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ORIGINAL ARTICLE

Simultaneous determination of amlodipine, valsartan and hydrochlorothiazide by LC–ESI-MS/MS and its application to pharmacokinetics in rats

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Abstract Polypill is a fixed-dose combination that contains three or more active ingredients used as a single daily pill to achieve a large effect in preventing cardiovascular disease with minimal adverse effects. A novel and accurate liquid chromatography tandem mass spectrometry method using electrospray ionization mode has been developed and validated for the simultaneous determination of amlodipine (AMD), valsartan (VAL) using losartan (LOS) as an internal standard (IS), and hydrochlorothiazide (HCT) using furosemide (FSD) as an IS. The separation was carried on Aquasil C_{18} (50 mm × 2.1 mm, 5 μm) reversed phase column using acetonitrile and water containing 0.1% formic acid (50:50, v/v) as the mobile phase. The method was validated in terms of linearity, accuracy and precision over the concentration range of 1–1000 ng/mL. The intra and inter-day precision and accuracy, stability and extraction recoveries of all the analytes were in the acceptable range. This method can be successfully applied to the pharmacokinetic study of AMD, VAL and HCT when given as a polypill.

1. Introduction

Control of hypertension is important for the prevention of cardiovascular risk factors. Calcium channel blockers have been widely used in the treatment of hypertension and are often prescibed in the treatment of hypertension and/or angina pectoris [1]. Combination therapy of calcium channel blockers with an angiotensin II receptor blocker and a diuretic would be expected to provide enhanced

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anti-hypertensive activity [2]. Exforge hydrochlorothiazide (HCT)™ is a triple drug polypill approved by US-FDA [3] available in market that contains amlodipine (AMD), valsartan (VAL) and hydrochlorothiazide (HCT) in a single pill [4,5]. Polypills are the fixed-dose combinations (FDC) of three or more active ingredients in a single pill with the intention of reducing the number of tablets or capsules that need to be taken to achieve a large effect in preventing cardiovascular disease with minimal adverse effects [6,7].

AMD, chemically known as 2-[(2-amino ethoxy)-methyl]-4-(2-cholophenyl)-1,4-dihydro-6-methyl-3,5-pyridine dicarboxylic acid 3-ethyl-5-methyl ester, benzosulfonate, is a dihydropyridine derivative [8–10] with calcium antagonist activity used in the management of hypertension, chronic stable angina pectoris and prinzmetal variant angina [11]. After oral administration AMD is slowly and almost completely absorbed and peak plasma concentrations are attained within 6–12 h. AMD has a relatively high oral bioavailability of 60–65%, which is not influenced by food. Moreover, AMD appears to have a linear pharmacokinetic profile, with strong positive correlations between oral dosage, C_{max} and AUC_{0-72}. In healthy volunteers, steady-state plasma concentrations are achieved after 7 once daily oral doses, without evidence of accumulation. AMD has a large volume of distribution of 21 L/kg, and is more than 95% bound to plasma proteins [12]. VAL is an effective nonpeptide angiotensin II receptor antagonist, chemically N-(1-oxopentyl)–N-[[2′-(1H-tetrazol-5-yl) [1,1′-biphenyl]-4-yl] methyl]-L-valine [13,14]. VAL displaces angiotensin II from the AT1 receptor and produces its blood pressure lowering effects by antagonizing AT1-induced vasoconstriction, aldosterone release, catecholamine release, arginine vasopressin release, water intake and hyper tropic responses, which results in blocking of the cardiovascular effects of Angiotensin II [15,16]. Peak plasma concentration was reached 2 h after oral administration and then declined with a terminal half-life of 3–7 h [17–19]. The maximum plasma concentrations after single oral dose of VAL (160 mg) reach 2–4 μg/mL. The drug is only minimally metabolized and excreted largely (about 80%) as unchanged compound [20,21]. HCT is a benzathiadiazine diuretic, chemically known as 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide, used in hypertension often prescribed in combination with other antihypertensive drugs such as beta blockers, angiotensin-converting enzyme inhibitors, or angiotensin II receptor blockers [22,23]. The bioavailability of HCT in human is 60–80%, and is independent of the dose over the range of 25–200 mg. After oral administration, peak plasma concentration is achieved at 2 h and the half-life of elimination averages 10 h [24,25].

