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Determination of apovincaminic acid in human plasma by high-performance liquid chromatography using solid-phase extraction and ultraviolet detection

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Abstract

A new, simple and rapid high-performance liquid chromatography (HPLC) method with UV detection has been developed for the determination of apovincaminic acid in human plasma. Apovincaminic acid and internal standard were isolated from plasma samples by solid-phase extraction with OASIS® HLB cartridges. The chromatographic separation was accomplished on a reversed-phase C_{18} column and UV detection was set at 311 nm. The calibration curves were linear in the concentration range of 2.4–240.0 ng/ml, and the limits of quantification was 2.4 ng/ml. The precision and accuracy ranged from 0.84 to 8.54% and 91.5 to 108.3%, respectively. The developed method was subsequently applied to study the pharmacokinetics of apovincaminic acid in a group of 20 human subjects at a single oral dose of 10 mg of vinpocetine tablet.
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Keywords: Apovincaminic acid; High-performance liquid chromatography (HPLC)

1. Introduction

Vinpocetine (VP) is a vincamine derivative used for the treatment of disorders arising from cerebrovascular and cerebral degenerative diseases [1]. VP is claimed to increase the cerebral flow in the ischaemia affected area of patients with cerebrovascular disease, to decrease platelet aggregability in patients with transient ischaemic attack or stroke, to increase red cell deformability in stroke patients, to have neuroprotective abilities and protective effect against brain ischaemia [2]. In humans, vinpocetine is rapidly absorbed and undergoes extensive metabolism, during which approximately 75% of the substance is hydrolysed into its main active metabolite, apovincaminic acid (AVA), leading to an absolute bioavailability of $(6.2 \pm 1.9)\%$ in man [3]. Since VP is rapidly metabolized into its active primary metabolite AVA, its concentration in the plasma was very low, C_{max} less than 5 ng/ml when VP was administered orally in a single dose of 10 mg [4]. Therefore, it is equally effective but more simple and convenient to monitor AVA alone instead of VP.

Several methods have been developed for the determination of AVA. GC methods based on prior derivatization have been used for the determination of AVA in biological samples [5,6]. However, all these methods needed a derivatization procedure and the sample preparation was very laborious. Determination of AVA by HPLC with ultraviolet detection [7–9] was more common. In the published methods, liquid–liquid extraction (LLE) with chloroform as the extraction solvent has been used for sample preparation. The disadvantages of these methods were that they involved several extraction steps yielding poor separation from the blood endogenous interferences. More importantly, all the extraction efficiency described in these articles was low (no more than 45%). Therefore, we have developed a new HPLC method suitable for the determination of AVA in human plasma employing solid-phase extraction for sample preparation, which enables simple and rapid isolation and concentrations of the AVA. This method has been successfully used for clinical VP pharmacokinetic studies in human.

2. Experimental

2.1. Reagents and chemicals

AVA and VP were obtained from Gedeon Richter Ltd. (Budapest, Hungary). Naproxen, used as internal standard (I.S.), was obtained from Shanghai Institute of Pharmaceutical

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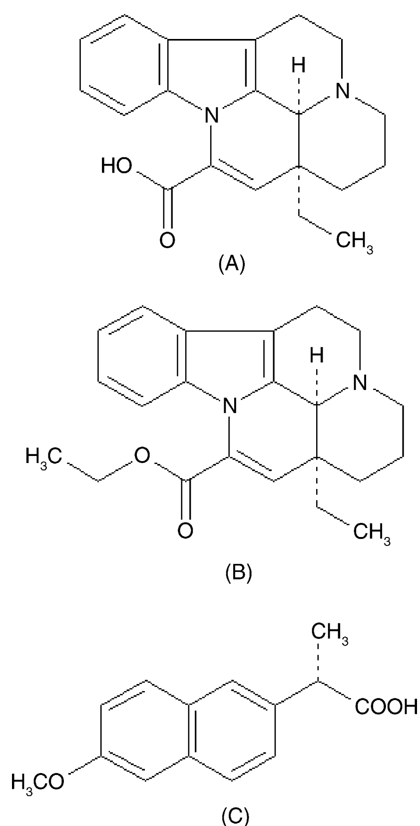

Fig. 1. Chemical structures of apovincaminic acid (A), vinpocetine (B) and naproxen (C).

