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Bioanalytical Method for Vinpocetine and Apovincamine Acid from Human Plasma by LC-MS/MS

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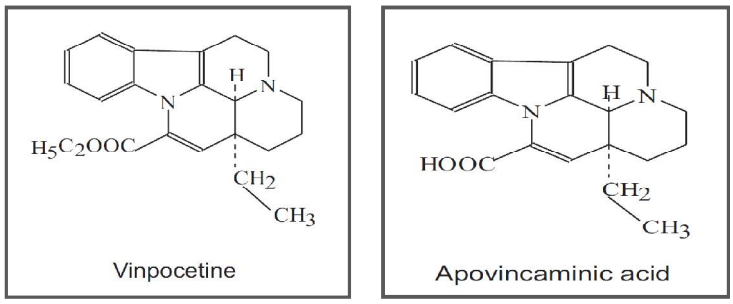
ABSTRACT

A sensitive and reproducible liquid chromatography-electro spray ionization-tandem mass spectrometry (LC-MS/MS) method was developed for the quantification of vinpocetine (VP) and its active metabolite apovincamine acid (AVA) in human plasma, with racem propyl vinpocetine(VP-IS) and racem propyl apovincamine acid (AVA-IS) as an internal standard (IS). The analyte was extracted with Solid Phase Extraction using ion exchange cartridges and analyzed on a Zorbax SB-CN (250 mm × 4.6 mm, 5 µm) column. The mobile phase was composed of methanol 10 mM ammonium acetate with 0.1% Formic Acid (70:30). Vinpocetine, apovincamine acid and IS racem propyl vinpocetine, racem propyl apovincamine acid were ionized by positive ion pneumatically assisted electro spray and detected in the multi-reaction monitoring (MRM) mode using LC-MS/ MS (API 5500 QTrap) →productions of m/z 351.4→280.1, m/z 323.2→279.2, m/z 365.3→294.1 and m/z 337.1→293.2 respectively. The specificity, matrix effect, recovery, sensitivity, linearity, accuracy, precision, and stabilities were all validated over the concentration range of 0.5 –250.0 ng mL^{-1} for both vinpocetine and apovincamine acid. The method developed was successfully and demonstrated for evaluation of pharmacokinetic profile of vinpocetine and apovincamine acid in human plasma.

Graphical Abstract



Bioanalytical method for vinpocetine and apovincamine acid

Keywords: LC-MS/MS-Liquid Chromatography Tandem Mass Spectroscopy, Solid Phase Extraction, Vinpocetine, Apovincamine acid.

INTRODUCTION

Vinpocetine (VP) is derivative of Vinca minor alkaloid, vincamine, is widely used in the treatment of various disease states of cerebrovascular origin. VP has higher biological activity and fewer side effects than vincamine. It improves the utilization of oxygen by the cerebral cells and protects the brain cells against ischemic anoxia [**1-3**]. In addition, VP can treat various cerebrovascular diseases such as acute cerebral infarction, vascular dementia and other related diseases [**4–6**]. In human body, VP is rapidly absorbed and metabolized into its primary active metabolite, apovincaminic acid (AVA). VP is rapidly undergoes extensive metabolism, during which approximately 75% of VP is hydrolyzed into its main active metabolite AVA, leading to a low bioavailability of (6.2 ± 1.9) % in vivo [**7-8**]. Several methods have been developed for the determination of VP and AVA. GC methods based on prederivatization have been used for the determination of AVA in biological samples [**9, 10**]. However, these methods require derivatization procedure and the sample preparation is very laborious. Analytical methods of VP and AVA in biological samples for pharmacokinetic application have been documented using LC-UV [**11–13**], LC-MS [**14**], GC [**15**] or GC-MS [**16, 17**] methods. These methods have various drawbacks such as long chromatographic run time within 14min [**12**], large sample volume with 1mL [**15**] for processing, or a tedious extraction procedure [**11**], which prevent their use in the routine biological sample analysis. Moreover, simultaneous estimation of VP and AVA in rat, beagle plasma and brain tissue using LC-MS/MS are also reported [**18–21**]; however, the sample pre-treatment was multi-step and costly [**19**], the linearity ranges for AVA were 10.61–240.0 ng mL^{-1} [**18**] and 1.0–200.0 ng mL^{-1} [**20**] in their methods, which sensitivity could not meet the requirement of the bioanalysis VP and AVA in human plasma. Sample processing by only protein precipitation using methanol and injected supernatant after centrifugation create problem during analysis clogging of HPLC column, Source capillary and Orifices [**20**]. Simultaneous determination of VP and its active metabolite AVA in human plasma by HPLC-MS/MS has not been reported at present. It is well known that the LC-MS/MS method could improve the selectivity and sensitivity, and shorten the analytical time compared to traditional HPLC and GC-MS method. Therefore, a LC-MS/MS method to simultaneously determine VP and AVA in human plasma is developed and validated.