Several chromatographic techniques have been reported for AMD [1,9,26–28], VAL [15,21,22,29,30] and HCT [22,31–33] individually and in combination with other drugs. However, so far, no single method has been reported for the simultaneous estimation of AMD, VAL and HCT in rat plasma by LC–MS/MS. Therefore, the aim of this study was to evaluate the pharmacokinetic parameters of these three drugs when used as a polypill. The developed bioanalytical method has been validated according to ICH guidelines [34] and successfully applied to pharmacokinetic study in rats. This method can also be useful in estimating the plasma samples of patients receiving Exforge HCT. The structures of analytes are presented in Fig. 1.

2. Experimental

2.1. Chemicals and apparatus

The reference standards of AMD, VAL, HCT, losartan (LOS), and furosemide (FSD) were obtained from Aurobindo Pharma Ltd. (Hyderabad, India). Their structures and purities were confirmed by HPLC, nuclear magnetic resonance and high resolution mass spectrometric methods. All the chemicals were more than 95% pure. High purity deionized water was obtained using a Direct Q ultra-pure water system from Millipore (Milford, MA, USA). HPLC grade acetonitrile and methanol were purchased from E-Merck (Mumbai, India). Analytical grade formic acid and glacial acetic acid were purchased from SD-Fine Chemicals Ltd. (Mumbai, India). 0.45 Syringe filters with nylon membrane was obtained from Pall scientific, Bangloor (KA, USA). Spinix Vortex shaker was obtained from Jaibro Scientific Works (New Delhi, India). Biofuge refrigerated centrifuge was purchased from Heraeus (Germany).

2.2. Standard solutions and fortification

Standard stock solutions of AMD, VAL, HCT were prepared by accurately weighing 10 mg of each standard on a closed electronic

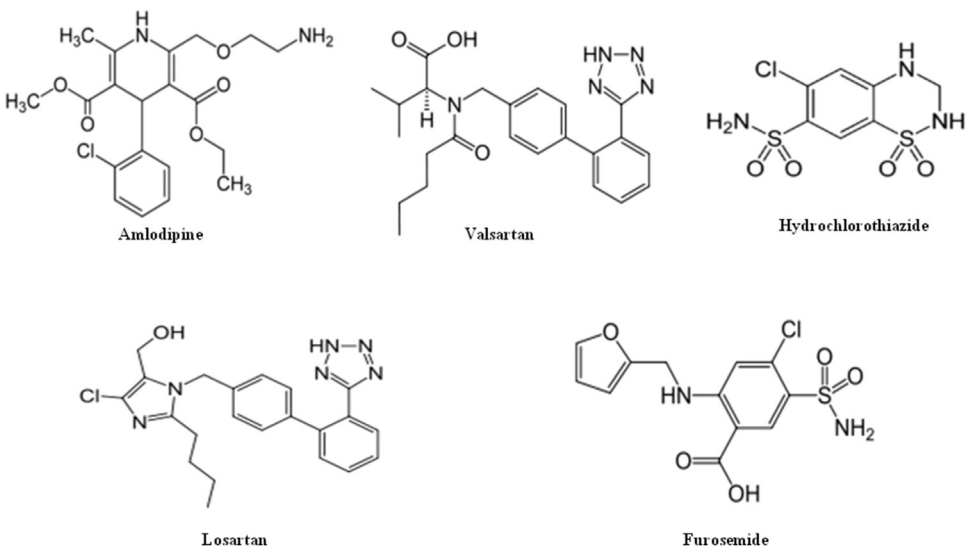

Fig. 1 Chemical structures of analytes.

microbalance (Sartorius, Berlin, Germany) and dissolving them separately in 10 mL of acetonitrile. Calibration standard and quality control (QC) samples in plasma were prepared by adding corresponding working solutions with drug-free rat plasma.

