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Application of an LC–MS/MS method for the analysis of amlodipine, valsartan and hydrochlorothiazide in polypill for a bioequivalence study

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ABSTRACT

A sensitive and selective method has been proposed for the simultaneous determination of amlodipine (AML), valsartan (VAL) and hydrochlorothiazide (HCTZ) in human plasma by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The analytes and their deuterated analogs were quantitatively extracted from 100 µL human plasma by solid phase extraction on Oasis HLB cartridges. The chromatographic separation of the analytes was achieved on a Chromolith RP_{18e} (100 mm × 4.6 mm) analytical column within 2.5 min. The resolution factor between AML and VAL, AML and HCTZ, and VAL and HCTZ was 2.9, 1.5 and 1.4, respectively, under isocratic conditions. The method was validated over a dynamic concentration range of 0.02–20.0 ng/mL for AML, 5.00–10,000 ng/mL for VAL and 0.20–200 ng/mL for HCTZ. Ion-suppression/enhancement effects were investigated by post-column infusion technique. The mean IS-normalized matrix factors for AML, VAL and HCTZ were 0.992, 0.994 and 0.998, respectively. The intra-batch and inter-batch precision (% CV) across quality control levels was ≤ 5.56% and the recovery was in the range of 93.4%–99.6% for all the analytes. The method was successfully applied to a bioequivalence study of 5 mg AML + 160 mg VAL + 12.5 mg HCTZ tablet formulation (test and reference) in 18 healthy Indian males under fasting. The mean log-transformed ratios of C_{max}, AUC_{0-120h} and AUC_{0-inf} and their 90% CIs were within 90.2%–102.1%. The assay reproducibility was demonstrated by reanalysis of 90 incurred samples.

1. Introduction

Hypertension is considered as a major risk factor for an array of cardiovascular and related diseases, which is primarily responsible for the cause of death worldwide [1]. The biggest challenge in the treatment of hypertension is to effectively control the blood pressure (BP), using potent drug regimen with high efficacy and tolerability. Monotherapy is a rational therapeutic approach in patients with mild BP elevation and low-to-moderate cardiovascular risk. For uncontrolled BP levels, increase in dose strength in monotherapy is a rational alternative; however, several antihypertensive drugs including calcium antagonists have a dose-dependent tolerability profile. Thus, by increasing the dose strength there is an increased risk of adverse events which reduce patient compliance with therapy. In such a scenario, low-dose combination therapy with drugs having different and complementary mechanisms of action can substantially improve tolerability and at the same time minimize individual side effects [2].

Commonly available two-drug combinations have a renin secretion agent (beta blocker, angiotensin converting enzyme inhibitor, angiotensin II receptor blocker) and another component which is effective in renin-independent hypertension like diuretic, dihydropyridine or non-dihydropyridine calcium channel blocker. Based on these considerations, addition of a diuretic to a combination of an antagonist of the renin-angiotensin system and a calcium channel blocker can enhance the effect of other antihypertensive agents as well as benefit individuals with salt-sensitivity of BP [3]. One such fixed-dose combination therapy that has provided enhanced BP control is that of amlodipine (AML), a calcium channel blocker, valsartan (VAL), an angiotensin II receptor blocker and hydrochlorothiazide (HCTZ), a diuretic. This combination is available in the market as Exforge HCT®, containing 5/10 mg AML, 160/320 mg VAL and 12.5/25 mg HCTZ [4].

AML is a third-generation, long-acting dihydropyridine calcium channel antagonist, prescribed for the treatment of angina pectoris, hypertension, cardiac arrhythmias and coronary heart failure. It

Table 1

Comparison of methods developed for simultaneous determination of amlodipine, valsartan and hydrochlorothiazide.

Sr. No.	Detection technique; linear range (ng/mL)	Human plasma (μL); extraction technique; internal standard	Column; mobile phase	Retention time (AML/VAL/HCTZ); run time (min)	Application	Ref.
1	HPLC–UV (227 nm); 4000–28,000 for AML, 5000–40,000 for VAL and 1000–12,000 for HCTZ	1000; PP with acetonitrile; –	Kinetex C_{18} (150 mm × 4.6 mm); acetonitrile and phosphate buffer, pH 2.8 (40:60, v/v)	3.169/11.193/2.265; 20	Analysis of Exforge HCT®, containing 10 mg AML, 160 mg VAL and 12.5 mg HCTZ and in spiked plasma samples	[42]
2	HPLC–UV (237 nm); 100–18,500 for AML, 300–15,500 for VAL and 300–22,000 for HCTZ	1000; LLE with *n*-hexane-ethyl acetate-isoamyl alcohol (88:10:2, v/v/v); –	ACE CN (200 mm × 4.6 mm, 5 μm); acetonitrile-methanol-10 mM phosphoric acid, pH 2.5 (7:13:80, v/v/v)	9.02/10.02/4.00; 15	Analysis of Exforge HCT®, containing 10 mg AML, 320 mg VAL and 25 mg HCTZ and in spiked plasma samples	[43]
3	HPLC–PDA (254 nm); 6–200 for AML, 50–4000 for VAL and 5–400 for HCTZ	500; PP with methanol/acetonitrile (50:50, v/v) mixture; telmisartan	Gemini C_{18} (250 mm × 4.6 mm, 5 μm); acetonitrile and 10 mM ammonium formate, pH 3.5 under gradient conditions	11.61/17.64/5.92; 20	Determination of AML, VAL and HCTZ in spiked plasma samples	[44]
4	LC–MS/MS; 1.0–1000 for AML, VAL and HCTZ	100 (rat plasma); PP with acetonitrile; losartan and furosemide	Aquasil C_{18} (50 mm × 2.1 mm, 5 μm); acetonitrile and 0.1% formic acid in water (50:50, v/v)	0.79/2.19/1.11; 3.0	Pharmacokinetic study in rats with 1.0, 15 and 2.5 mg/kg of AML, VAL and HCTZ, respectively	[45]
5	LC–MS/MS; 0.02–20 ng/mL for AML, 5.00–10,000 ng/mL for VAL and 0.20–200 ng/mL for HCTZ	100; SPE on Oasis HLB cartridges; AML-d4, VAL-d9 and HCTZ-13C,d2	Chromolith RP_{18e} (100 mm × 4.6 mm); acetonitrile and 2 mM ammonium formate, pH 4.0 (90:10, v/v)	1.80/1.08/1.43; 2.5	Bioequivalence study after oral administration of Exforge HCT®, containing 5 mg AML, 160 mg VAL and 12.5 mg HCTZ to 18 healthy subjects	PM

