mass spectrometer (matrix effects)[23]. It was proven that, the cleanness of sample was very high with solid phase technique. The chromatography was developed with the aim of obtaining separation of the analytes from potentially interfering endogenous compounds as well as a fast run time. This was obtained using a relatively short (50mm) analytical column and a high percent

acetonitrile usage in mobile phase system with a total analysis time of 2.8 min.

The MS/MS parameters were optimised to produce the most abundant product ions for flunarizine, IS using electrospray ionisation. The highest signal to noise ratios were achieved in the positive mode. The obtained dominant fragments 203.1 for flunarizine and 167.1 were used for quantification.

Cinnarizine was chosen as internal standard because isotope labelled compound was costly. It was found to be suitable as an internal standard due to its similar chromatographic, ionisation properties to the analyte (i.e. which is a fluorinated derivative of IS) and also the extraction recovery was found to be the same.

Method validation

System suitability

During method validation, the precision (coefficient of variation, CV, %) of a system suitability test was found to be in the range 0.00–0.22% for retention time and 0.24–1.33% for the area ratio of flunarizine and IS.

Carryover effect

Carryover evaluation was performed to ensure auto sampler performance that it does not affect the accuracy and precision of the developed method. No significant carryover was observed in blank sample after injection of ULOQ (upper limit of quantitation) which indicates no carryover effect.

Screening and selectivity

The selectivity of the method was examined by analyzing extracted blank human plasma samples of different lots, no significant direct interference at the retention times of flunarizine and IS was observed from endogenous substances. Similarly, no interference was observed from commonly used concomitant medications such as acetaminophen, ibuprofen, metamizole, ketorolac and ondansetron.

Matrix effect

Matrix effect assessment was done to check the effect of biological matrix on quantification method. Matrix effect was assessed by the calculating the IS normalized matrix factor and also based on back calculated concentrations of 60 QC samples (quantification matrix effect). The matrix factor data results were shown in Table 1. The results of quantification and matrix factor were within the acceptable limits. No significant matrix effect was observed in all lots of human plasma at low and high quality control concentrations.

Linearity, precision and accuracy

The 8-point calibration curve was linear over the concentration range of 0.2–50 ng/mL for flunarizine. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ was found to produce best linear fit for the concentration–response relationship. The mean correlation coefficient of the weighted calibration curves generated during the validation was ≥0.99.

The results for intra-day and inter-day precision and accuracy in quality control samples are summarized in Table 2. The intra-day and inter day precision (%CV) values were all within 15% at LQC, MQC, HQC levels, and within 20% at LLOQQC level. The intra- and inter-day accuracy values were in the range of 92.6-99.1%. The results revealed that method has good reproducibility. The mean back-calculated concentrations for 1:2 and 1:4 factor dilution integrity samples were within 85–115% of their nominal value and the coefficients of variations (%CV) were <3.2 %. The extended precision and accuracy run was carried out with each 50 QC samples at LQC, MQC, HQC level. The samples were injected from low to high concentration consecutively, the results were found acceptable with accuracy in the range of 92.4-108.6%. IS trend analysis was evaluated considering IS response in all QC samples. The obtained % CV was 6.4, indicates highly consistent and reliable method performance. The representative chromatograms of flunarizine and IS in extracted blank, LLOQQC and HQC were shown in figures 1 (a,b), 2 (a,b) and 3 (a,b) respectively.

Extraction efficiency

Six replicates at low, medium and high quality control concentrations for flunarizine were prepared to determine the relative recovery. The overall mean recovery for flunarizine was 59.2 % with the precision range of 1.2–3.6 %. The IS recovery was found to be 61.8 % at MQC level. Hence, the assay has been proved to be rugged and robust in high-throughput bioanalysis when applied to real subject sample analysis. The recovery results were shown in Table-3.

Stability studies and dilution integrity

Different stability experiments were carried out, viz. bench-top stability (14 h), auto sampler stability (42 h), dry extract stability (51 h), repeated freeze–thaw cycles (seven cycles), whole blood stability (3 h) and long-term stability at 70 °C for 41 days. The mean back calculated concentration values of the analyte were found to be within ±15% to the nominal concentrations for the analyte at LQC

and HQC levels. The stability data results at LQC, HQC level were shown in Table 4. Thus, the results were found to be within the acceptable limits during the entire validation. Stock solutions of flunarizine and IS were found to be stable for 15 days at 2–8°C.