A volume of 10 μL of appropriate diluted stock solutions of mixture of drugs (AMD, VAL and HCT) at different concentrations and 10 μL of ISs (LOS and FSD) at a fixed concentration were spiked into 100 μL of drug-free rat plasma to yield final concentrations of calibration samples 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL for AMD, VAL and HCT, respectively. The final concentration of ISs (LOS and FSD) was 20 ng/mL. Similarly, QC samples were prepared at four concentration levels LLOQ (1 ng/mL), two MLOQs (5 and 50 ng/mL) and HLOQ (500 ng/mL) for AMD, VAL and HCT.

2.3. Sample preparation

Analytes were extracted from plasma by employing the protein precipitation method. 200 μL of acetonitrile was added as a protein precipitating agent, vortexed for 1 min and then centrifuged at 10,000 rpm for 10 min on Biofuge refrigerated centrifuge at 4 °C. The supernatant layer was separated and filtered through 0.45 μm syringe filters and 20 μL of the solution was injected for LC–MS/MS analysis.

2.4. Instrumentation

The LC–MS/MS analysis was carried out in electrospray ionization (ESI) positive mode for AMD and VAL using LOS as IS and in negative ion ESI mode for HCT using FSD as IS on a Thermo Finnigan LCQ Advantage Max ion trap mass spectrometer coupled to a Thermo Finnigan HPLC system containing surveyor LC quaternary pump plus, surveyor auto-sampler plus (Thermo Scientific, USA). The Xcalibur software (version 2.1) was used for data acquisition and analysis. The separation of all the analytes was carried out on an Aquasil-C_{18} (50 mm length × 2.1 mm internal diameter and 5 μm particle size) column. Temperature was set to 20 °C. The mobile phase composed acetonitrile and water (50:50, v/v) containing 0.1% formic acid for separation of AMD and VAL (LOS as IS) at a flow rate of 0.2 mL/min for 3 min and the isocratic mobile phase comprised acetonitrile and water (60:40, v/v) containing 0.1% glacial acetic acid used for HCT (FSD as IS) at a flow rate of 0.2 mL/min for 5 min. The full scan MS and MS/MS spectra of each analyte were obtained by direct infusion of the respective sample solution at a concentration of 10 μg/mL solution prepared in the mobile phase. The flow rates of sheath gas and auxiliary gas were optimized and set to 30 psi and 5 psi, respectively. The needle spray voltage was set to 4.5 kV. Helium was used as collision gas tuned for each analyte to obtain good signal intensity in MS^2 experiment. The drugs were analyzed using multiple reactions monitoring (MRM) mode. The precursor ions, product ions, and LC–MS/MS parameters are depicted in Table 1.

2.5. Method validation

The bioanalytical method was validated according to the FDA guidelines (US Food and Drug Administration, May 2001). The method was validated in terms of selectivity, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, recovery, matrix effect and stability.

The selectivity and specificity were assessed by comparing the chromatograms of six different sources of blank rat plasma with those of the corresponding spiked plasma. Each blank plasma sample was tested using the proposed extraction procedure and LC–MS/MS conditions to ensure no interference of AMD, VAL, HCT, and the ISs from blank plasma. The linearity of the assay was evaluated by constructing calibration curves with different concentrations ranging from 1 to 1000 ng/mL for AMD, VAL and HCT. The calibration curves were constructed by plotting each respective peak area ratios of AMD and VAL to LOS (IS) and HCT to FSD (IS) against the concentrations of AMD, AVL and HCT, respectively, using the weighting factor of $1/x^2$.

LODs of the drugs were determined based on signal intensity three times more than baseline noise ($S/N=3$) and LOQs of the drugs were determined based on intensity of signal which was ten times more than the noise ($S/N=10$).

QC samples were prepared in blank plasma at the concentrations of 1 (LLOQ), 5, 50 (MLOQs) and 500 (HLOQ) ng/mL for AMD, VAL and HCT in six replicates ($n=6$) for assessing the accuracy, intra- and inter-day precisions (reproducibility) of the method. All QC samples were prepared freshly on three consecutive days and analyzed in each analytical batch along with the unknown samples.