Industry (Shanghai, China). Chemical structures are presented in Fig. 1. The purity of AVA, VP and naproxen were all >99.5% (as determined by HPLC). HPLC grade methanol was purchased from Tedia Company (Fairfield, OH, USA). Trifluoroacetic acid (HPLC grade reagent) was obtained from Merck Company (Germany). Triethylamine and phosphoric acid (all were of analytical reagent grade) were purchased from Shanghai Chemical Reagent Company (Shanghai, P.R. China). Double distilled water was purified by Millipore Simplicity™ (Millipore, Bedford, MA, USA). The drug-free human heparinized plasma was obtained from Shanghai Blood Center (Shanghai, P.R. China).

2.2. Preparation of standard solution

Stock solution of AVA was prepared by dissolving the appropriate amount of powder in a solution containing methanol, to yield the concentration of 0.24 mg/ml. Working solutions of AVA were prepared by appropriate dilution of the stock solution with methanol at the concentrations of 0.024, 0.24, 2.4 μg/ml. The internal standard stock solution was prepared by dissolving 10.0 mg of naproxen in 10 ml methanol, producing a concentration of 1.0 mg/ml and was stored at 4 °C. This solution was further diluted with water to prepare the internal standard working solution containing 2 μg/ml of naproxen. All these solutions were stored at 4 °C and no change in stability over a period of 1 month was observed.

2.3. Apparatus and chromatographic conditions

A Shimadzu HPLC system (Kyoto, Japan) consisting of LC-10AT VP pump, SPD-10A VP ultraviolet detector, CTO-10 AS VP column oven and SIL-10ADvp auto sampler was utilized. Empire HS-2000 software was used for data acquisition. Analyses were performed on an YMC® ODS-A reverse phase column (5 μm particle size, 150 mm × 4.6 mm i.d., YMC Co., Ltd., Kyoto, Japan). The mobile phase was composed of 0.1% (v/v) trifluoroacetic acid in water (adjusting to pH 2.3 with triethylamine) and methanol (45:55, v/v). The flow rate was set at 1.2 ml/min, and the total run time was 12 min. The column was maintained at 40 °C. Ultraviolet detection was performed at 311 nm.

2.4. Sample preparation

A 12-tube solid-phase extraction vacuum manifold (Agilent, U.S.A.) was used for sample preparation. Satisfactory values for recovery of AVA were obtained with a single extraction with OASIS® HLB solid-phase extraction cartridge (1 cc, 30 mg, Waters Corporation) for isolation of the drug and the internal standard from plasma samples. The cartridge was conditioned sequentially by rinsing with 2 ml methanol and 2 ml water. For sample preparation, 500-μl aliquot of plasma was added into a 2-ml Eppendorf microvial, then 20 μl of concentrated phosphoric acid and 500 μl of I.S. solution were added. The resulting solution was vortexed and centrifuged for exactly 5.0 min at 10,000 × *g* at 4 °C. The spiked sample (approximate volume 1.0 ml) was introduced into the cartridge under vacuum. The cartridge was washed with 1 ml of water. AVA and I.S. were then subsequently desorbed with 1 ml of methanol. The eluted solution was evaporated to dryness in a water bath at 40 °C under nitrogen steam and the residue reconstituted in 100 μl of mobile phase. The resulting solutions were carefully vortexed and centrifuged at 10,000 × *g* for 10 min at 4 °C then a volume of 50 μl was subsequently injected into the chromatographic system for HPLC analysis.

2.5. Validation of the assay method

2.5.1. Specificity

Six randomly selected control blank human plasma samples and samples from healthy subjects who participated in this clinical pharmacokinetic study of VP were processed by the this solid–liquid extraction procedure and chromatographed to determine the extent to which endogenous plasma components may contribute to the interference at retention time of analyte and internal standard.

2.5.2. Linearity

Plasma samples were spiked in five replicates at concentrations of 2.4, 4.8, 24, 48, 96, 240, 480 ng/ml. The samples were assayed using the method described above. The standard calibration curves for AVA were constructed using the analyte/I.S. peak–area ratios versus the nominal concentrations of the analytes. Linear least-squares regression analysis with weighting

factor of $1/x^2$ was performed to assess the linearity as well as to generate the standard calibration equation: $y = ax + b$, where y is the peak–area ratio, x the concentration, a the slope and b is the intercept of the regression line.