CONCLUSION

This paper demonstrates the successful development and validation of a simple, sensitive and rapid LC-MS/MS method for the determination of flunarizine in human plasma samples according to USFDA, ANVISA, EMA bioanalytical method validation guidelines with LLOQ of 0.2 ng/mL. The method employs a low plasma volume (300 µL) and the total analysis time was 2.8 min per sample, so relatively greater number of samples can be analyzed in short time. The simple SPE, saves considerable time and simplifies the sample preparation procedure. From the results of all the validation parameters, developed method could be useful for bioavailability and bioequivalence studies and routine therapeutic drug monitoring with the desired precision and accuracy.

Table 1: Matrix factor data results

Blank Plasma Lots	LQC			HQC		
	MF Analyte	MF ISTD	ISNMF	MF Analyte	MF ISTD	ISNMF
LOT-1	0.67	0.75	0.89	0.64	0.71	0.90
LOT-2	0.65	0.71	0.92	0.66	0.74	0.90
LOT-3	0.66	0.73	0.90	0.65	0.69	0.94
LOT-4	0.62	0.66	0.95	0.65	0.66	0.99
LOT-5	0.67	0.74	0.90	0.65	0.71	0.91
LOT-6	0.65	0.69	0.94	0.65	0.70	0.92
LOT-7 hemolytic	0.64	0.69	0.92	0.66	0.71	0.93
LOT-8 hemolytic	0.68	0.67	1.01	0.64	0.69	0.92
LOT-9 lipemic	0.64	0.62	1.03	0.62	0.61	1.01
LOT-10 lipemic	0.63	0.70	0.90	0.62	0.68	0.91
Mean			0.937	Mean		0.934
SD			0.0485	SD		0.0380
%CV			5.2	%CV		4.1
MF-Matrix factor		ISNMF-Internal standard normalised matrix factor				

Table 2: Intra- and inter day precision and accuracy results

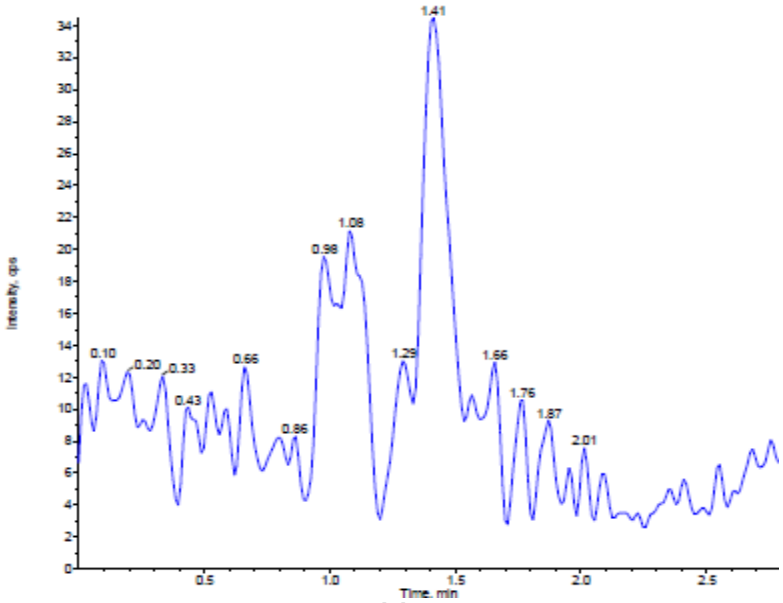
Sample name	INTRA-DAY For mean concentrations at each level (n=12)		INTER-DAY For mean concentrations at each level (n=36)	
	%Accuracy	% CV	%Accuracy	% CV
FLUNARIZINE				
LLOQQC	95.8	2.0	99.1	4.2
LQC	92.6	3.8	94.6	2.7
MQC	98.6	1.0	99.5	1.1
HQC	97.3	1.2	98.2	1.2