The matrix effect and recoveries of analytes were quantitatively measured by comparing the signal intensities and the peak area ratios (analyte/IS) obtained from postextraction spiking (A) (extracting 100 μL of rat plasma with 1 mL of acetonitrile. The residues, after evaporation of solvent by nitrogen purging, were reconstituted with 10 μL of standard solution containing AMD, VAL and HCT at concentrations of 5, 50 and 500 ng/mL and internal standards LOS and FSD at 20 ng/mL) and standard solutions (B) (samples prepared in mobile phase) at the same concentrations in six replicates ($n=6$). The ratio ($A/B \times 100\%$) was used to evaluate the matrix effect.

The stabilities of AMD, VAL and HCT in plasma at different storage conditions were evaluated and the results were expressed as mean percentage accuracies. The short-term stability was determined by keeping QC samples in six replicates ($n=6$) at room temperature for 24 h. The autosampler stability was evaluated by keeping the QC samples at 4 °C for 24 h in autosampler before analysis. Freeze–thaw stability of QC samples was analyzed after

Table 1 Optimized LC–MS/MS conditions for AMD, VAL, LOS, HCT and FSD.

Analyte	RT (min)	ESI mode	MRM transitions	CE (eV)
AMD	0.79	Positive	409 → 238	25
VAL	2.19	Positive	436 → 418	30
LOS	1.11	Positive	423 → 405	40
HCT	1.34	Negative	296 → 268	30
FSD	3.60	Negative	328 → 285	35

three freeze–thaw cycles by freezing at −80 °C for 24 h and thawing at room temperature for 24 h.

2.6. Animal study

Six healthy female Sprague-Dawley rats with an average weight of 200 ± 10 g were procured from National Institute of Nutrition, Tarnaka, Hyderabad (India). The animals were housed in BIOSAFE, under standard (22 ± 2 °C, 60–70% humidity) laboratory conditions, maintained on a 12-h natural day–night cycle, with free access to standard food and water. Animals were acclimatized to laboratory conditions before the study. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of IICT, Hyderabad (India) and was conducted according to the CPCSEA guidelines on the use and care of experimental animals. After overnight fasting of animals, the mixture of AMD, VAL and HCT was prepared and administered orally to the rats as gum acacia suspension at a dosage of 1, 15 and 2.5 mg/kg, respectively. A volume of 0.25 mL of blood was collected by retro-orbital puncture into EDTA coated glass tubes at time intervals of 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24 and 48 h after drug administration. Blood was centrifuged at 5000 rpm for 10 min and the plasma was separated and stored at −80 °C until analysis. Pharmacokinetic parameters were estimated using non-compartmental analysis with RAMKIN GW-BASIC (Ver. 3.22) software.

3. Results and discussion

3.1. Mass spectrometric and chromatographic conditions

To optimize peak shape with appropriate retention time various combinations of mobile phases were investigated. Separation of these drugs was attempted using various combinations of acetonitrile and water with different percentage of modifiers. The best separation was achieved with acetonitrile and water in the ratio of 50:50 (v/v) containing 0.1% formic acid for estimating AMD, VAL and LOS. The optimized mobile phase for HCT and FSD was acetonitrile and water in the ratio of 60:40 (v/v) containing 0.1% glacial acetic acid. The reversed-phase Thermo Aquasil C_{18} column (50 mm × 2.1 mm, 5 μm) protected by Phenomenex security guard column C_{18} (4 mm × 2 mm i.d.) (Phenomenex, India) was used with a flow rate of 0.2 mL/min in both the methods. The retention times of AMD, VAL and LOS were found to be 0.79, 2.19 and 1.34 min, respectively. The retention times of HCT and FSD were 1.11 and 3.60 min, respectively. The total chromatographic run time was 3.0 min in estimating AMD, VAL and LOS whereas 5.0 min for HCT and FSD.