2.5.3. Recovery

Spiked plasma samples were prepared in triplicate at concentrations of 2.4, 24 and 240 ng/ml, and assayed as described above. Recovery (extraction efficacy) was calculated by comparing the peak–area of the extracted sample to that of the unextracted standard solution containing the same concentration.

2.5.4. Precision and accuracy

The precision and accuracy of this method were evaluated using quality control samples at concentrations of 2.4, 24 and 240 ng/ml. For intra-day assay precision and accuracy, five replicates of quality control samples at each concentration were assayed all at once within a day. The inter-day assay precision and accuracy was determined by analyzing the quality control samples on five different days. Five replicates at each concentration were assayed per day.

2.5.5. Sensitivity

The lower limit of quantification (LLOQ) was determined for AVA, based on the criteria that: (1) the analyte response at LLOQ is four times of baseline noise; (2) the analyte response at LLOQ can be determined with sufficient precision and accuracy, i.e. precision of 20% and accuracy of 80–120%. The limit of detection (LOD) was determined as the lowest concentration, which gives a signal-to-noise ratio of 3 for AVA.

2.5.6. Stability of analytes

2.5.6.1. Freeze–thaw stability. The stability of AVA in plasma samples subjected to multiple freeze–thaw cycles was assessed. Aliquots of plasma spiked with AVA at 2.4, 24 and 240 ng/ml, underwent three freeze–thaw cycles: frozen samples were allowed to thaw at ambient temperature for 2 h and were subsequently refrozen. The AVA levels in samples collected at each step of the freeze–thaw cycles were analyzed in the same series, to eliminate the inter-assay variability. The variations of AVA concentrations were expressed in percentage of the levels of samples not subjected to the freeze–thaw cycles.

2.5.6.2. Reconstitution solution stability. Reconstitution solution stability of AVA in the mobile phase was verified at three concentrations (2.4, 24, 240 ng/ml) during 24 h of storage at room temperature and the results were compared.

2.5.6.3. Storage stability. The stability of AVA plasma sample was assessed by spiking AVA standard solution into blank plasma from healthy volunteer at three different concentrations (2.4, 24, 240 ng/ml). Samples were kept in freezer at −20 °C for 30 days. After 30 days of storage, the samples were analyzed by HPLC as described above.

2.6. Clinical application

This method developed was used to investigate the plasma profile of AVA after single 10 mg oral dose of VP tablet. A clinical study on 20 Chinese healthy male volunteers (age from 18 to 24 years old) was conducted under fasting conditions. Informed consent was obtained from the subjects after explaining the nature and purpose of the study. The protocol was approved by the Base for Drug Clinical Trial of SDA of Shanghai Zhongshan Hospital (Shanghai, China). Following written informed consent, each volunteer received the VP tablet with 250 ml of water. No food was allowed until 4 h after dose administration. Water intake was allowed after 2 h and low-fat standard meals were provided at 4 h and 10 h post dose. Blood samples were drawn into tubes through an indwelling cannula before (0 h) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 h after oral administration. The blood samples were centrifuged at $1643 \times g$ for 15 min, plasma was separated and kept frozen at −20 °C in coded polypropylene tubs until analysis.

3. Results

3.1. Separation and specificity

Fig. 2 shows the representative chromatograms of blank plasma, plasma samples spiked with AVA at 48 ng/ml and at LLOQ (2.4 ng/ml), and plasma sample obtained from a healthy subject following an oral 10 mg dose of VP. The analytes were well separated using the present chromatographic conditions. The retention times were 7.2 min for AVA and 10.7 min for I.S. No interfering peaks from the endogenous plasma components were observed at the retention time of AVA or I.S. In addition, the retention time of VP was 30 min under this chromatographic condition, so it should take about 30 min for each analytical run.

3.2. Calibration and linearity

The method exhibited a good linear response for the range of concentrations from 2.4 to 480 ng/ml with a coefficient of determination of 0.9998. Results of five representative calibration curves for AVA determination are given in Table 1.

3.3. Recovery

For plasma concentrations at 2.4, 24, 240 ng/ml, the mean recovery of AVA were 93.2 ± 3.1, 88.6 ± 2.5 and $87.3 \pm 2.0\%$ ($n = 3$), respectively, and the mean recovery of I.S. was $95.5 \pm 2.4\%$ ($n = 9$).