MATERIALS AND METHODS

Chemicals and Reagents: Vinpocetine, apovincamine acid, racem propyl vinpocetine and racem propyl apovincamine acid working standard of purity 99.7, 92.9, 98.53 and 86.89 was obtained from Gedeon Richter Ltd. Water (LC–MS grade), methanol (HPLC grade), and acetonitrile (HPLC grade) were acquired from Biosolve (Valkenswaard, The Netherlands). Analytical grade of ammonium formate, Hydrochloric acid, Ammonia solution and formic acid were used. SPE cartridges of Phenomenex (30 mg1 mL^{-1}) were used.

Equipments: LC-MS/MS analysis was performed on Applied Biosystems/MDS SCIEX API 5500 LC-MS/MS triple quadrupole mass spectrometry with ESI source and Analyst software 1.6.1 version. The LC part consists of Shimadzu auto sampler LC-10 series chromatographic system (Shimadzu Corporation, Kyoto, Japan) equipped with dual pump (LC10AD-VP). The column oven employed (CTO-10AS VP). Positive pressure Solid Phase extraction unit of Orochem Ezypress 48 was used for extraction of plasma samples.

LC-MS/MS Conditions: Chromatographic separations were achieved by using Agilent Zorbax SB-CN column (250 x 4.6 mm, 5µm).The mobile phase consisted of methanol and 10 mM ammonium formate buffer with 0.1%v/v formic acid in the ratio of 70:30 v v^{-1}. The rinsing solution used was made of methanol and water (70:30, v v^{-1}). All separations were performed isocratically at a flow rate of 1.00 mL min^{-1} and the injection volume was 20 µL. The column oven was kept at 40°C throughout the analysis. The effluent was ionized by mass spectrometry interfaced with the HPLC via turbo ion source positive electrospray ionization mode and detected by mass spectrometry multiple reaction

monitoring (MRM) mode. MS/MS optimization for vinpocetine, apovincamine acid and IS Racem propyl vinpocetine, racem propyl apovincamine were performed using 100 ng mL^{-1} concentrations in mobile phase. The precursor ions and LC-MS/MS parameters are displayed in table 1.

Table 1. Mass Spectrometric condition

Name of compound	VP	AVA	VP-IS	AVA-IS
Molecular weight	350.46	322.45	364.48	336.43
Parent Mass(Da)	351.400	323.200	365.300	337.100
Product Mass(Da)	280.100	279.200	294.100	293.200
Declustering Potential (DP)	100.00	120.00	100.00	100.00
Entrance Potential (EP)	10.00	10.00	10.00	10.00
Collision Energy (CE)	40.00	30.00	40.00	32.00
Collision cell Exit Potential (CXP)	15.00	15.00	15.00	10.00

The source parameters were optimized for analyte and internal standard by infusing by 100 ng mL^{-1} standard solution containing all four compounds in mobile phase at 30µL/min, via an external syringe pump directly connected to the mass spectrometer. The turbo ion spray source temperature was optimized at 5000C, Ion Spray Voltage optimized at 5500 volt, curtain gas at 40(arbitrary unit) and Nebulizer Gas at 50(arbitrary unit).