Table 3: Recovery results

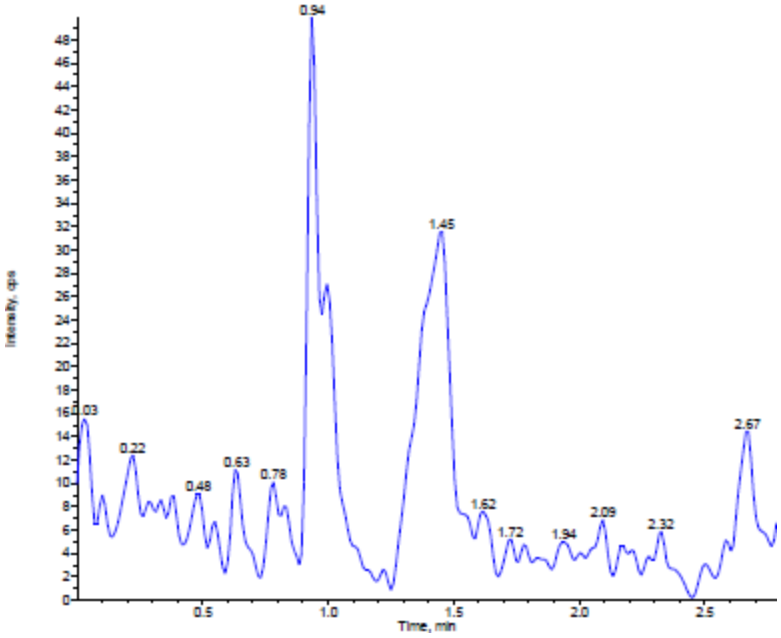
Sample name	% Recovery
LQC	56.2
MQC	58.9
HQC	62.6
Mean	59.2
IS	61.8

Table 4: The stability data results

Stability Experiment	stability condition	%Mean stability FLUNARIZINE	
		LQC	HQC
Auto sampler stability	42 h at 5° C	99.2	105.6
Free and thaw stability	7 cycles at -70° C ± 15°C	93.4	102.7
Dry extract stability	51 h at 2-8° C	89.9	96.6
Room temperature stability	14 h at Room temperature at 25° C ± 5°C	104.8	99.9
Long term stability	41 days at -70° C ± 15°C	95.7	95.4
Whole blood stability	3 h at 25° C ± 5°C	100.5	95.4

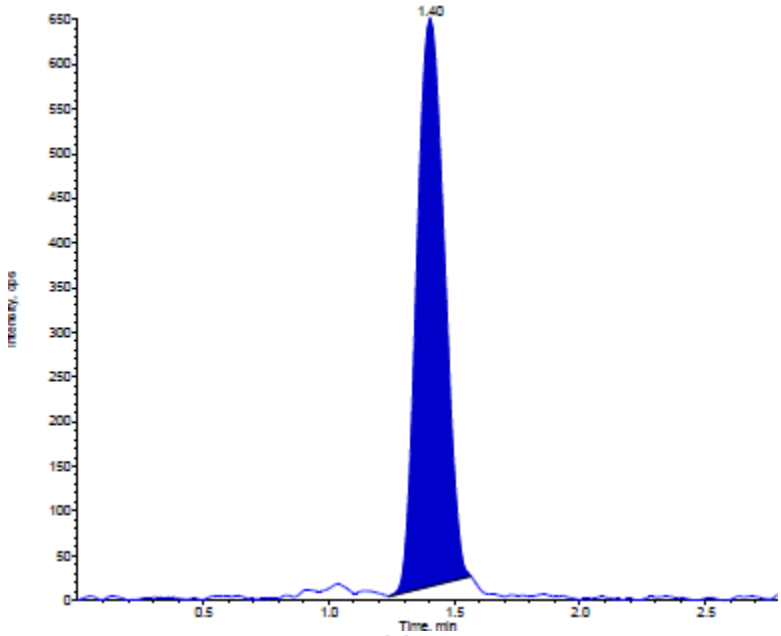


(a)