3.4. Precision and accuracy

The precision and accuracy for the measurement of AVA is summarized in Table 2. The RSD of AVA ranged from 1.63 to 8.54% for intra-day and 0.84 to 3.38% for inter-day, respectively. The accuracy of AVA ranged from 100.2 to 104.7% for intra-day and 100.1 to 106.3% for inter-day, respectively.

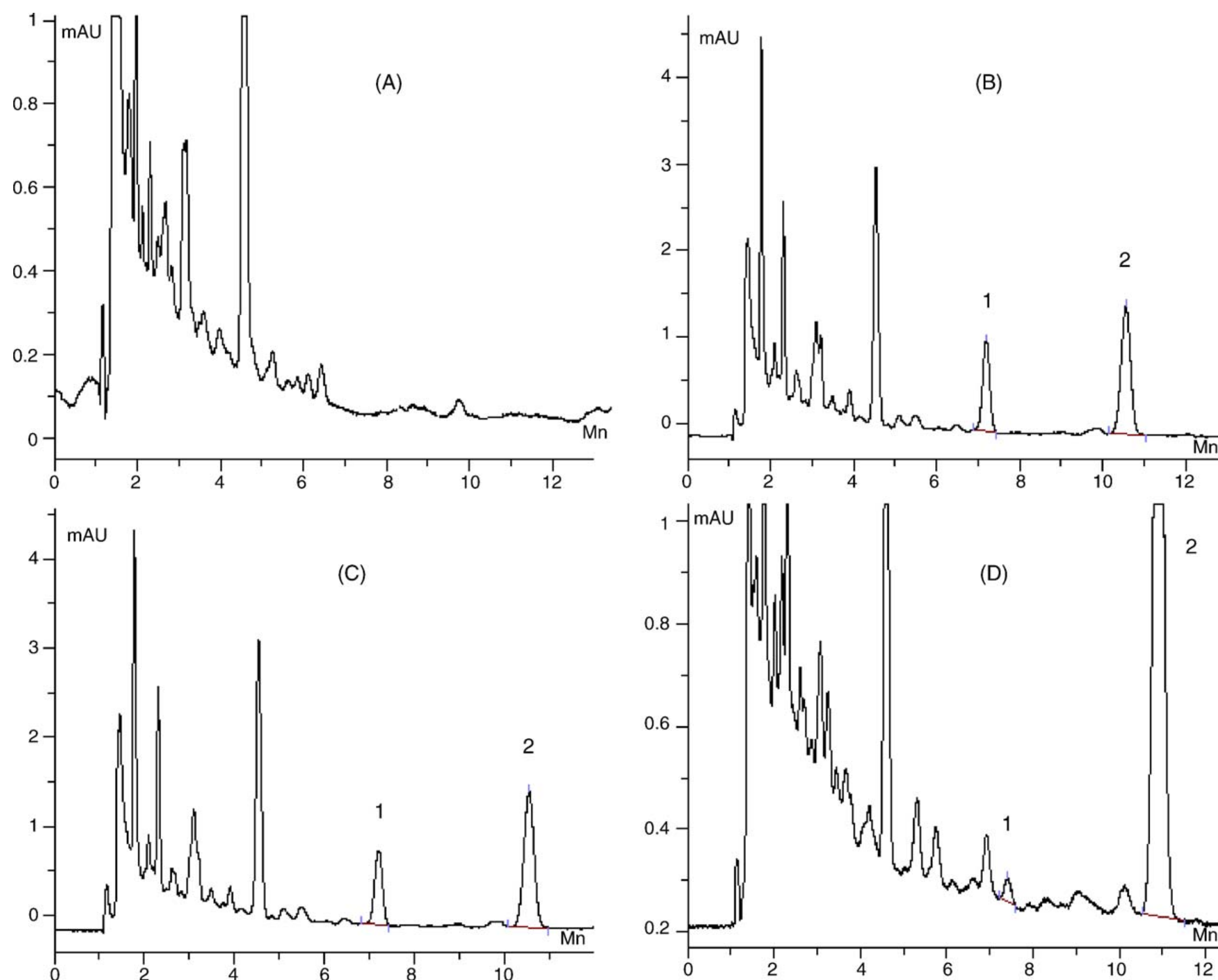

Fig. 2. Chromatograms of: (A) blank plasma; (B) plasma sample spiked with 48 ng/ml of apovincaminic acid and I.S.; (C) plasma sample from a healthy subject following a 10 mg oral dose of VP, the plasma concentration was determined to be 37.9 ng/ml for AVA; (D) spiked plasma sample at LLOQ (2.4 ng/ml). 1: Apovincaminic acid; 2: naproxen.