AML: amlodipine; VAL: valsartan; HCTZ: hydrochlorothiazide; UV: ultraviolet. PDA: photodiode array; PP: protein precipitation; LLE: liquid-liquid extraction. SPE: solid phase extraction; PM: present method.

inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. By acting as a peripheral arterial vasodilator, it reduces the peripheral vascular resistance, thereby lowering the blood pressure and relieving angina pain [3]. AML is extensively metabolized in the liver to inactive metabolites, with 10% of the parent drug and 60% of the metabolites excreted in the urine [4]. It is highly protein bound (98%), with a bioavailability of 60%–65%. AML has a long elimination half life in humans, ranging from 35 to 45 h due to the large volume of distribution (21 L/kg). Like most other dihydropyridine calcium channel blockers, it is used as a racemic mixture [5]. VAL is a potent, nonpeptide, highly selective and orally active antihypertensive drug belonging to the family of angiotensin II type I receptor antagonists. It has been used clinically for the treatment of hypertension and heart failure [6]. VAL is rapidly absorbed after oral administration, reaching peak plasma concentration within 2–4 h and has a terminal half life in the range of 3–7 h. It is extensively protein bound (~95%) and has an absolute average bioavailability of 23% for a capsule formulation and 39% for a buffered solution [7]. The thiazide diuretic HCTZ is responsible for reabsorption of electrolytes in the tubules of the kidney, facilitating an increase in the secretion of sodium and chloride and thereby leads to reduction in plasma volume. The antihypertensive activity of HCTZ begins within 2 h after oral administration and peaks at about 4 h [4].

Several methods are reported for the quantitation of AML alone [8–14], and in presence of other antihypertensive agents like metoprolol [15], nicardipine [16], telmisartan [17], bisoprolol [18], losartan [19] and benazepril [20] in plasma samples. Similarly, numerous bioanalytical methods are presented for the determination of VAL alone [21–23], in presence of drugs prescribed in cardiovascular therapy [24,25], along with other angiotensin II receptor antagonists [26–28]. Few other methods describe simultaneous determination of VAL and HCTZ [29,30] and VAL and AML in human plasma [31–33]. Likewise, several methods are described for the estimation of HCTZ as a single analyte [34,35] and together with other antihypertensive drugs [36–41] in biological fluids.

To the best of our knowledge, there are only four methods which describe their simultaneous analysis in pharmaceutical preparations [42,43] and human plasma [42–45]. The salient features of these methods are summarized in Table 1. Only one method presents the application of the method for pharmacokinetics in rats [45]. Moreover, there are no reports on the pharmacokinetics of polypill containing these drugs. Thus, the objective of this work was to develop and fully validate a selective and highly sensitive method for the simultaneous estimation of AML, VAL and HCTZ in human plasma by LC–ESI–MS/MS. The method presents an efficient extraction procedure based on solid-phase extraction with quantitative recovery for all the analytes. The sensitivity and overall analysis time are encouraging compared to previous reports [42–45]. The method is selective in quantifying AML, VAL and HCTZ in the presence of commonly prescribed cardiovascular drugs like losartan, telmisartan, candesartan, irbesartan and nifedipine. Systematic evaluation of matrix effect was done by calculating IS-normalized matrix factors and also by post-column infusion technique. The proposed method was successfully applied to a bioequivalence study of 5 mg (AML) + 160 mg (VAL) + 12.5 mg (HCTZ) fixed-dose combination (FDC) tablet formulation in 18 healthy Indian males under fasting.

2. Experimental

2.1. Chemicals and materials

Amlodipine besylate (AML, 99.50%) and amlodipine-d4 besylate (AML-d4, 99.10%) were purchased from SynFine Research Inc. (Ontario, Canada), valsartan (VAL, 99.80%) and valsartan-d9 (VAL-d9, 99.30%) were obtained from TLC Pharmachem Inc. (Ontario, Canada) and hydrochlorothiazide (HCTZ, 99.88%) and hydrochlorothiazide-13C,d2 (HCTZ-13C,d2, 99.29%) were procured from Clearsynth Labs Ltd. (Mumbai, India). HPLC grade methanol, acetonitrile, ammonium formate, formic acid and analytical reagent grade ammonia solution (30%) were obtained from Merck Specialties Pvt.

Ltd. (Mumbai, India). Oasis HLB (1 mL, 30 mg) extraction cartridges were from Waters Corporation (Milford, MA, USA). Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at −70 °C.

