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ORIGINAL ARTICLE

Simultaneous determination of asenapine and valproic acid in human plasma using LC–MS/MS: Application of the method to support pharmacokinetic study

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Abstract Combination of asenapine with valproic acid received regulatory approval for acute treatment of schizophrenia and maniac episodes of bipolar disorders. A simple LC–MS/MS method was developed and validated for simultaneous quantification of asenapine and valproic acid in human plasma. Internal standards were added to 300 μL of plasma sample prior to liquid–liquid extraction using methyl tertiary butyl ether (MTBE). Chromatographic separation was achieved on Phenomenex C18 column (50 mm × 4.6 mm, 5 μm) in isocratic mode at 40 °C. The mobile phase used was 10 mM ammonium formate–acetonitrile (5:95, v/v) at a constant flow rate of 0.8 mL/min monitored on triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) mode. The injection volume used for LC–MS/MS analysis was 15 μL and the run time was 2.5 min. These low run time and small injection volume suggest the high efficiency of the proposed method. The method was validated over the concentration range of 0.1–10.02 ng/mL and 10–20,000 ng/mL for asenapine and valproic acid respectively. The method recoveries of asenapine (81.33%), valproic acid (81.70%), gliclazide (78.45%) and benzoic acid (79.73) from spiked plasma samples were consistent and reproducible. The application of this method was demonstrated by a pharmacokinetic study in 8 healthy male volunteers with 5 mg asenapine and 250 mg valproic acid administration.

1. Introduction

The common psychiatric disorders such as schizophrenia and bipolar disorder are very devastating, characterized by manic/hypomanic, depressed or mixed states, and associated with a high risk for suicide [1,2]. Although manic episodes are considered as the hallmark state of bipolar disorder and it is depression that primarily contributes to functional disability and high rates of

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suicide [3–5]. In 2001, the World Health Organization reported that bipolar affective disorders rank within the top 10 causes of disability among all medical conditions, as measured in years lived with disability [6]. Although a number of treatment options have been established for acute manic or mixed episodes, including most currently used atypical antipsychotics. Asenapine is indicated in the European Union for the treatment of moderate to severe manic episodes associated with bipolar I disorder [7]. The multi-receptor pharmacologic profile of asenapine includes antagonism at serotonergic 5-HT_{2A} and adrenergic receptors [8] suggesting that it may effectively treat depressive symptoms. Asenapine is an antipsychotic with a unique pharmacologic profile indicated in the United States in adults for treatment of schizophrenia and as monotherapy or adjunctive therapy with valproate/valproic acid in the treatment of manic or mixed episodes associated with bipolar I disorder [9,10]. Asenapine is approved in August 2009 by US FDA for the acute treatment of schizophrenia and maniac episodes of bipolar disorders. Asenapine belongs to the class dibenzo-oxepino pyrroles, it is a white to off-white powder. The chemical designation is (3aRS, 12bRS)-5-Chloro-2-methyl2, 3, 3a, 12b-tetrahydro-1Hdibenzo [2, 3:6, 7] oxepino [4,5c] pyrrole (2Z)-2-butenedioate (1:1). Its molecular formula is $C_{17}H_{16}ClNO \cdot C_4H_4O_4$ and its molecular weight is 401.84 (free base: 285.8). Valproic acid is one of only a few anticonvulsants that are approved by the FDA for the treatment of mania in bipolar disorder. The chemical structures of asenapine and valproic acid are shown in Fig. 1.

The coupling of HPLC with mass spectrometry (LC–MS/MS) is now generally accepted as the preferred technique for quantifying small molecule drugs, metabolites, and other xenobiotic biomolecules in biological matrices, since this technique is highly selective and sensitive [11–14]. Literature survey revealed that only one LC–MS/MS study has been reported for the quantification of asenapine in human plasma with solid phase extraction [15]. But best of our knowledge no analytical method has been reported for simultaneous determination of asenapine and valproic acid. Hence, now we presented a sensitive and selective LC–MS/MS study for simultaneous determination of asenapine and valproic acid in human plasma using a simple and cost-effective liquid–liquid extraction method. The developed bio-analytical method was validated as per the FDA guidelines and applied to human pharmacokinetic study in healthy male volunteers after oral administration of asenapine and valproic acid [16].