3.5. Sensitivity

The lower limit of quantification (LOQ) was 2.4 ng/ml for AVA. The limit of detection (LOD) was 1.2 ng/ml for AVA.

3.6. Stability

Table 3 summarizes the stability data of the freeze and thaw stability, reconstitution stability, long-term stability of AVA. The results obtained after three freeze–thaw cycles demonstrated that 95.2–101.3% of the initial content of AVA were recovered and that the analytes were stable under these conditions. AVA in reconstitution solution was found to be stable for approximately 24 h since the found concentrations of analytes were within 97.7–102.5% of the initial concentrations. The residual percentages of AVA stored in plasma at −20 °C for 30 day ranged from 94.7 to 103.3%, indicating no stability problems occurred.

Table 1
Results of five representative calibration curves for AVA determination

	Added concentration (ng/ml)						
	2.4	4.8	24	48	96	240	480
Back-calculated concentration	2.54	4.88	22.11	48.21	101.23	236.5	489.1
	2.51	4.52	21.18	46.21	95.42	238.5	471.8
	2.63	4.83	21.17	46.44	98.24	240.4	496.8
	2.74	5.05	23.11	46.25	96.20	235.4	477.5
	2.58	4.94	22.25	50.24	93.87	245.6	480.0
Mean	2.60	4.85	21.96	47.47	96.99	239.3	483.0
SD	0.09	0.20	0.82	1.76	2.84	4.0	9.9

Table 2
Intra- and inter-day precision and accuracy of AVA spiked in human plasma ($n = 5$)

Added concentration (ng/ml)	Intra-day			
	Detected concentration (ng/ml)	Mean ± SD (ng/ml)	Mean accuracy (%)	RSD (%)
2.4	2.43 2.22 2.54 2.56 2.81	2.51 ± 0.21	104.7	8.54
24	24.7 24.8 23.6 23.7 23.8	24.14 ± 0.60	100.6	2.49
240	234.0 241.7 240.6 241.6 244.6	240.5 ± 3.9	100.2	1.63
Added concentration (ng/ml)	Inter-day			
	Detected concentration (ng/ml)	Mean ± SD (ng/ml)	Mean accuracy (%)	RSD (%)
2.4	2.58 2.60 2.53 2.54 2.51	2.55 ± 0.04	106.3	1.45
24	25.79 23.73 23.88 24.36 24.14	24.38 ± 0.82	101.6	3.38
240	242.4 241.5 239.1 237.3 240.5	240.2 ± 2.0	100.1	0.84

Table 3
Stability of AVA in plasma

	2.4 ng/ml		24 ng/ml		240 ng/ml	
	Recovery (mean ± SD) (%)	RSD (%)	Recovery (mean ± SD) (%)	RSD (%)	Recovery (mean ± SD) (%)	RSD (%)
Freeze–thaw stability	95.2 ± 4.2	4.44	101.3 ± 1.2	4.13	98.3 ± 3.8	3.89
Reconstitution stability	97.7 ± 2.3	2.30	102.5 ± 2.8	2.71	99.6 ± 1.8	1.81
Storage stability	103.3 ± 8.2	7.95	94.7 ± 5.3	5.57	98.4 ± 2.4	2.47

Table 4
Liquid chromatographic methods for the analysis of AVA in human plasma

Sample pretreatment	Internal standard	Detection (nm)	LLOQ (ng/ml)	Reference
LLE (two-step ion pair extraction combined with back extraction)	Vincaminic	254	20	Kozma et al. [7]
	Yohimbine	224	10	Kraus et al. [8]
	Primidone	254	10–20	Maya et al. [9]
SPE (single step extraction)	Naproxen	311	2.5	Present method