2.2. Liquid chromatographic and mass spectrometric conditions

A Shimadzu LC-VP HPLC system (Kyoto, Japan) consisting of LC-10ADVP pump, SIL-HTc autosampler, CTO 10 ASvp column oven and a DGU-14A degasser was used for setting the reverse-phase liquid chromatographic conditions. The separation of AML, VAL and HCTZ was achieved on a Chromolith RP$_{18e}$ (100 mm × 4.6 mm) analytical column from Merck KGaA (Darmstadt, Germany) and maintained at 35 °C in a column oven. For isocratic separation, the mobile phase consisted of acetonitrile and 2 mM ammonium formate, pH 4.0 adjusted with formic acid (90:10, v/v). Ionization and detection of AML, VAL, HCTZ and ISs was carried out on a triple quadrupole mass spectrometer, MDS SCIEX API-4000 (Toronto, Canada), equipped with turbo ion spray interface and operated in positive ionization mode for AML and VAL and negative mode for HCTZ. The set chromatographic conditions and mass parameters are described in Supplementary material.

2.3. Calibrators and quality control samples

Calibration standards (CSs) were made at 0.02, 0.04, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, 10.0 and 20.0 ng/mL for AML, 5.00, 10.00, 50.00, 100.0, 200.0, 500.0, 1000, 2000, 5000 and 10,000 ng/mL for VAL and 0.20, 0.40, 1.00, 2.50, 5.00, 10.0, 25.0, 50.0, 100 and 200 ng/mL for HCTZ. Quality control (QC) samples were prepared at five levels, 16.0/8000/160 ng/mL (HQC, high quality control), 8.00/3500/80.0 ng/mL (MQC-2, medium quality control), 1.50/750.0/15.0 ng/mL (MQC-1, medium quality control), 0.40/150.0/4.00 ng/mL (LQC-2, low quality control), 0.06/15.00/0.60 ng/mL (LQC-1, low quality control) and 0.02/5.00/0.20 ng/mL (LLOQ QC, lower limit of quantification quality control) for AML/VAL/HCTZ, respectively. The details of solution preparation for analytes, ISs and their storage conditions are described in Supplementary material.

2.4. Extraction procedure

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 100 μL of spiked plasma sample, 25 μL of internal standard solution containing 1.0 μg/mL of each was added and vortexed for 10 s. Furthermore, 400 μL of 1.0% formic acid was added and vortexed for another 10 s. The samples were loaded on Waters Oasis HLB (1 mL, 30 mg) extraction cartridges which were preconditioned with 1.0 mL of methanol followed by 1.0 mL of water. The cartridges were washed with 1.0 mL of MilliQ water followed by 1.0 mL of 10% methanol. Drying of cartridges was done for 2 min by applying 20 psi pressure. Elution of analytes and ISs from the cartridges was carried out with 1 mL of methanol and the eluate was evaporated to dryness in a thermostatically controlled water-bath maintained at 45 °C under a gentle stream of nitrogen gas. The dried samples were reconstituted in 100 μL of mobile phase solution and 5 μL was used for injection in the chromatographic system.

2.5. Validation procedures

Validation of the method was done for system suitability, selectivity, carryover, linearity, accuracy and precision, recovery, matrix effect, stability, dilution integrity and ruggedness as per the USFDA guidance [46] and was similar to that described in our previous work [47]. The

detailed procedures are summarized in Supplementary material.

2.6. Bioequivalence study and incurred sample reanalysis

The validated method was successfully applied to quantify AML, VAL and HCTZ concentration in human plasma samples after oral administration of 5/160/12.5 mg respectively in FDC formulation. The design of the study comprised of "an open label, balanced, randomized, two-treatment, two-period, two-sequence, single dose, crossover oral bioequivalence study for a FDC of 5/160/12.5 mg AML/VAL/HCTZ test (Indian Pharmaceutical Company) and a reference (Exforge HCT® tablets distributed by Novartis Pharmaceuticals Corporation, New Jersey, USA) formulation in 18 healthy adult Indian males under fasting. The study was conducted as per the International Conference on Harmonization, E6 Good Clinical Practice guidelines [48]. Incurred sample reanalysis (ISR) was performed as discussed in our previous report [49]. The details for both the experiments are provided in Supplementary material.

3. Results and discussion

3.1. Method development

The present study was intended to develop a highly sensitive, selective and a high throughput method to determine AML, VAL and HCTZ in a single run for a clinical study involving healthy subjects. Due to significant difference in the ionization constants values of AML (pKa, 8.7) [50], VAL (pKa, 3.9 and 4.7) and HCTZ (pKa,7.9 and 9.2) [51], calibration range and their dose strength used in combination therapy, it was imperative to suitably optimize the extraction procedure, mass detection and the liquid chromatographic conditions for their simultaneous determination. During method development, electrospray ionization of the analytes and their deuterated analogs was carried out in the positive ionization mode for AML and VAL [32], while negative mode was selected for HCTZ [39]. AML which has a primary amino group and a pyridine nitrogen atom was readily protonated, while VAL having secondary nitrogen atoms as well as a carboxylic acid group exhibited favorable sensitivity in the positive ionization mode under the established experimental conditions. On the other hand, HCTZ which possesses a secondary amine and a sulphonamide group showed greater sensitivity in the negative mode. The Q1 MS full scan spectra for AML, VAL, AML-d4 and VAL-d9 predominantly contained protonated precursor $[M+H]^+$ ions at m/z 409.4, 436.3, 413.3 and 445.2 respectively, while deprotonated precursor $[M-H]^-$ ions at m/z 296.1 and 299.0 were observed for HCTZ and HCTZ-13C,d2, respectively. The most abundant and consistent product ion in Q3 MS spectra for AML was observed at m/z 238.2 due to the formation of a very stable aromatic 1-azabutadiene derivative. In the case of VAL, a stable, intense and reproducible product ion was observed at m/z 291.2, which corresponded to the valine containing substructure. For HCTZ, the product ion at m/z 204.9 was formed due to elimination of HCN and SO$_2$ from the deprotonated molecular ion. The product ion mass spectra for all the analytes and their ISs are shown in Fig. S1. The source dependent and compound dependent parameters were suitably optimized to obtain a consistent and adequate response for all the analytes. A dwell time of 200 ms was sufficient and no cross talk was observed between the multiple reaction monitoring (MRM) of AML and AML-d4 having identical product ions.