2. Materials and methods

2.1. Chemicals and reagents

All reagents used were of analytical grade or HPLC grade. Ammonium formate and formic acid were purchased from Merck® (Darmstadt, Germany). Methyl tertiary butyl ether was obtained from Vetec® (Rio de Janeiro, Brazil). Acetonitrile was acquired from Merck® (Darmstadt, Germany). Asenapine, valproic acid, benzoic acid and gliclazide (assigned purity 99.9%) were purchased from United States Pharmacopoeia (USP). HPLC grade water was prepared by Milli-Q reverse osmosis (Millipore®, Bedford, USA) and meets United States Pharmacopoeia requirements. Modified hydrophilic polytetra-fluoroethylene membranes with a pore size of 0.22 µm were used to filter the mobile phase (Millipore®, Bedford, USA) and Millex® syringe filters hydrophilic polytetra-fluoroethylene membranes (13 mm, 0.45 µm pore size) from Millipore® (Bedford, USA) were used to filter the samples.

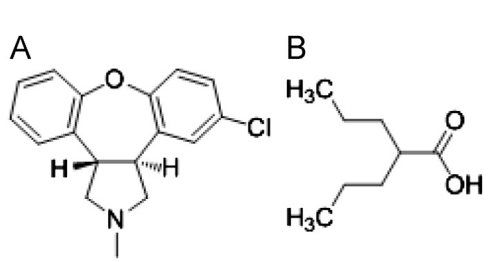

Fig. 1 Chemical structure of (A) asenapine and (B) valproic acid.

2.2. Instrumentation

The LC–MS/MS system used for method development and validation was an Applied Biosystems MDS Sciex API 4000 model (Switzerland), coupled with HPLC system consisted of LC-20AD binary gradient pump, SIL-10HTC auto sampler and a column oven CTO-10ASVP (Shimadzu Corporation, Kyoto, Japan). Data acquisition and processing were conducted using the Analyst 1.5.1 software on a Dell computer (Digital equipment Co). The analytical column used was Phenomenex C18 column (50 mm × 4.6 mm, 5 µm). The LC solvent program was operated in isocratic mode at 40 °C. Mobile phase used was 10 mM ammonium formate–acetonitrile (5:95, v/v) at a constant flow rate of 0.8 mL/min. The LC eluate was split down to 0.2 mL/min and introduced into triple quadrupole MS/MS system equipped with an electro spray ionization source. The MS/MS system was operated in positive ion mode for asenapine and in negative mode for valproic acid. The detailed mass spectrometer conditions are as follows: vaporizer temperature (TEM) 500 °C, ionization spray voltage +5500 V (asenapine) and −5000 V (valproic acid), collision gas (CAD, nitrogen) 7 psi, curtain gas (CUR) 40 psi, nebulizer gas (GS1) 35 and (GS2) 35 psi. The mass spectrometer was operated in both positive and negative ion multiple reaction monitoring (MRM) modes. The MRM transitions for analytes and IS are shown in Table 1. The declustering potential (DP), collision energy (CE) and other compound parameters for the analytes and IS were optimized individually and are shown in Table 1 (It is anticipated that small deviations in the masses ($m/z \pm 0.5$) could occur because of tuning differences and the MS system used).

2.3. Preparation of calibration standards and quality control samples

Standard stock solution and IS stock solutions of benzoic acid (IS of valproic acid) and gliclazide (IS of asenapine) were prepared by dissolving reference substances in mobile phase. Standard stock solutions (asenapine 1.0 mg/mL, valproic acid 4.0 mg/mL) and IS stock solution (benzoic acid 1.06 mg/mL, gliclazide 1.0 mg/mL) were stored at approximately 4 °C. Standard curve and QC samples were prepared by diluting stock solutions with blank human plasma. The concentrations of standard curve and QC samples are summarized in Table 2.

2.4. Preparation of mobile phase

Accurately weighed 0.639 g of ammonium formate was dissolved in 1000 mL of Milli Q-water and filtered through 0.22 µm membrane filter paper. The mobile phase solution consisting of ammonium formate and acetonitrile (5:95%, v/v) was prepared and

Table 1 Optimized parameters for MRM analysis of asenapine, valproic acid, gliclazide and benzoic acid.