The standard solutions of 10 μg/mL with respect to AMD, VAL and LOS in 50% acetonitrile containing 0.1% formic acid were infused directly into the mass spectrometer ESI source in positive ion mode and HCT and FSD in negative ion mode. The observed full scan mass spectra in positive mode showed prominent protonated molecular ions $[M+H]^+$ of *m/z* 408, 435, and 422 for AMD, VAL and LOS, respectively, and prominent deprotonated molecular ions $[M-H]^-$ of *m/z* 297 and 330 for HCT and FSD, respectively, in negative ion mode. The $[M+H]^+$ ions and $[M-H]^-$ ions of respective analytes were subjected to collision- induced dissociation (CID) at average collision energy of 30%. The collision energies were optimized for each analyte to obtain the most intense fragment ions. The molecules underwent fragmentation to yield the following fragment ions of *m/z* 238, 418, 405, 268, and 285. The MS/MS spectra of analytes are presented in Fig. 2. Based

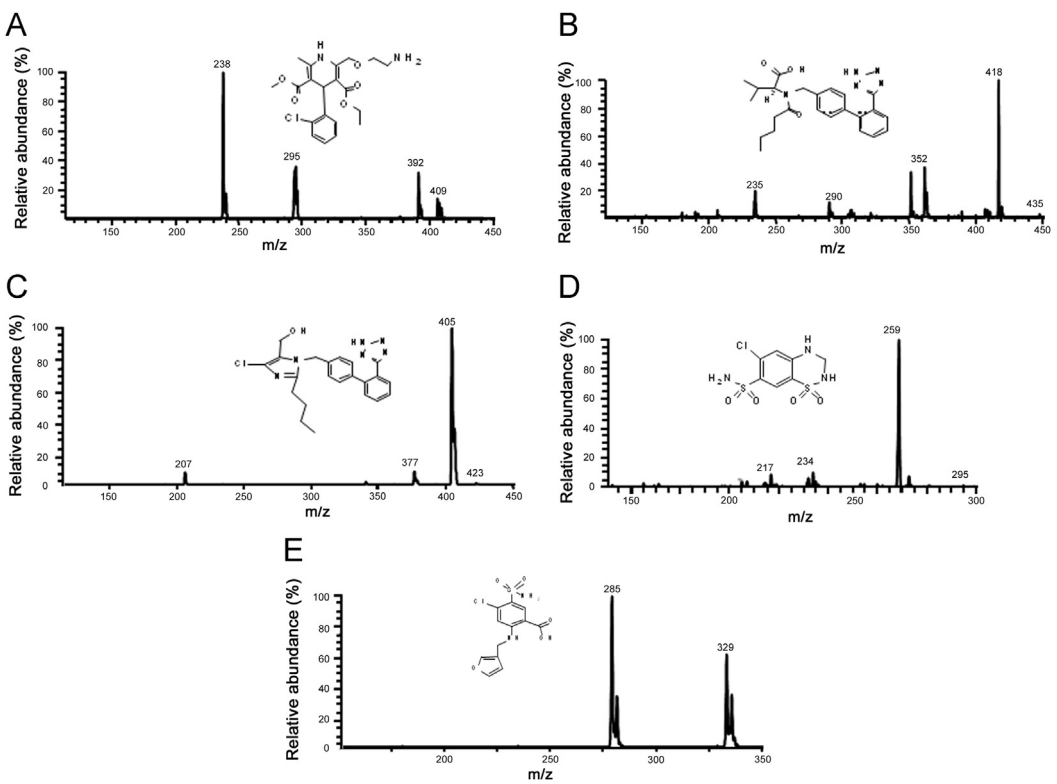

Fig. 2 LC–ESI-MS/MS spectra of (A) amlodipine, (B) valsartan, (C) losartan, (D) hydrochlorothiazide and (E) furosemide.