Standard and Quality control Solutions: The stock solutions of VP, AVA and IS, all were prepared in methanol at concentrations of 500 µg mL^{-1}, 500 µg mL^{-1} and 100µg mL^{-1} respectively. The stock solutions were then serially diluted with methanol-water (50:50 v v^{-1}) to get the standard working solutions: 2.0, 4.0, 20.0, 100.0, 200.0, 400.0, 600.0, 800.0 and 1000.0 ng mL^{-1} for VP and 10.0, 20.0, 100.0, 500.0, 1000.0, 2000.0, 3000.0, 4000.0 and 5000.0 ng $m^{-1}L$ for AVA. Three levels of quality-control samples at concentration of 6, 500 and 750 ng mL^{-1} for VP and 30, 2500 and 3750 ng mL^{-1} for AVA were prepared separately in the same fashion. The Plasma samples of calibration standard and quality control samples were prepared by spiking 5% of above spiking solutions in plasma. Final plasma concentrations of calibration standard and quality control samples for VP are 0.1 to 50.0 ng mL^{-1} and for AVA are 0.5 to 250.0 ng mL^{-1} The IS working solution of 50.0 ng mL^{-1} (VP-IS) and 100.0 ng mL^{-1} (AVA) was prepared by dilution of the stock standard solution with methanol-water (50:50, v/v).

Sample preparation: Plasma sample of 200 µL was spiked in the pre-labeled RIA vials and added50 µL IS dilution solution mixture of racem propyl vinpocetine (approx.50 ng mL^{-1}) and racem propyl apovincamine acid (approx.100 ng mL^{-1}) and 500 µL of 3% hydrochloric acid in water was added in each sample and vortexed to mix properly. The cartridges {Strata XC (30 mg $1cc^{-1}$)} were conditioned with 1.0 mL methanol, equilibrated with the1.0 mL of 3% hydrochloric acid in Milli-Q water and samples were loaded, cartridges were washed with 1.0 mL water and 1.0 mL of methanol. Dried the cartridges under full vacuum and elute with 1mL of 5% ammonia solution in methanol. Evaporated the eluted samples in nitrogen evaporator under stream of nitrogen and reconstituted the dry residue with 1.0 mL of mobile phase.

Analytical method Validation

Selectivity: Ten different Human plasma lots including Lipemic and Haemolysed plasma with K3EDTA as anticoagulant were evaluated for selectivity. All the lots were found to be free from any significant interfering peak at the retention time of vinpocetine, apovincamine acid, racem propyl vinpocetine (IS) and racem propyl apovincamine acid (IS) presented in figure1 and 2.