Compound	Transition (*m/z*)	DP (V)	EP (V)	CE (eV)	CXP (V)
Asenapine	286.3/229.2	80	10	33	22
Valproic acid	143.4/143.4	–65	10	–30	–20
Gliclazide (IS)	324.3/110.1	30	10	25	6
Benzoic acid (IS)	121.1/77.1	–35	–10	–25	–10

Table 2 Standard solution concentrations of asenapine and valproic acid.

Standards and QCs	Asenapine (ng/mL)	Valproic acid (ng/mL)
STD 1	0.10	10
STD 2	0.20	20
STD 3	0.34	50
STD 4	0.67	200
STD 5	1.34	800
STD 6	2.68	2000
STD 7	4.01	6000
STD 8	8.02	13,000
STD 9	10.02	20,000
LQC	0.29	50
MQC	3.54	7200
HQC	7.51	14,300

filtered through nylon (0.22 μm) membrane sample filter paper and degassed and then the solutions were stored at ambient temperature.

2.5. Sample procedure

Before to assay, frozen human plasma samples were thawed at ambient temperature and centrifuged at 4500 rpm for 4 min at 5 °C to precipitate solids. In the following order, 0.03 mL of IS work solution (50 ng/mL gliclazide and 10,000 ng/mL benzoic acid) was added into each glass tube except for blank plasma. 0.3 mL of standards, QCs, study samples and blank plasma were transferred into the glass tubes. After vortex for 3 min, 2.0 mL of methyl tertiary butyl ether (MTBE) was transferred inward then tubes were stoppered well and shaken vigorously for approximately 5 min. Following centrifugation at 6000 rpm for 10 min, upper organic phase was transferred out. Another 3.0 mL MTBE was added to aqueous layer and the same extraction procedure was repeated. The organic layers from two extraction procedures were mixed together and evaporated to dryness under a slightly heated stream of nitrogen at approximately 40 °C. The residue was reconstituted with 0.2 mL of mobile phase and 15 μL of sample was injected into LC–MS/MS.

2.6. Method validation

The developed analytical method was validated in accordance to the recommendations published by the FDA.

System suitability experiment was performed by injecting six consecutive injections at least once in a day with aqueous MQC solution. System performance experiment was performed by injecting sequence of injections at the beginning of analytical batch. Specificity was determined by analyzing six blank human plasma samples without adding IS to determine the interference with the analytes. Matrix effects for analytes were evaluated by comparing the peak areas of analyte in extracted samples of blank plasma from six different drug-free volunteers spiked with known concentrations with the corresponding peak areas obtained by direct injection of standard solutions. Matrix effects for the IS were also investigated. The interference between asenapine and valproic acid was observed. The lowest standard on the calibration curve was accepted as the limit of detection (LOD). The LOD was the concentration with signal-to-noise ratio of 3.0. Six sets of calibration curves ranging from 0.1 to 10.02 ng/mL for asenapine, from 10 to 20,000 ng/mL for valproic acid, respectively, were constructed by plotting the peak area ratios of analyte/IS versus analyte concentrations in blank human plasma. The intra-day precision and accuracy were estimated by analyzing six replicates at three different QC levels. The inter-day precision was determined by analyzing three level QC samples on six different runs. The accuracy was expressed by (mean observed concentration)/(spiked concentration) × 100% and the precision by relative standard deviation (R.S.D.). The extraction recoveries of asenapine and valproic acid at three QC levels were determined by comparing peak area of the analytes obtained from plasma samples with the analytes spiked before extraction to those spiked after the extraction. QC samples prepared to test stability were subjected to short-term (12 h) at room temperature, 48 h autosampler at 18 ± 2 °C, 48 h refrigeration at 4 °C, two freeze-thaw cycles at –70 ± 5 °C, and long-term at –70 ± 5 °C for 30 days stability tests.

2.7. Application of the method to pharmacokinetic study

A pharmacokinetic study was conducted in healthy male subjects ($n = 8$). The ethics committee approved the protocol and the volunteers provided informed written consent. Blood samples were obtained following oral administration of 5 mg of asenapine and 250 mg of valproic acid. EDTA solution was used as an anti-coagulant and the blood samples were collected at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 12 and 18 h after oral administration of both drugs. Plasma was separated from whole blood by centrifuging the blood using a Heareus® model 1.0 R at 4000 rpm for 10 min, at 4 °C and stored frozen at –70 ± 5 °C until analysis. Along with study samples, QC samples at LQC, MQC, and HQC levels were assayed in duplicate and were distributed among unknown samples in the analytical run. Plasma concentration–time data of asenapine and valproic acid were analyzed by noncompartmental method using WinNonlin version 5.1 (Pharsight Corporation, Mountain View, CA, USA).