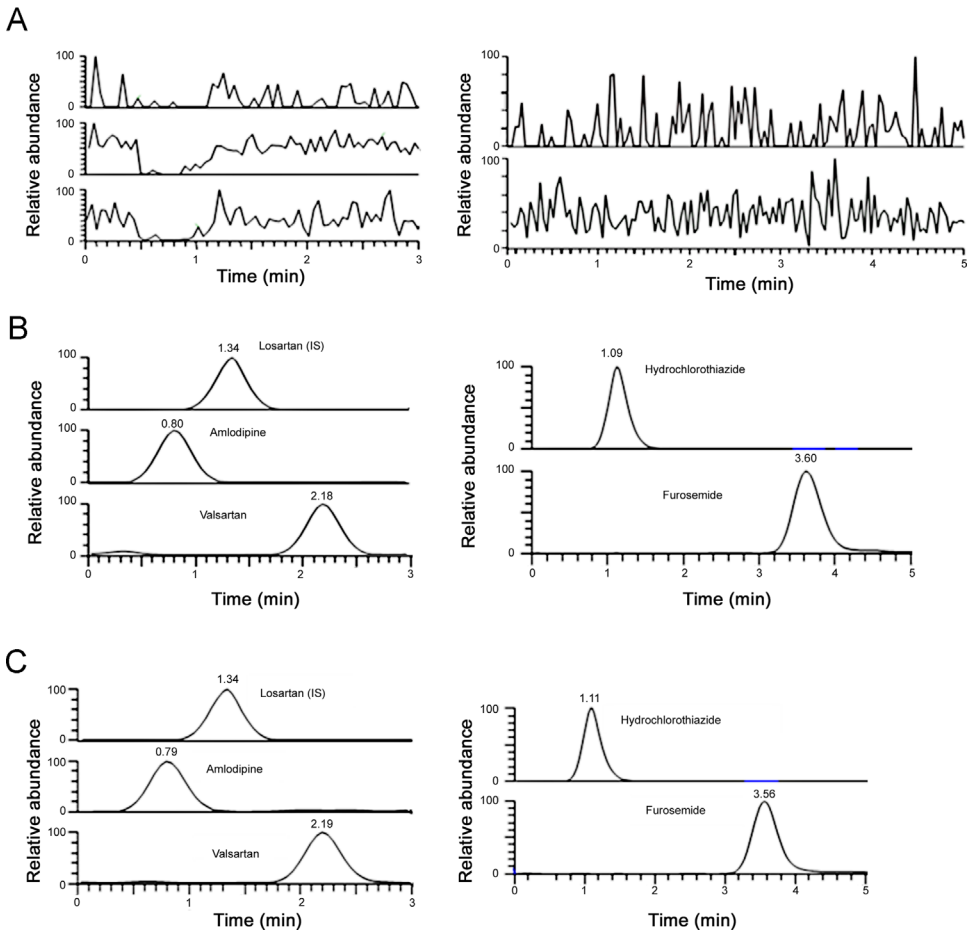

Fig. 3 LC–ESI-MRM chromatograms of (A) blank plasma, (B) spiked plasma at 1 ng/mL, and (C) plasma extracted from rats after 1 h of oral administration.

Table 2 Intra-day and inter-day variation for AMD, VAL and HCT in six replicates ($n=6$) at each concentration.

Analyte	Concentration (ng/mL)	Intra-day		Inter-day	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
AMD	1	93.4	8.3	86.2	11.4
	5	87.9	5.8	92.4	7.2
	50	98.2	9.3	105.7	9.3
	500	106.3	5.8	110.0	8.3
VAL	1	89.3	12.4	85.7	13.5
	5	91.8	6.2	93.6	11.4
	50	103.4	9.4	107.0	9.2
	500	95.3	6.7	89.3	6.2
HCT	1	88.7	12.2	86.4	14.3
	5	89.9	9.4	88.2	9.1
	50	92.9	8.3	90.2	3.9
	500	95.3	8.4	96.8	5.3

on their mass spectra and tandem mass spectra, the following MRM transitions: m/z 409→238, m/z 436→418, m/z 296→268, m/z 423→405, m/z 329→285, were selected for analysis of AMD, VAL, LOS, HCT and FSD, respectively. The structures of these drugs and their putative fragments were also confirmed through high resolution mass spectrometry (HRMS)

by measuring their accurate masses. The HRMS analysis was carried out on an Exactive bench top Orbitrap mass spectrometer (Thermo Scientific, USA).

Table 3 Recovery values of AMD, VAL and HCT ($n=6$).