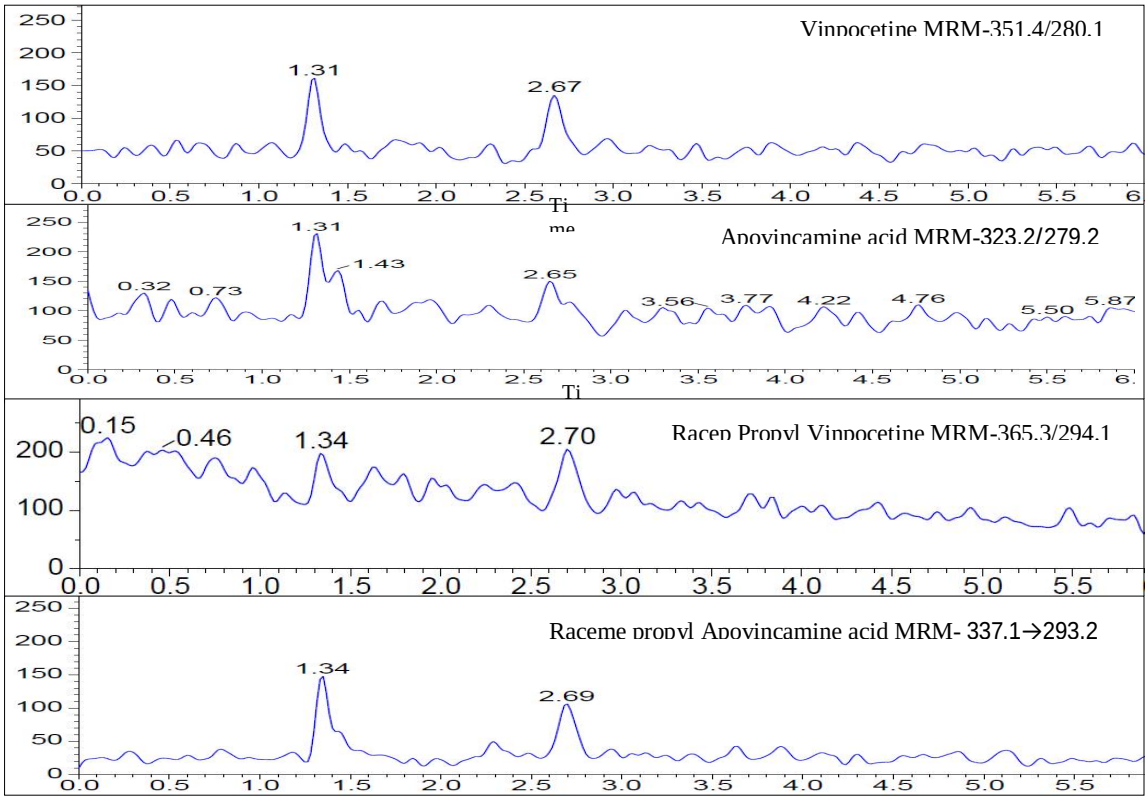


Figure 1. Blank Plasma Sample Peak for VP, AVA, VP-IS and AVA-IS.

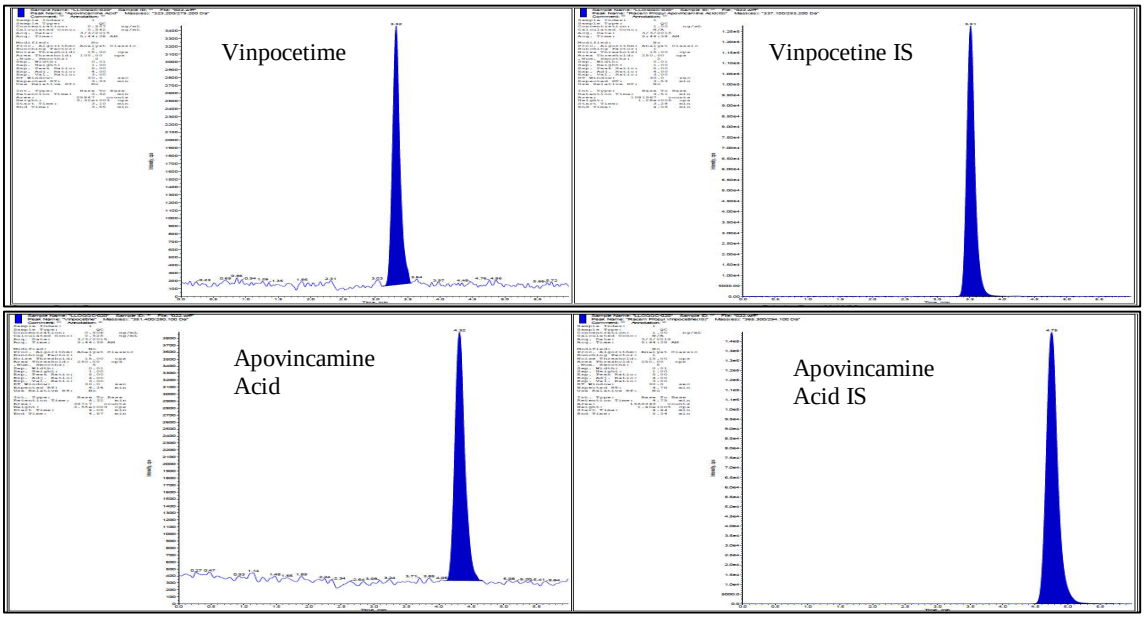


Figure 2. LLOQ Sample peak of vinpocetine and apovincamine acid with IS.

Calibration Curve: A linear equation was established to provide the best fit for the concentration vs. detector response using 1/2 as weighting factor for vinpocetine and apovincamine Acid. Nine point calibration curves were found to be linear from 0.102 ng mL^{-1} to 50.317 ng mL^{-1} for vinpocetine and 0.505 ng m^{-1} to 252.285 ng mL^{-1} for apovincamine Acid. The goodness of fit (r) was consistently

≥0.9994 for vinpocetine and ≥0.9981 for apovincamine acid during the course of validation. The representative calibration curve was presented in figure 3.