3. Results and discussion

3.1. Sample preparation and LC–MS/MS analysis

The aim of the present study was to develop a rapid, selective and sensitive analytical method including an efficient and reproducible

sample extraction step for quantitative analysis of asenapine and valproic acid in human plasma. Based on our previous experience on sample extraction for analyses, a simple and inexpensive liquid–liquid extraction (LLE) procedure has been implemented for the extraction of asenapine, valproic acid and IS from human plasma. Ethyl acetate, MTBE, *n*-hexane, diethyl ether, *n*-hexane–isopropanol (95:5, v/v), ethyl acetate–*n*-hexane (1:1, v/v), and ethyl acetate–*n*-hexane (1:2, v/v), were all tested, and finally MTBE was adopted because of its high extraction efficiency and less interference.

Liquid chromatography method development begins with optimizing the mobile phase composition and column type. The feasibility of several mixtures of solvents such as acetonitrile and methanol using different buffers including ammonium acetate, ammonium formate, acetic acid and formic acid were tested for complete chromatographic resolution of asenapine, valproic acid and IS. The versatility, suitability and robustness of the method were checked with several C18 and Cyano (CN) columns. The Phenomenex C18 column (50 mm × 4.6 mm, 5 μm) provided very good selectivity, sensitivity and peak shape for both analytes and

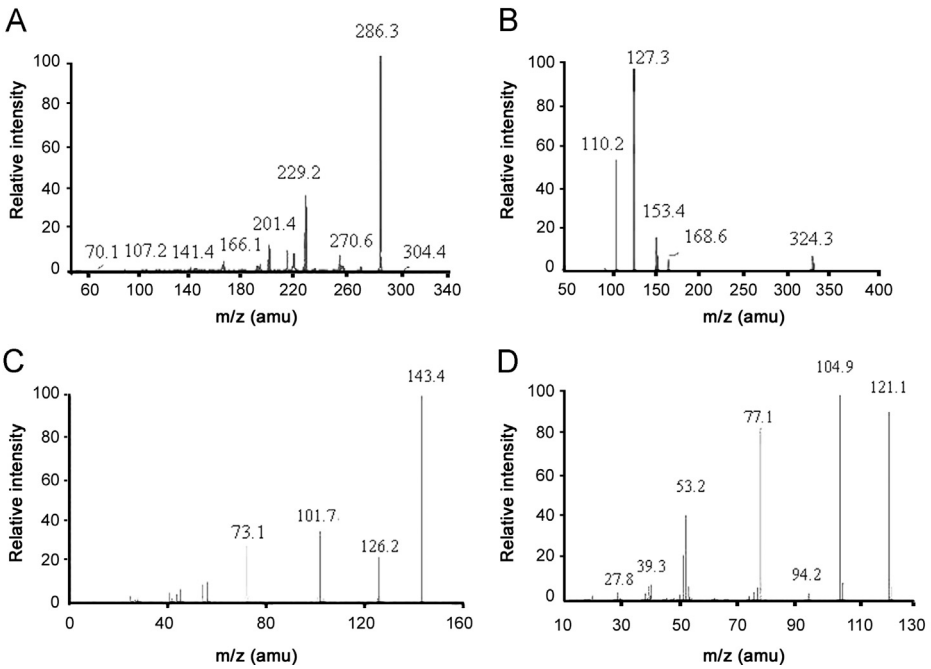

Fig. 2 Representative mass spectra of (A) asenapine, (B) gliclazide, (C) valproic acid and (D) benzoic acid.