Analyte	Concentration (ng/mL)	Mean recovery ± SD (%)	RSD (%)
AMD	5	86.3 ± 7.5	6.1
	50	89.2 ± 5.7	9.5
	500	92.5 ± 6.2	4.7
VAL	5	86.4 ± 8.2	5.2
	50	88.7 ± 4.2	8.4
	500	90.1 ± 9.1	5.2
HCT	5	89.3 ± 10.5	9.2
	50	90.2 ± 6.4	8.4
	500	86.7 ± 5.2	8.4

3.2. *Method validation*

The specificity of this method was confirmed by comparing chromatograms of blank plasma, spiked plasma with analytes at a concentration of 1 ng/mL and plasma sample obtained after 1 h of oral administration shown in Fig. 3. AMD, VAL and LOS (IS) in positive ESI experiment and HCT and FSD (IS) in negative ESI experiment were well separated under the described chromatographic conditions. No interfering endogenous peaks were observed around their retention times.

The calibration curves of the analytes showed a good linearity over the studied concentration range of 1–1000 ng/mL for AMD, VAL and HCT with correlation coefficients (r^2) 0.9993, 0.9997 and 0.9997, respectively. The LODs for all studied analytes were found to be 0.5 ng/mL. The LLOQs for all analytes were 1 ng/mL with acceptable precision and accuracy, which was sufficient to perform pharmacokinetic studies of AMD, VAL and HCT in rats.

The intra- and inter-day precisions for AMD, VAL and HCT were less than 14.3%. The obtained intra-day accuracies were in the range of 87.9–106.3% and inter-day accuracies were in the range of 85.7–110%. The validation parameters are depicted in Table 2.

Table 4 Stability studies of AMD, VAL and HCT in rat plasma at three QC levels ($n=6$).

Storage conditions	AMD		VAL		HCT	
	Mean accuracy (%)	RSD (%)	Mean accuracy (%)	RSD (%)	Mean accuracy (%)	RSD (%)
Short term stability (24 h, room temperature)	92.5	9.7	104.6	6.1	89.4	11.5
Freeze/thaw stability (3 cycles)	95.4	7.4	91.5	9.8	87.3	7.3
Pre-preparative stability at 4 °C for 12 h (autosampler)	94.3	8.6	106.2	4.7	91.6	8.4