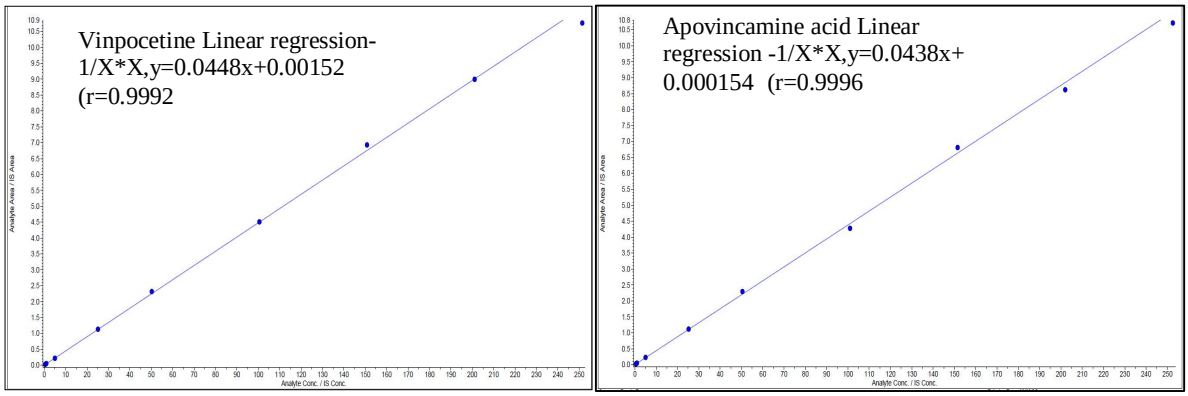


Figure 3. Calibration curve of Vinpocetine and Apovincamine Acid.

Precession and accuracy: The within batch accuracy for the LQC, M1QC, MQC, and HQC samples of Vinpocetine was 96.03 to 103.36%, which is within the acceptance criteria 85–115% of nominal concentration. The between batch accuracy was 94.84 to 105.65%, which is within the acceptance criteria 85–115% of nominal concentration. The within batch accuracy for the LQC, M1QC, MQC, and HQC samples of apovincamine acid was 97.58 to 103.66%, which is within the acceptance criteria 85–115% of nominal concentration. The between batch accuracy was 99.28 to 102.64%, which is within the acceptance criteria 85–115% of nominal concentration. Precision is measured by percent coefficient of variation over the concentration range of quality control samples during the course of validation. The within batch precision (%CV) for the LQC, M1QC, MQC, and HQC samples of Vinpocetine was 0.89 to 5.68%. The between batch precision (%CV) was 1.76 to 4.11%, which are within the acceptance criteria of ≤ 15%. The within batch precision (%CV) for the LQC, M1QC, MQC, and HQC control samples of apovincamine acid was 0.89 to 5.46%. The between batch precision (%CV) of apovincamine acid was 4.89 to 7.70%, which are within the acceptance criteria of ≤15% for precision.

Recovery and Matrix effect: The recovery of VP and AVA was evaluated by comparing peak area of plasma quality control samples with the area of respective aqueous quality control samples. Recovery for VP was 90.8% and precision (%CV) of recovery at LQC, MQC and HQC level was 3.11%, recovery of VP-IS was 88.2% and the precision (%CV) of recovery at LQC, MQC and HQC level was 2.93%, recovery of AVA was 91.8% and the precision (%CV) of recovery at LQC, MQC and HQC level was 2.36%, recovery of AVA-IS was 93.9% and the precision (%CV) of recovery at LQC, MQC and HQC level was 0.87%,This was within the acceptance criteria of ≤15% for precision. Matrix effect was evaluated using ten different lots of plasma including haemolysed and Lipemic plasma. Each lot was extracted as per extraction procedure to obtain blank sample, which was spiked with analyte and IS to achieve the concentration equivalent to LQC and HQC. Simultaneously aqueous LQC and HQC was prepared and analyzed as per method. Matrix factor was calculated by comparing the drug peak area of LQC and HQC prepared using extracted plasma blank (in presence of matrix ions) with drug peak area of aqueous LQC and HQC (in absence of matrix ions). IS normalized matrix factor was calculated by taking the ratio of matrix factor of analyte to matrix factor of IS.