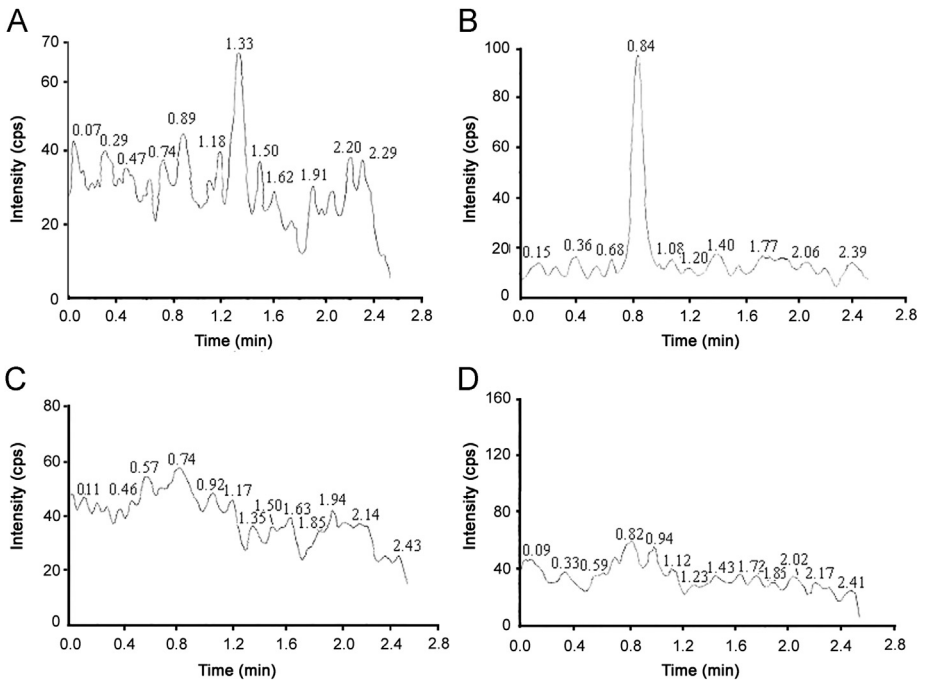

Fig. 3 Representative chromatograms for blank plasma of (A) asenapine, (B) gliclazide, (C) valproic acid and (D) benzoic acid.

IS, as compared to other columns. The mobile phase consisting of 10 mm ammonium formate–acetonitrile solution (5:95, v/v) with a flow rate of 0.8 mL/min was found to be suitable during LC optimization. The retention times for asenapine and gliclazide (IS for asenapine) were observed at 1.32 and 0.84 min, whereas for valproic acid and benzoic acid they were 1.57 and 1.43 min respectively.

In the MS method development, full-scan mass spectra of both analytes and their IS were obtained in combination of positive and negative ion modes with ESI source. Multiple reaction monitoring mode was considered to obtain better selectivity. However, the response found was much higher in negative ionization mode for valproic acid compared to that in positive mode due to its acidic nature. Valproic acid and benzoic acid (IS) were monitored by MRM of deprotanated molecules at *m/z* 143.4–143.4 and *m/z* 121.1–77.1 respectively. The mass spectrometer was operated in the positive ion MRM mode for asenapine in the LC–MS/MS analysis. ESI spectra revealed higher signals at *m/z* 286.3 for asenapine and *m/z* 324.3 for gliclazide. The full scan spectra were dominated by protonated molecules $[M+H]^+$. Additional tuning of ESI source and collision-induced dissociation (CID) parameters onto the transition *m/z* 286.3–229.2 (asenapine) and *m/z* 324.3–110.1 (gliclazide) further improved the sensitivity. The representative mass spectra of analytes and internal standards are shown in