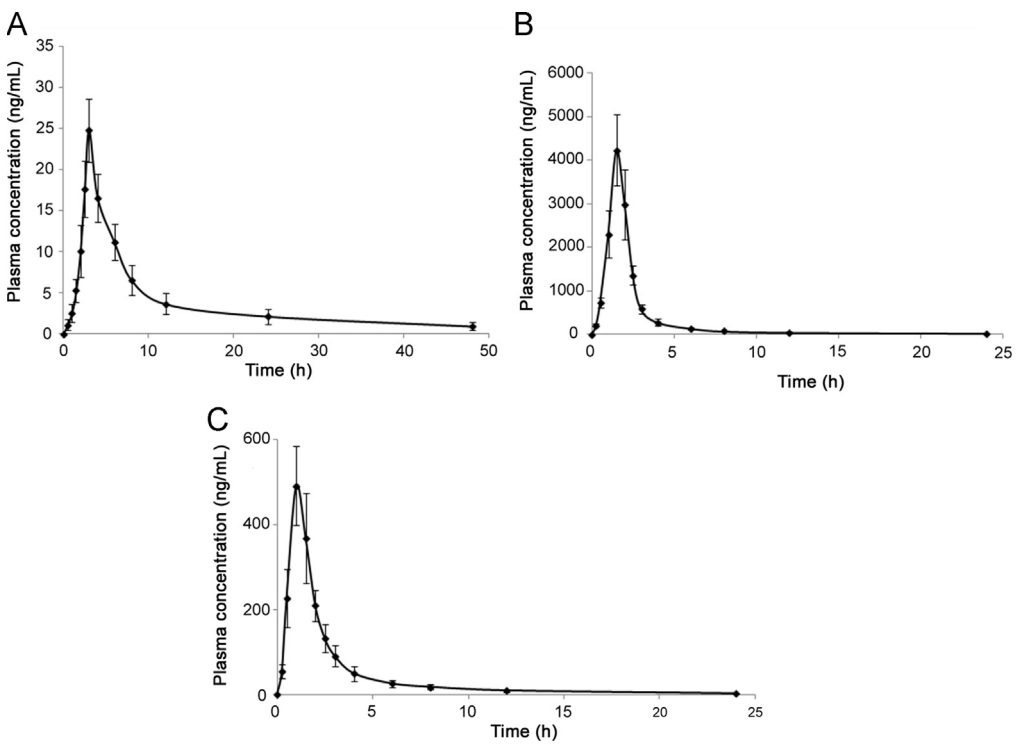

Fig. 4 Mean plasma concentration–time profile of (A) amlodipine, (B) valsartan and (C) hydrochlorothiazide in rats. Each point represents the mean ± SD ($n=6$).

Table 5 Pharmacokinetic parameters of AMD, VAL, and HCT in rats ($n=6$).

Parameter	AMD	VAL	HCT
C_{max} (ng/mL)	24.76±3.85	4390.91±678.54	516.13±74.18
T_{max} (h)	3.20±0.45	1.60±0.22	1.10±0.22
AUC_{0-t} (ng mL/h)	181.51±44.60	7517.63±847.70	1037.61±175.24
$AUC_{0-\infty}$(ng mL/h)	200.31±56.47	7633.50±824.10	1053.14±182.42
$t_{1/2}$ (h)	13.03±3.20	4.11±0.28	3.90±0.47

C_{max}, the maximum plasma concentration; T_{max}, the time to reach C_{max}; $t_{1/2}$, elimination half-life; $AUC_{0\rightarrow t}$, the area under the plasma concentration–time curve from time zero to the last sampling time; $AUC_{0\rightarrow\infty}$, the area under the plasma concentration–time curve from time zero to infinity.

The extraction recoveries of all drugs from rat plasma were in the range of 86.3–92.5% with relative standard deviations less than 10%, which indicates the sample preparation technique is suitable for extracting the studied drugs from rat plasma. The recovery results are displayed in Table 3.

The stability studies of these drugs were performed at three QC concentration (low, medium and high) levels in six replicates ($n=6$). The predicted concentrations for each analyte deviated within ±13% of nominal concentrations after storage of plasma samples at room temperature for 24 h, three freeze–thaw cycles and in autosampler for 12 h at 4 °C. The mean accuracies were found to be more than 87% with relative standard deviations less than 12%, which are summarized in Table 4.

3.3. *Application to pharmacokinetic study*

The established method was successfully applied to analysis of plasma samples after an oral administration of 1, 15 and 2.5 mg/kg of AMD, VAL and HCT simultaneously. The mean plasma concentration–time profile of AMD, VAL and HCT is shown in Fig. 4. A non-compartmental model was used to estimate the pharmacokinetic parameters of AMD, VAL and HCT in rat plasma, the results are shown in Table 5. After oral administration of the three drugs, peak plasma concentrations (C_{max}) were reached at the time to reach maximum concentration (T_{max}) of 3.20±0.45 h, 1.60±0.22 h and 1.10±0.22 h with an elimination half-life ($t_{1/2}$) of 13.03±3.20 h, 4.11±0.28 h and 3.90±0.47 h for AMD, VAL and HCT, respectively. Thus the developed method was successfully applied for pharmacokinetic study in rats after oral administration of AMD, VAL and HCT in combination.

4. Conclusion

We have developed and validated a highly sensitive, specific, reproducible and high throughput LC–MS/MS assay to quantify AMD, VAL and HCT simultaneously in rat plasma. Simple and single step protein precipitation was used to extract analytes from rat plasma. The major advantages of the assay are simple sample preparation and short run time. The obtained LODs and LOQs of all the drugs were adequate to perform the pharmacokinetic study in rat plasma. Based on the results, we can conclude that the present method is not only suitable for assessing the pharmacokinetics of Exforge HCT™ polypill in preclinical but also applicable for clinical pharmacokinetics.

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