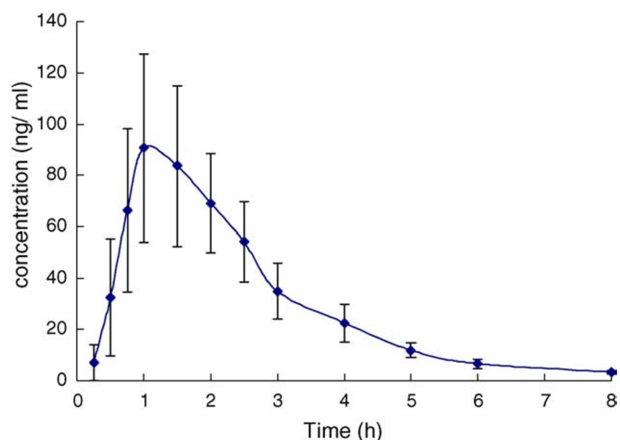

Fig. 3. Mean concentration–time profiles of AVA in 20 healthy subjects following a 10 mg oral dose of VP.

3.7. Application to clinical study

The present HPLC method of AVA was for the first time employed to determine the pharmacokinetic parameters of AVA in volunteers' plasma sample. After a single oral dose of 10 mg VP tablet in 20 healthy volunteers, concentration versus time profiles were constructed for up to 8 h for AVA determination. Fig. 3 shows the mean concentration–time curves of AVA in 20 subjects following a 10 mg oral dose of VP under fasting condition. The maximum AVA plasma concentration was 99.9 ± 29.8 ng/ml, T_{max} was 1.3 ± 0.5 h, AUC was 238 ± 51 h ng/ml and $t_{1/2}$ in the terminal elimination phase was 1.8 ± 0.5 h. The parameter values were in good agreement with those reported previously [4,10].

4. Discussion

AVA is a hydrophilic acid and very soluble in water, so it cannot be extracted with organic solvents from human plasma. All the previous papers used two-step ion pair extraction with tetrabutylammonium as the counter ion to extract AVA from plasma. In order to clean-up of the final extract, those methods described a successive back extraction procedure, which made the sample preparation more tedious and time-consuming. Although ion pair extraction was used, the extraction efficiency reported was all very low (no more than 45%). In the present study, we develop a solid–liquid extraction method. Different cartridges such as C_{18}, C_2, (which the sorbents were porous silica particles surface-bonded with C_{18} and C_2, respectively) and OASIS® (which the sorbent was poly(divinylbenzene-*N*-vinylpyrrolidone) were used for solid-phase extraction for the purpose of obtain satisfactory values for recovery of AVA. High recovery of AVA (>75%) and internal standard were obtained when solid-phase extraction was performed on OASIS® and C_2 cartridges, especially OASIS® cartridge obtained the best results, whereas the absolute recovery of AVA on C_{18} cartridges was less than 45%.

In optimizing the chromatographic conditions, the pH of the mobile phase was explored. When the pH of mobile phase >4.0, AVA, VP and I.S. were not separated from each other, but they were well separated at pH < 3.5. However, at pH ranged from 3.5 to 2.8, the retention time of AVA was found very sensitive to pH variation. Only when the pH decrease to 2.2–2.5 was the retention time of AVA not sensible to pH variation and also the peaks were sharp and symmetric. So the pH value of the mobile phase was finally adjusted to 2.3.

The detection wavelength was 311 nm in our study, which was not identical to previous study. UV absorption spectra revealed absorption maxima at 227, 264 and 311 nm for AVA. Although higher sensitivity was attained at 227 and 264 nm as compared to 311 nm (only 0.35 times that of 227 nm and 0.59 times that of 264 nm), more significant interferences from endogenous plasma substances rendered the two former detection wavelengths inappropriate in the analysis of plasma samples. Moreover, the noise at 311 nm was quite low and LLOQ could be lower by inject more volumes of sample. So the LLOQ could be obtained to a lower degree by using only 0.5 ml of plasma compared with other previous methods (Table 4).

5. Conclusion

In conclusion, we have described a novel method for the quantitative determination of AVA in human plasma, which is specific, accurate and precise, and can be easily implemented in routine practice. The sample pretreatment procedure is based on a simple and efficient solid-phase extraction, thereby eliminating the need of ion pair extraction combined with back extraction. The detection limit of this method for AVA is 2.4 ng/ml, which is enough to detect terminal phase concentrations of AVA after oral administration of 10 mg dose of VP to healthy volunteers. It proved to be superior in sensitivity and speed of analysis as compared to the analytical methods reported previously, so the method is more suitable for high-throughput quantitative analysis such as human pharmacokinetic studies.

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