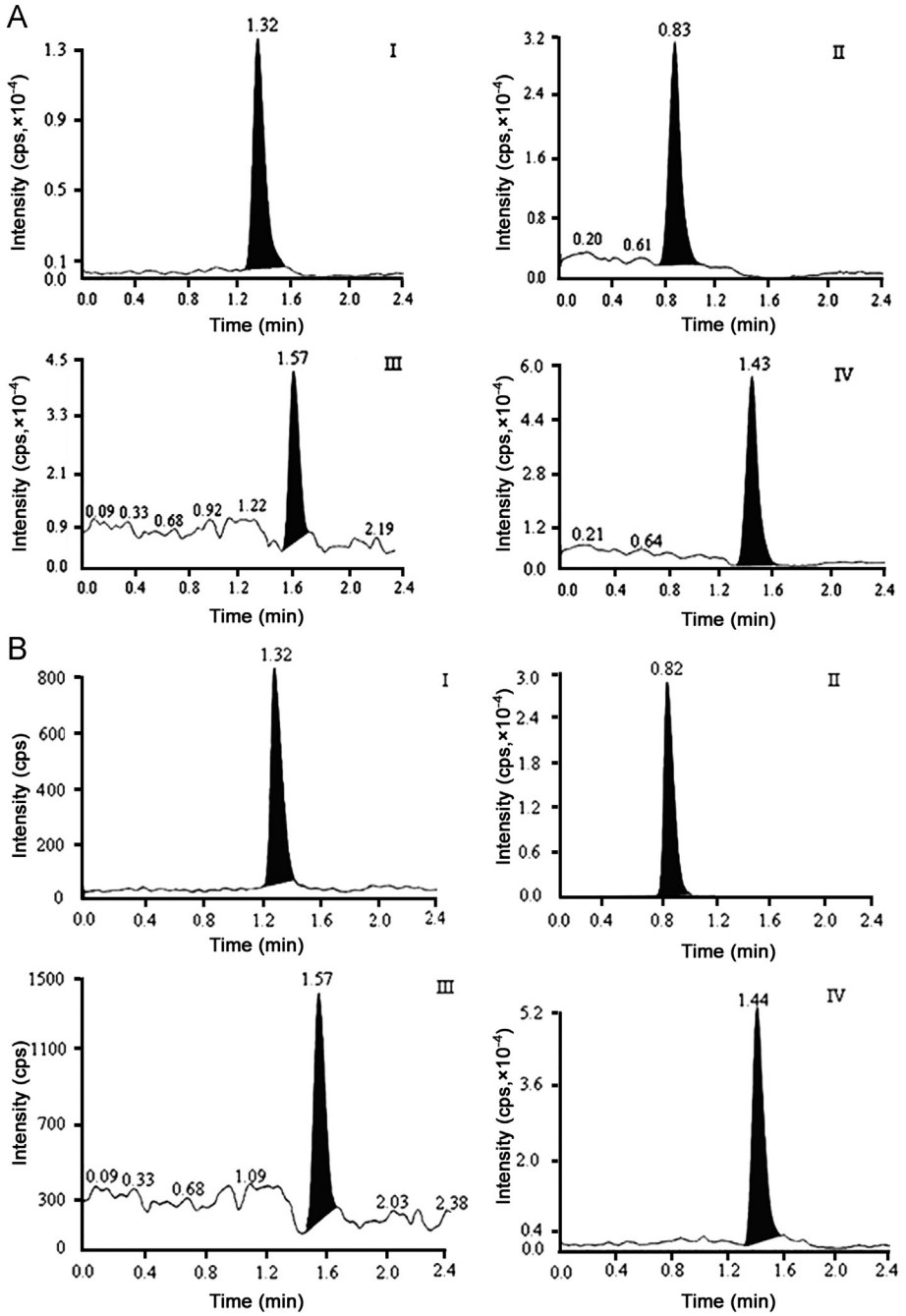

Fig. 4 Chromatograms of (A) plasma sample at LLOQ concentration and (B) plasma sample 1.0 h after oral dose of 5 mg asenapine and 250 mg valproic acid. (I) Asenapine, (II) gliclazide, (III) valproic acid and (IV) benzoic acid.

Fig. 2. Although the stable isotope labeled compounds of the analytes would be the ideal IS, due to the lack of commercial availability, the compounds with similar chromatographic, mass spectrometric behavior and extraction characteristics were chosen as IS. Therefore, gliclazide was chosen as IS for asenapine and benzoic acid as IS for valproic acid.

3.2. Method validation parameters

3.2.1. Selectivity/specificity

Nanopure water was used to assess exogenous substances in blank plasma with the lowest calibration curve sample. No significant interfering peaks were observed at the retention times of asenapine, valproic acid and IS in blank plasma extraction, which are shown in Fig. 3. No matrix effect for analytes (asenapine R.S.D.=2.9%, valproic acid R.S.D.=3.4%) and IS (gliclazide R.S.D.=4.4%, benzoic acid R.S.D.=5.1%) was observed for six different plasma pools indicating that no undetected co-eluting compounds could influence the ionization of the analytes. There was no interference between asenapine and valproic acid from Fig. 4.