As evident from the methods listed in Table 1, only protein precipitation (PP) [42,44,45] and liquid-liquid extraction (LLE) [43] have been used to extract these drugs simultaneously from plasma samples. Thus, during initial trials these approaches were tested for selective extraction of the analytes using different solvent systems. PP was studied using methanol and acetonitrile as precipitants in 2:1 and 3:1 (v/v) with respect to the sample; however, the samples were not clear in either of the solvents with poor recovery for AML (18%) and

considerable ion suppression. LLE was then initiated with diethyl ether, dichloromethane, methyl *tert*-butyl ether (MTBE) and ethyl acetate under acidic (1.0%–5.0%, formic/acetic acid), basic (10–50 mM NaOH) and neutral conditions. Compared to other solvents, better results (66.5%–82.3%) were obtained with MTBE in presence of 5% formic acid for AML and VAL. However, the recovery was relatively poor for HCTZ (~ 22%) and inconsistent at all QC levels. Previous studies have reported use of Oasis hydrophilic-lipophilic balance (HLB) [10] and a monolithic weak cation exchange (WCX) column [16] for AML. Similarly, Oasis HLB [30] and Oasis mixed mode anion exchange (MAX) [28] have been employed for the extraction of VAL. Also, SPE on Oasis HLB has been used previously for quantitative recovery of HCTZ [39]. Thus, SPE was tried on Oasis HLB and also on MCX and MAX cartridges, which provide dual mode of retention (reversed-phase and ion-exchange) to have a reproducible and quantitative recovery with minimum matrix interference. Using the recommended Waters protocol for extraction of acidic and basic compounds, it was possible to separate AML (70%), HCTZ (66%) and VAL (37%) under acidic conditions of 5% formic acid on MCX cartridge. However, in presence of 5% NH_4OH the recovery was less for AML (51%) and HCTZ (56%) compared to VAL (73%) on MCX cartridge. Extraction on MAX cartridge resulted in much improved recovery for VAL (~ 85%), but was poor for AML (21%) and HCTZ (37%).

Thus, optimization of the sample clean-up procedure was done with HLB (1 mL, 30 mg) extraction cartridge to achieve satisfactory recovery for all the analytes, as it has hydrophilic and lipophilic sites. In addition to the difference in the ionization constants of the analytes, AML (log*P* ~ 2.6) and VAL (log*P* ~ 1.5) are relatively more lipophilic than HCTZ (log*P* −0.07) [51,52]. These dissimilarities were effectively controlled on HLB instead of MCX or MAX extraction cartridges. The extraction was tried under acidic as well as neutral conditions on HLB cartridges. Precise and quantitative recoveries with minimum matrix interference were obtained in both the cases. However, due to comparatively higher recoveries in acidic (400 µL, 1.0% formic acid) media, the latter conditions were finalized in the present work.

Optimization of chromatographic conditions was equally critical for their adequate retention and simultaneous determination in a single run. This was thoroughly investigated by considering the column type, organic modifier, mobile phase composition (aqueous and organic part), buffer pH and strength, acidic and basic additives and flow rate. Based on this approach, the chromatographic separation was initiated by changing these parameters to achieve a short run time, symmetric peak shapes, minimum matrix interference and solvent consumption. Previous work on the simultaneous determination of AML, VAL and HCTZ have used different reversed-phase columns like Kinetex C_{18} [42], ACE CN [43], Gemini C_{18} [44] and Aquasil C_{18} [45].

In all these methods, the analysis time was appreciably long (≥ 15 min) except for the work of Gadepalli et al. [45], in which it was 3.0 min. As a wide variety of columns with different dimensions (50–250 mm) have been reported for their simultaneous analysis, the chromatographic separation was tried initially on different columns like Inertsil ODS-3C_{18} (50 mm × 4.6 mm, 3 µm), Phenomenex Gemini C_{18} (100/150 mm × 4.6 mm, 5 µm), Waters X-Terra MS C_{18} (100/150 mm × 4.6 mm, 5 µm), ACE C_{18} (150 mm × 4.6 mm, 5 µm), Grace Kromasil C_{18} (150 mm × 4.6 mm, 3.5 µm), Thermo Hypurity C_{18} (100 mm × 4.6 mm, 5 µm) and Zorbax Eclipse XDB C_{18} (150 mm × 4.6 mm, 5 µm). To find the best eluting solvent system, various combinations of methanol/acetonitrile along with buffers (ammonium formate/formic acid, ammonium acetate/acetic acid) having different ionic strengths (1.0–10 mM) in the pH range of 3.0–6.5 and volume ratios (70:30, 75:25, 80:20, 85:15, 90:10 and 95:05, v/v) were tested. Furthermore, the effect of flow rate was also studied from 0.5 to 1.1 mL/min, which was also responsible for acceptable chromatographic separation. In general, the use of methanol in the mobile phase resulted in broad peaks with considerable tailing and caused saturation in the response for higher calibration standards (CS-7 and