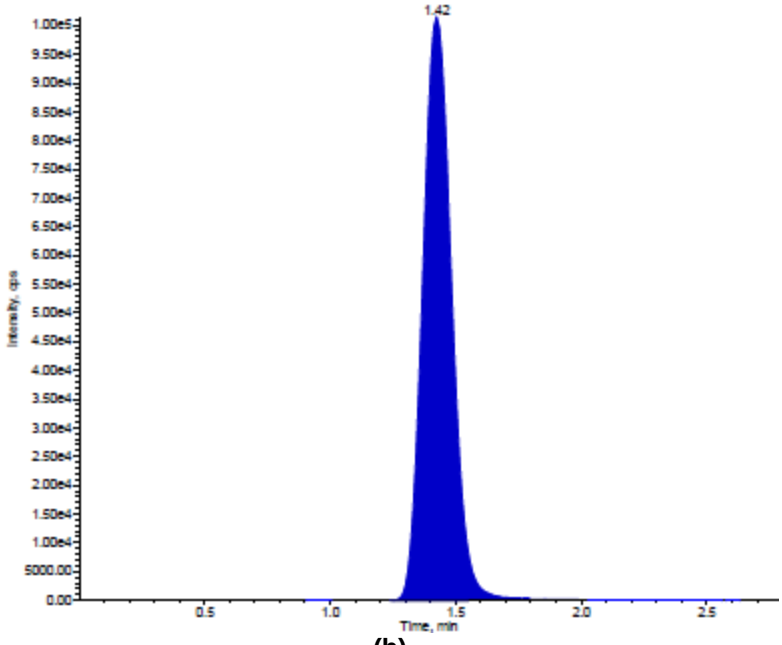


(b)

Fig. 1: Representative chromatograms of extracted blank (a) flunarizine and (b) IS

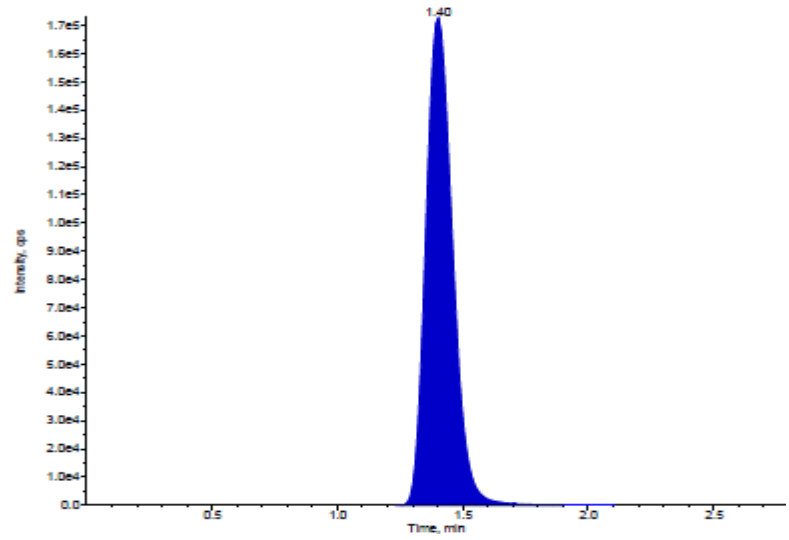


(a)

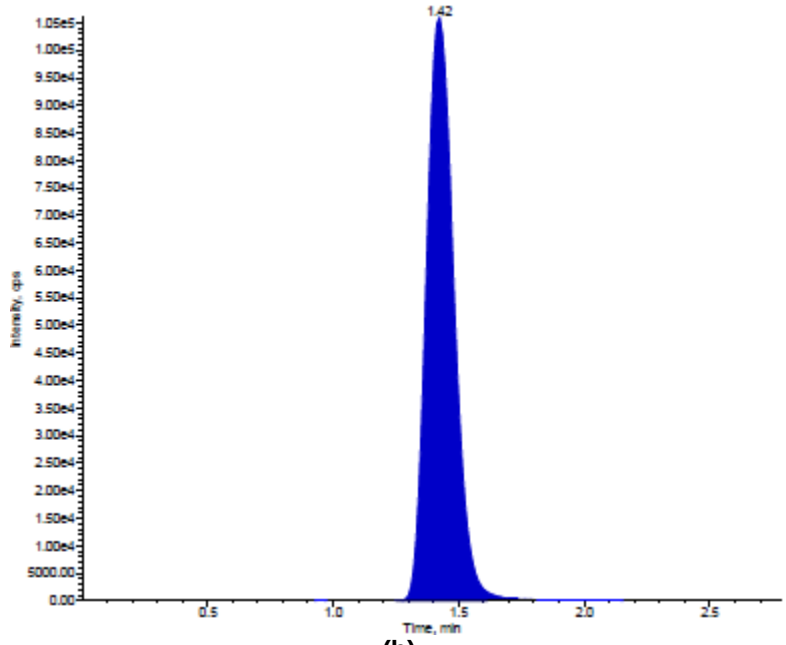


(b)

Fig. 2: Representative chromatograms of LLOQQC (a) flunarizine and (b) IS



(a)



(b)

Fig. 3: Representative chromatograms of HQC (a) flunarizine and (b) IS

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