3.2.2. Limit of quantification and linearity

For this method, the lower limit of quantitation (LLQQ) was 0.1 ng/mL for asenapine, and 50 ng/mL for valproic acid. Representative chromatograms of LLOQ and subject samples are shown in Fig. 4. The correlation coefficient (r^2) value during the validation ($n=6$) was >0.998 for both asenapine and valproic acid. The 9-point calibration curve was found to be linear over the concentration range of 0.1–10.02 ng/mL for asenapine and 10–20,000 ng/mL for valproic acid. After comparing the three weighting models (x, $1/x$ and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the analyte to IS concentration was found to produce the bestfit for the concentration–detector response relationship for asenapine and valproic acid in human plasma.

3.2.3. Extraction recovery

The mean extraction recoveries of asenapine and valproic acid were more than 81.3% and 81.7%, respectively. To IS, the mean recoveries for gliclazide and benzoic acid were 78.4% and 79.7%, respectively. The results are presented in Table 3. The extraction recoveries were consistent over the entire concentration range and comparable to the respective internal standard.

3.2.4. Accuracy and precision

Data for intra-day and inter-day precision and accuracy of the assay are summarized in Table 4. The intra-day accuracy ranged

Table 3 Extraction recoveries of asenapine, valproic acid and internal standards from human plasma[a].

QCs	Concentration (ng/mL)	Recovery[b] (%)	CV (%)
LQC			
Asenapine	0.29	84.72	2.11
Valproic acid	50	81.21	4.37
MQC			
Asenapine	3.54	80.40	4.01
Valproic acid	7200	82.60	5.74
HQC			
Asenapine	7.51	78.91	3.48
Valproic acid	14,300	81.34	5.21
IS			
Gliclazide	50	78.45	4.12
Benzoic acid	10,000	79.73	3.24

[a] $n=6$.
[b] Extraction recovery (%) calculated as ratio of mean peak area of analytes spiked into plasma before extraction to that of after extraction.

Table 4 Intra-day and inter-day precision and accuracy data of asenapine and valproic acid.

QCs	Concentration (ng/mL)	Intra-day ($n=6$)			Inter-day ($n=6$)		
		Mean conc. (ng/mL)	CV (%)	Accuracy (%)	Mean conc. (ng/mL)	CV (%)	Accuracy (%)
LQC							
Asenapine	0.29	0.260	1.93	89.6	0.252	2.54	86.8
Valproic acid	50.00	44.65	2.27	89.3	46.80	2.15	93.9
MQC							
Asenapine	3.54	3.11	5.16	87.8	3.20	3.61	90.3
Valproic acid	7200.00	6364.80	4.27	88.4	6595.20	2.48	91.9
HQC							
Asenapine	7.51	6.82	3.13	90.8	6.53	5.31	86.9
Valproic acid	14,300.00	13,098.80	3.82	91.6	12,626.90	4.81	88.3

between 87.8% and 90.8% with a precision of 1.93–5.16%, the inter-day accuracy between 86.8% and 90.3% with a precision of 2.54–5.31% for asenapine. And the intra-day accuracy ranged between 88.4% and 91.6% with a precision of 2.27–4.27%, the inter-day accuracy between 88.3% and 93.9% with a precision of 2.15–4.81% for valproic acid.

3.2.5. *Stability*

The stability of asenapine and valproic acid was evaluated under the conditions described in Table 5. The samples were stable under these conditions. Therefore, in all cases the international acceptance criteria (variation values for area smaller than 15%) were met. Thus, the result presented was found to be within the acceptable limits during the entire validation.

3.3. *Application of the developed LC–MS/MS method*

In order to verify the sensitivity and selectivity of the developed method in a real-time situation, the method described in this paper was applied to a human pharmacokinetic study that generated over 112 human plasma samples. Comparison of peak area ratio from the unknown samples with that from calibration curve allowed quantitation of the assayed samples. After oral administration of 5 mg of asenapine and 250 mg of valproic acid tablets to the volunteers, the

Table 5 Summary of stability studies of asenapine and valproic acid in human plasma.