CS-8) and at HQC level for VAL on majority of the columns except for X-Terra MS C_{18} and Zorbax Eclipse XDB-C_{18}. Substituting methanol with acetonitrile gave much better chromatography; however, on some of these columns the run time was either too long (5–10 min) or too short (0.6–1.2 min) with very little retention for AML or VAL. Use of ammonium formate/acetate buffer having higher strengths (5.0–10 mM) did not help in obtaining linear response for calibration standards, especially for VAL. Similarly, flow rates above 0.8 mL gave inadequate resolution, while lower flow rate resulted in longer run time. Based on the outcome of these trials, further experiments were done on a highly porous monolith reversed phase column Chromolith RP_{18e} (100 mm × 4.6 mm) which is made from a single piece of high-purity polymeric silica gel. It has a unique combination of macropores and mesopores, which affords rapid flow of the mobile phase with minimum back pressure, at the same time, creates a uniform surface area for adsorption process, and thereby enhances the column performance. Two previous reports have successfully demonstrated the excellent performance of Chromolith columns for the simultaneous determination of AML with nicardipine [16] and VAL in combination with losartan and telmisartan [27]. After suitable optimization of chromatographic parameters on Chromolith RP_{18e}, it was possible to get the desired sensitivity, good linearity for the calibration curves, superior retention, resolution, run time and acceptable peak shapes. A mobile phase consisting of acetonitrile and 2 mM ammonium formate, pH 4.0 adjusted with formic acid (90:10, v/v), at a flow rate of 1.2 mL/min ensured baseline separation of AML, VAL and HCTZ at 1.80, 1.08 and 1.43 min respectively in a total run time of 2.5 min. The capacity factor (*k*) which shows the relative rates of migration of analytes on the column was 1.53, 0.52 and 1.01 for AML, VAL and HCTZ, respectively. The resolution factor between AML and VAL, AML and HCTZ, and VAL and HCTZ was 2.9, 1.5 and 1.4, respectively under isocratic conditions. The reproducibility of retention time for the analytes, expressed as % CV was ≤ 1.5% for 100 injections on the same column. The use of deuterated internal standards helped in maintaining the overall accuracy and precision of the method.

Fig. 1 shows representative chromatograms of the blank plasma, analytes at LLOQ level, real subject sample at C_{max} and their respective ISs. None of the antidepressant medications studied interfered in the determination of the analytes. Under the optimized experimental conditions, the retention time for losartan, telmisartan, candesartan, irbesartan and nifedipine was observed at 1.16, 1.31, 1.23, 1.28 and 1.54 min, respectively. However, due to their different MRM transitions, there was no interference in the quantification of the analytes. Results of post-column infusion experiment in Fig. S2 indicate no ion suppression or enhancement at the retention time of analytes and ISs. The IS-normalized matrix factor values ranged from 0.975 to 1.008, 0.965 to 1.014, and 0.979 to 1.018 for AML, VAL and HCTZ, respectively.

3.2. Method validation results

Carry-over evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. There was negligible carry over (≤0.12%) observed during auto-sampler carryover experiment. No enhancement in the response was observed in extracted blank plasma (without ISs and analytes) after sub-sequent injection of highest calibration standard at the retention time of both analytes and IS.

All five calibration curves were linear over the concentration range of 0.02–20.0 ng/mL for AML, 5.00–10,000 ng/mL for VAL and 0.20–200 ng/mL for HCTZ. The calibration lines were drawn using least square regression analysis to give the mean linear equation $y = (1.0083 ± 0.0179)x − (0.0314 ± 0.0031)$, $y = (0.9738 ± 0.0480)x − (17.6019 ± 13.1280)$ and $y = (0.9760 ± 0.0123)x − (0.3104 ± 0.1875)$ for AML, VAL and HCTZ, respectively, where *y* is the peak area ratio of the analyte/IS and *x* the concentration of the analyte. The correlation
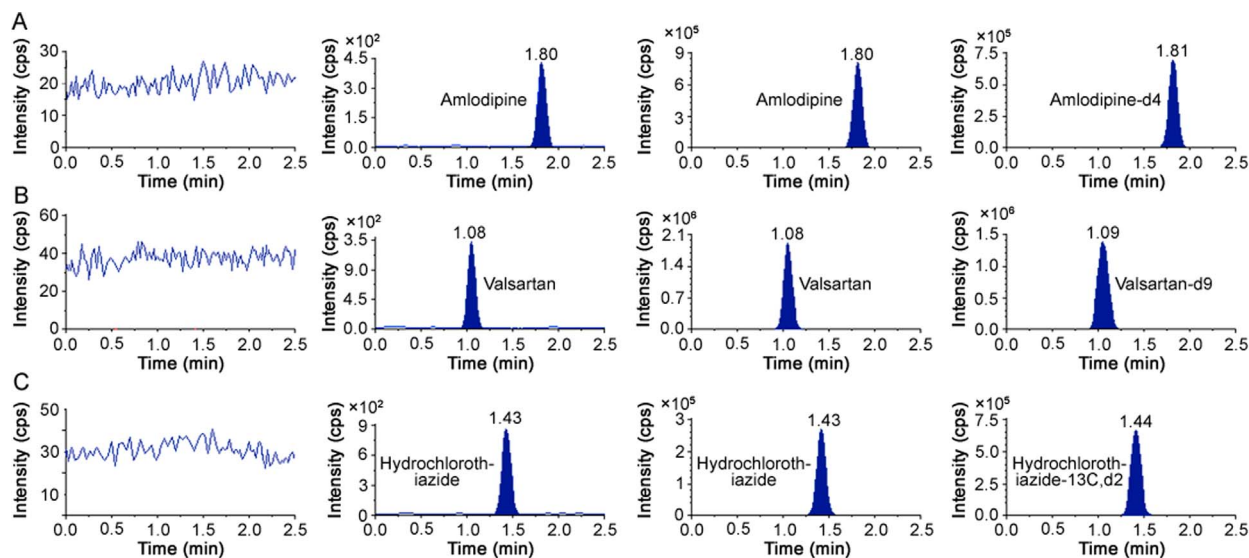
Fig. 1. Representative MRM chromatograms of double blank plasma (without analyte and IS), analyte at LLOQ concentration, analyte at C_{max} concentration and blank plasma with working solution of labeled ISs for (A) amlodipine, (B) valsartan and (C) hydrochlorothiazide, respectively.