The mean IS normalized matrix factor for VP was 1.0167at LQC and 0.9531 at HQC and for AVA it was 0.9920at LQQC and 0.9937at HQC. The %CV of mean IS normalized matrix factor for VP at LQC and HQC was 1.89 and 1.25% and for AVA at LQC and HQC was 2.43 and 1.65%, which ware within the acceptance criteria of ≤15%.

Stability: Stock solution stability and dilution stability were evaluated at room temperature and refrigerator (2-8°C) at working concentration. Aqueous solution stabilities were assessed by comparing with the freshly prepared stock solutions and dilutions. Matrix (human plasma) and post preparative stabilities were evaluated at LQC and HQC Concentration level by comparing with freshly spiked calibration standards. Stability results are presented in table 2.

Table 2. Stability results of vinpocetine and apovincamine acid

Stability Conditions	Duration	Sample Analyzed	% Stability	
			VP	AVA
Stock Solution Stability	23 h at room temperature	MQC	98.48	98.07
Dilution Stability	23 h at room temperature	LQC	100.93	99.71
		HQC	98.97	100.42
Stock Solution Stability	3 days at 2-8°C	MQC	100.01	100.05
Dilution Stability	3 days at 2-8°C	LQC	101.93	101.03
		HQC	99.81	99.17
Wet extract stability	For 45 h at 2–8°C	LQC	100.84	102.25
		HQC	92.60	100.41
	17 h at Room Temperature	LQC	101.86	102.96
		HQC	94.09	101.22
Auto sampler stability	59 h at 100C	LQC	102.12	104.86
		HQC	93.51	100.98
Bench top stability	14 h at Room Temperature	LQC	102.67	103.54
		HQC	93.67	100.47
Freeze thaw stability	Four FT Cycle	LQC	102.22	104.63
		HQC	94.91	101.28
Evaporation stability	50 mins at 50°C	LQC	101.40	104.30
		HQC	93.40	101.23

RESULTS AND DISCUSSION

Method Development: As the literature reveals, there is no report yet on the simultaneous determination of VP and AVA in human plasma by LC–MS/MS. Thus, in the present study method development was initiated to sensitive and specific LC–MS/MS method with a short overall analysis time for the simultaneous quantification of VP and AVA inhuman plasma. To accomplish this aim it was imperative to have a simple, efficient extraction procedure, with a short chromatographic run time. Also, the sensitivity should be adequate enough to monitor at least three to five half-lives of VP and AVA concentration with good accuracy and precision for human plasma samples. The tuning of MS parameters was carried out in positive as well as negative ionization modes for VP, AVA and IS using 100 ng m^{-1} tuning solution in 50% Methanol in water. The response observed was much higher in positive ionization mode for all four compounds compared to the negative mode. Moreover, use of ammonium formate and formic acid in the mobile phase further enhanced the response for both the analytes and the IS with low background noise, resulting in higher sensitivity. The analytes and IS gave predominant singly charged protonated precursor $[M+H]^+$ions at m/z of 351.4, 323.2, 365.3 and 337.1 for VP, AVA VP-IS and AVA- IS respectively in Q1 MS full scan spectra. The most abundant ions found in the product ion mass spectra were m/z 280.1, 279.2, 294.1and 293.2 at 40, 30, 40 and 32V collision energy for VP, AVA, VP-IS and AVA-IS respectively. It was observed that ion spray voltage had a significant impact on the response of both the analytes and the IS. At high voltage the response was enhanced and hence an optimum potential of 5500V was kept which gave consistent and stable signal. The probe capillary temperature had little effect on the signal and thus was maintained at 500◦C. Dwell time of 200ms was kept and no interference due to cross stock was observed between the MRMs of analytes and IS. To develop an accurate, valid and optimal chromatographic condition, the different HPLC parameters including mobile phase(methanol–0.1% formic acid aqueous solution, methanol–water, methanol–2 mM ammonium formate aqueous solution containing0.1% formic acid, acetonitrile–0.1% formic acid aqueous solution), category of column