Stability	LQC (ng/mL)		MQC (ng/mL)		HQC (ng/mL)	
	Asenapine 0.29	Valproic acid 50	Asenapine 3.54	Valproic acid 7200	Asenapine 7.51	Valproic acid 14,300
Room temp (12 h)						
Mean conc ($n=5$)	0.264	46.04	3.61	6796.35	6.79	13,372.70
CV (%)	3.11	3.41	1.84	4.22	5.78	4.21
Bias (%)	–8.96	–7.90	1.97	–5.61	–9.58	–6.47
Auto sampler (48 h)						
Mean conc ($n=5$)	0.273	48.27	3.47	7305.22	7.34	13,795.24
CV (%)	3.41	2.54	5.21	1.68	1.41	4.17
Bias (%)	–5.86	–3.45	–1.97	1.47	–2.63	–3.51
Short-term (12 h)						
Mean conc ($n=5$)	0.310	50.86	3.76	7293.72	7.12	13,658.20
CV (%)	1.33	1.61	2.78	1.23	3.14	2.19
Bias (%)	6.89	1.72	6.21	1.31	–5.19	–4.48
Freeze-thaw						
Mean conc ($n=5$)	0.284	50.43	3.41	6658.36	7.29	13,735.28
CV (%)	3.49	1.96	2.69	3.21	2.54	2.77
Bias (%)	–2.06	0.85	–3.67	–7.50	–2.92	–3.87
Long-term						
Mean conc ($n=5$)	0.271	48.70	3.29	6908.48	7.62	13,461.20
CV (%)	5.73	2.57	3.32	1.94	2.61	5.81
Bias (%)	–6.55	–2.59	–7.06	–4.03	1.46	–5.85

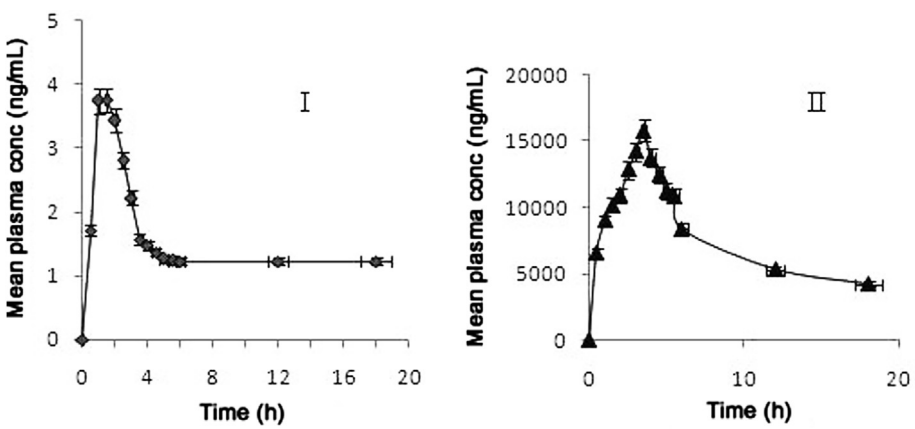

Fig. 5 Concentration–time profile for 8 subjects after (I) 5 mg dosage of asenapine and (II) 250 mg dose of valproic acid.

Table 6 Pharmacokinetic data of asenapine and valproic acid after their oral administration in 8 healthy male volunteers.

Parameter	Asenapine	Valproic acid
C_{max} (ng/mL)	3.76±1.18	15,820±3270
T_{max} (h)	1.00±0.16	1.07±0.48
$t_{1/2}$ (h)	23.63±11.8	3.46±0.48
AUC_{0-18} (ng h/mL)	13.42±2.91	19,485±640
$AUC_{0-\infty}$ (ng h/mL)	18.15±5.73	106,270±2140

C_{max}: maximum plasma concentration.
T_{max}: time point of maximum plasma concentration.
$t_{1/2}$: half life of drug elimination during the terminal phase.
AUC_{0-18}: area under the plasma concentration–time curve from zero hour to 18 h.
$AUC_{0-\infty}$: area under the plasma concentration–time curve from zero hour to infinity.

concentration versus time profiles for asenapine and valproic acid are presented in Fig. 5. The resulted values are in good agreement with the previously reported methods [15,17,18] and the pharmacokinetic data of both asenapine and valproic acid are presented in Table 6.

4. Conclusions

The selective LC–MS/MS method for the simultaneous determination of asenapine and valproic acid in human plasma has been successfully developed and validated. A simple and inexpensive liquid–liquid extraction procedure and an isocratic chromatography condition using a reversed-phase column provided an assay well suited for real-time analyses. The method is adhered to the regulatory requirements for selectivity, sensitivity, linearity, precision, accuracy, recovery, carry-over, matrix effect and stability using a small sample volume 300 μL. Because of the relative short chromatographic run time (2.5 min) and straightforward sample pre-treatment procedure, the method is easy to follow and can be adopted for clinical drug monitoring.

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