Table 2
Extraction recovery and matrix effect of amlodipine, valsartan and hydrochlorothiazide from human plasma ($n=6$).

QC level	Area response			Extraction recovery (B/A × 100)		Matrix factor		
	A (post extraction spiking)	B (pre-extraction spiking)	C (neat samples in mobile phase)	Analyte (B/A)	IS	Analyte (B/C)	IS	IS-normalized
Amlodipine								
LQC-1	9775	9944	10,126	98.3	98.8	0.982	1.007	0.975
LQC-2	65,422	65,883	65,490	99.3	99.6	1.006	1.011	0.995
MQC-1	246,391	253,750	253,243	97.1	97.8	1.002	1.009	0.993
MQC-2	1,314,621	1,329,243	1,330,573	98.9	99.3	0.999	0.991	1.008
HQC	2,637,984	2,722,378	2,744,332	96.9	97.5	0.992	1.003	0.989
Valsartan								
LQC-1	13,866	14,324	14,752	96.8	97.6	0.971	1.006	0.965
LQC-2	137,211	146,907	147,349	93.4	94.3	0.997	0.986	1.011
MQC-1	691,022	703,688	715,130	98.2	99.0	0.984	0.995	0.989
MQC-2	3,215,484	3,363,477	3,376,985	95.6	96.8	0.996	1.004	0.992
HQC	7,365,884	7,547,012	7,554,567	97.6	98.3	0.999	0.985	1.014
Hydrochlorothiazide								
LQC-1	11,524	11,699	11,688	98.5	98.9	1.001	1.010	0.991
LQC-2	76,854	77,552	78,813	99.1	99.5	0.984	1.005	0.979
MQC-1	287,662	290,861	287,413	98.9	99.4	1.012	1.007	1.005
MQC-2	1,532,778	1,567,258	1,554,819	97.8	98.3	1.008	0.990	1.018
HQC	3,061,121	3,073,415	3,058,124	99.6	99.8	1.005	1.006	0.999

LQC: low quality control; MQC: medium quality control; HQC: high quality control; IS: internal standard.

coefficient (r^2) observed was ≥ 0.9992, while the accuracy and precision (% CV) for the calibration curve standards ranged from 96.8% to 102.7% and 0.33%–6.36% respectively for all the analytes. The lowest concentration (LLOQ) in the standard curve that can be measured with acceptable accuracy and precision found was 0.02, 5.00 and 0.20 ng/mL for AML, VAL and HCTZ, respectively in plasma at a signal-to-noise ratio (S/N) of ≥ 18.

The intra-batch and inter-batch precision and accuracy were established from validation runs performed at HQC, MQC-1/2, LQC-1/2 and LLOQ QC levels (Table S1). The intra-batch precision (% CV) ranged from 1.30% to 5.56% and the accuracy was within 96.9%–102.3% for all three analytes. Similarly, for the inter-batch experiments, the precision varied from 0.57% to 3.75% and the accuracy was within 96.7%–102.4%.

The relative recovery and IS-normalized matrix factors for AML, VAL, and HCTZ at LQC-1/2, MQC-1/2 and HQC levels are presented in Table 2. The relative recovery of the analytes is the 'true recovery', which is unaffected by the matrix as it is calculated by comparing the area ratio response (analyte/IS) of extracted (spiked before extraction) and unextracted (spiked after extraction) samples. Furthermore, the relative matrix effect, which compares the precision (% CV) values of the slope of the calibration curves prepared in different lots (sources) of plasma samples, was 3.08, 1.55 and 2.67 for AML, VAL and HCTZ, respectively as shown in Table S2.

The stability of analytes and ISs in human plasma and stock solutions was examined under different storage conditions. Samples for short-term stability remained unchanged up to 74 h, while the long-term stability of the stock solutions for analytes and ISs were stable for minimum of 132 days at 5 °C. AML, VAL and HCTZ in control human plasma (bench top) were stable for at least 28 h at 25 °C and for minimum of six freeze and thaw cycles at −20 °C and −70 °C. Spiked plasma samples stored at −20 °C and −70 °C, for long-term stability experiment were found stable for a minimum period of 132 days. Autosampler stability (wet extract) of the spiked quality control samples maintained at 5 °C was determined up to 52 h without significant loss of the analytes. The percentage change for different

Table 3
Stability of amlodipine, valsartan and hydrochlorothiazide in plasma under various conditions (*n*=6).