(Agilent Zorbax SB-C18 column,100mm×3.0mm, 3m, Agilent Zorbax Eclipse XDB-C18 column,150mm×2.1mm, 5m, or Waters symmetry C18 column, 50mm× 2.1mm, 5m, Gemini C18 column, 100mm×2.0mm,3.0m), column temperature (30, 40, 45 or 50°C) and flow rate of mobile phase (0.2, 0.3 or 0.4 mL min^{-1}) were all examined and compared. Finally, plasma samples were separated by HPLC on a Zorbax SB-CN (250×4.6 mm) 5µm column using a solvent system consisting of methanol and 10mMammonium formate aqueous solution (containing 0.2% formic acid) at the flow rate was set at 1.00 mL min^{-1}. The system provides higher resolution, greater baseline stability and higher ionization.

Calibration Curve Precession and accuracy: The calibration curves for VP (0.1 to 50ng mL^{-1}) and AVA 0.5 to 500ng/mL) were linear from with correlation coefficient r≥0.99 across three regression curves. The within and between batch precision and accuracy were evaluated in six replicates, which was found within acceptance limit as per guideline [22-25].

Selectivity: The aim of performing selectivity check with 6 different types of plasma samples was to ensure the authenticity of the results for study sample analysis. Figure 2 and 3 demonstrate the selectivity results with the chromatograms of double blank plasma (without IS), and the peak response of VP at 0.10 ng mL^{-1} and AVA 0.50ng mL^{-1} concentration. The solid–liquid extraction method employed gave very good selectivity for the analytes and IS in the blank plasma. The chromatograms show excellent peak shape for both the analytes and the IS. No endogenous interferences were found at the retention times of VP (4.1 min), AVA (3.4min), VP-IS (4.7min) and AVA-IS (3.7min) in the blank plasma. The retention time was short for both the analytes, which made it suitable for routine analysis. Under the optimized conditions, the limit of detection (S/N≥4) and the limit of quantification (S/N≥5) of both VP and AVA observed to be 0.1ng mL^{-1} and 0.5ng mL^{-1} respectively.

Recovery and Matrix effect: The extraction recoveries for QC-high, medium, and low samples were>88.2% and precession <3.11%. There was no matrix effect was observed as the extraction cartridges used is inn exchange and washing was given to cartridges with methanol. The IS normalized matrix factors were for QC high and low levels. The inter relative matrix factor variation of the QC high and low levels both were below 15%, as required by the EMA guideline [25].

Stability: The stability experiments were performed thoroughly to evaluate their stability in stock solutions and in plasma samples under different conditions. The stability of spiked QC samples was compared with freshly prepared quality control samples. Stock solution of VP, AVA and IS were stable at room temperature for 13 h and at 2-8°C for 3 days for VP, AVA and IS with mean %stability well within 0.7–1.4%. The intermediate Dilution of VP, AVA and IS in methanol: water (50:50, v v^{-1}) was stable for 3 days. Both the analytes were found stable in controlled plasma at room temperature up to 4 h and for at least four freeze and thaw cycles. Bench top stability of extracted samples was also up to 24 h.

APPLICATION

This method was successfully validated and applied for the estimation of vinpocetine and its active metabolite apovincamine acid from 10mg dose of vinpocetine of test and reference formulation. Incurred sample reanalysis of the plasma samples was found within acceptance criteria as per guidelines [22-25].

CONCLUSION

The objective of this work was to develop a simple, cost effective, rapid bioanalytical method for simultaneous estimation of VP and its active metabolite AVA in human plasma, basically to meet there requirement for biological sample analysis. The simple solid phase extraction employed in the present work gave consistent and reproducible recoveries for both the analytes. The limit of

quantification was low enough to monitor at least four half-lives of VP and AVA concentration with good within and between batch reproducibility for the quality controls. From the results of all the validation parameters, the method also could be useful for VP pharmacokinetics study and for therapeutic drug monitoring in human with acceptable precision and accuracy. Our results show that the results of accuracy, precision, and stability fulfilled international guidelines acceptance criteria [22-25].

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