Storage conditions	QC Level	Nominal conc. (ng/mL)			Mean stability samples (ng/mL, ± SD)			Change (%)		
		AML	VAL	HCTZ	AML	VAL	HCTZ	AML	VAL	HCTZ
Bench-top stability at 25 °C, 30 h	HQC	16.0	8000	160.0	16.2 ± 0.54	7968 ± 94.7	159.7 ± 1.68	1.13	−0.40	−0.18
	LQC-2	0.400	150.0	4.00	0.402 ± 0.008	146.0 ± 6.53	4.08 ± 0.15	0.50	−2.65	1.90
	LQC-1	0.060	15.00	0.600	0.059 ± 0.002	15.09 ± 0.33	0.601 ± 0.018	−1.37	0.60	0.13
Freeze and thaw stability at −20 °C	HQC	16.0	8000	160.0	15.9 ± 0.38	7903 ± 83.4	156.5 ± 3.37	−0.86	−1.21	−2.18
	LQC-2	0.400	150.0	4.00	0.384 ± 0.021	148.6 ± 4.25	3.83 ± 0.21	−3.95	−0.91	−4.38
	LQC-1	0.060	15.00	0.600	0.061 ± 0.001	14.66 ± 0.17	0.611 ± 0.012	2.17	−2.30	1.87
Freeze and thaw stability at −70 °C	HQC	16.0	8000	160.0	15.7 ± 0.23	7883 ± 88.7	157.3 ± 1.80	−1.63	−1.46	−1.66
	LQC-2	0.400	150.0	4.00	0.386 ± 0.009	142.0 ± 5.73	3.89 ± 0.08	−3.50	−5.36	−2.85
	LQC-1	0.060	15.00	0.600	0.061 ± 0.002	14.50 ± 0.33	0.613 ± 0.001	1.67	−3.32	2.17
Wet extract stability at 2–8 °C, 56 h	HQC	16.0	8000	160.0	15.7 ± 0.47	8091 ± 135.9	163.0 ± 5.15	−2.19	1.14	1.86
	LQC-2	0.400	150.0	4.00	0.408 ± 0.017	152.8 ± 4.17	4.09 ± 0.20	2.05	1.89	2.32
	LQC-1	0.060	15.00	0.600	0.058 ± 0.003	14.80 ± 0.41	0.583 ± 0.018	−2.67	−1.34	−2.80
Long-term stability at −20 °C, 132 days	HQC	16.0	8000	160.0	16.5 ± 0.34	8105 ± 90.4	162.4 ± 1.50	3.00	1.31	1.50
	LQC-2	0.400	150.0	4.00	0.409 ± 0.006	154.9 ± 2.27	4.16 ± 0.12	2.15	3.25	3.95
	LQC-1	0.060	15.00	0.600	0.057 ± 0.002	15.42 ± 0.22	0.579 ± 0.012	−5.00	2.81	−3.46
Long-term stability at −70 °C, 132 days	HQC	16.0	8000	160.0	16.2 ± 0.35	7914 ± 298.3	164.6 ± 4.38	1.44	−1.07	2.89
	LQC-2	0.400	150.0	4.00	0.422 ± 0.007	151.7 ± 1.27	3.85 ± 0.15	5.50	1.16	−3.72
	LQC-1	0.060	15.00	0.600	0.059 ± 0.001	15.14 ± 0.16	0.605 ± 0.009	−1.90	0.95	0.87

AML: amlodipine; VAL: valsartan; HCTZ: hydrochlorothiazide; SD: standard deviation.
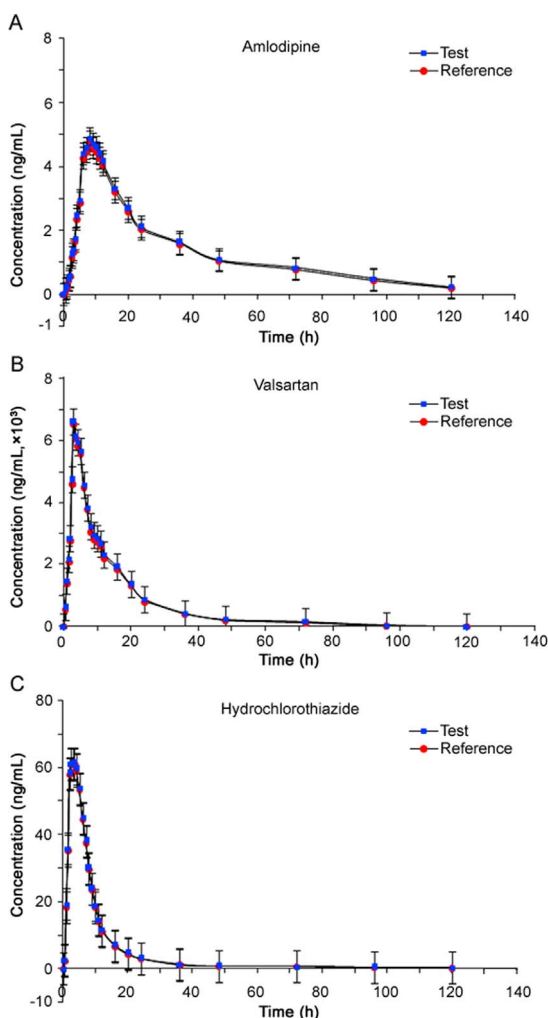

Fig. 2. Mean plasma concentration-time profiles of (A) amlodipine, (B) valsartan and (C) hydrochlorothiazide after oral administration of 5 mg amlodipine besylate, 160 mg valsartan and 12.5 mg hydrochlorothiazide fixed dose tablet formulation to 18 healthy Indian males.

stability experiments in plasma at two QC levels varied from −5.36% to 5.50% for the analytes as shown in Table 3.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis. However, none of the subject samples measured showed concentration above the ULOQ. The precision (CV) for dilution integrity of 1/5th and 1/10th was between 1.42% and 2.69%, while the accuracy results were within 97.1% and 102.8% respectively for all the analytes, which are within the acceptance limit of 15% for precision (% CV) and 85%–115% for accuracy.

Method ruggedness was evaluated using re-injection of analyzed samples on two different batches of the same stationary phase and also with different analysts. The precision (% CV) and accuracy values for two different columns ranged from 1.0% to 2.5% and 98.5% to 103.5% respectively at all five quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 1.7%–3.8% and 96.8%–101.5% respectively at these levels.

3.3. Application of the method and ISR results

The developed method was applied to enumerate the plasma concentration of AML, VAL and HCTZ for a bioequivalence study with a single fixed dose of a test and reference tablet formulation containing 5/160/12.5 mg AML/VAL/HCTZ in 18 healthy Indian male volunteers. The method was sufficiently sensitive to measure their concentration up to 120 h. Fig. 2 shows the plasma concentration-time profile of AML, VAL and HCTZ in human subjects under fasting condition after oral administration of FDC tablets. The mean pharmacokinetic parameters of the two formulations are summarized in Table 4. For a similar study in 30 healthy subjects with identical dose strength, the C_{max}, AUC_{0-inf} and T_{max} values were 2.7 ng/mL, 143.4 h·ng/mL and 6 h for AML, 3525 ng/mL, 26,327 h·ng/mL and 3 h for AML and 76.3 ng/mL, 517.6 h·ng/mL and 2 h for HCTZ, respectively [4]. Compared to the results obtained in the present work these values were somewhat lower for AML and VAL, while they were comparable for HCTZ. This can be attributed to difference in race, ethnicity and food, which may account for the observed differences in the pharmacokinetics of AML and VAL. The ratios of mean log-transformed parameters and their 90% confidence intervals for C_{max}, AUC_{0-120h} and AUC_{0-inf} were within the acceptance criterion of 80%–125%, proving that the test and reference formulations were pharmacokinetically equivalent in terms

Table 4
Mean pharmacokinetic (± SD) parameters following oral administration of 5 mg amlodipine + 160 mg valsartan + 12.5 hydrochlorothiazide fixed dose formulation in 18 healthy Indian subjects under fasting.

Parameter		C_{max} (ng/mL)	T_{max} (h)	$t_{1/2}$ (h)	AUC_{0-120h} (h·ng/mL)	AUC_{0-inf} (h·ng/mL)	K_{el} (h^{-1})
Amlodipine	T	4.90 ± 0.45	7.98 ± 0.52	32.46 ± 3.94	146.50 ± 22.54	161.15 ± 18.11	0.022 ± 0.003
	R	4.81 ± 0.34	8.01 ± 0.74	34.59 ± 3.48	153.87 ± 28.33	169.26 ± 15.95	0.020 ± 0.002
Valsartan	T	6492.2 ± 548.2	2.86 ± 0.81	7.52 ± 1.14	30,229 ± 4018	36,418 ± 5125	0.086 ± 0.021
	R	6582.5 ± 667.3	2.95 ± 0.69	7.18 ± 1.27	31,437 ± 3891	38,847 ± 6271	0.092 ± 0.018
Hydrochlorothiazide	T	63.48 ± 11.56	2.56 ± 0.15	9.58 ± 0.82	498.38 ± 57.44	528.33 ± 66.31	0.072 ± 0.012
	R	61.39 ± 19.66	2.73 ± 0.36	9.82 ± 0.63	475.91 ± 71.88	503.43 ± 58.31	0.071 ± 0.018

T: test; R: reference; 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_{0-120h}: area under the plasma concentration-time curve from zero hour to 120 h; AUC_{0-inf}: area under the plasma concentration-time curve from zero hour to infinity; K_{el}: elimination rate constant; SD: standard deviation.

of rate and extent of drug absorption.

ISR was also conducted by random selection of 90 subject samples. The selection criteria included samples which were near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug [48]. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the value was not more than ± 15% (Fig. S3).

4. Conclusions

The proposed validated method for the simultaneous estimation of AML, VAL and HCTZ in human plasma is highly sensitive compared to all published reports. The method offers significant advantages over those previously reported, in terms of lower sample requirements (100 μL), simplicity of extraction procedure and overall analysis time. The efficiency of SPE and a chromatographic run time of 2.5 min per sample make it an attractive procedure in high-throughput bioanalysis of AML, VAL and HCTZ. The limit of quantification is low enough to monitor at least five half-lives of AML, VAL and HCTZ concentration with good intra- and inter-assay reproducibility (% CV) for the quality controls. The linear dynamic range established was adequate to measure the plasma concentration of AML, VAL and HCTZ in a clinical study involving Indian subjects. The proposed method is rugged and selective in presence of five cardiovascular drugs used in combination therapy. The reproducibility of study data was effectively demonstrated through ISR.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpha.2017.06.001.

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