



Original Research Article

An improved LC–MS/MS method for the quantification of alverine and para hydroxy alverine in human plasma for a bioequivalence study



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ABSTRACT

A highly sensitive and selective high performance liquid chromatography–tandem mass spectrometry method was developed and validated for the quantification of alverine (ALV) and its active metabolite, para hydroxy alverine (PHA), in human plasma. For sample preparation, solid phase extraction of analytes was performed on Phenomenex Strata-X cartridges using alverine-d5 as the internal standard. The analytes were separated on Symmetry Shield RP₁₈ (150 mm×3.9 mm, 5 μm) column with a mobile phase consisting of acetonitrile and 10 mM ammonium formate (65:35, v/v). Detection and quantitation was done by electrospray ionization mass spectrometry in the positive mode using multiple reaction monitoring. The assay method was fully validated over the concentration range of 15.0–15,000 pg/mL for ALV and 30.0–15,000 pg/mL for PHA. The intra-day and inter-day accuracy and precision (% CV) ranged from 94.00% to 96.00% and 0.48% to 4.15% for both the analytes. The mean recovery obtained for ALV and PHA was 80.59% and 81.26%, respectively. Matrix effect, expressed as IS-normalized matrix factor ranged from 0.982 to 1.009 for both the analytes. The application of the method was demonstrated for the specific analysis of ALV and PHA for a bioequivalence study in 52 healthy subjects using 120 mg ALV capsules. The assay reproducibility was also verified by reanalysis of 175 incurred subject samples.

1. Introduction

Antispasmodics are a group of drugs that are used for the treatment of irritable bowel syndrome (IBS), a long-lasting gastrointestinal disorder which is characterized by abdominal pain/discomfort, bloating and altered bowel habits [1,2]. Patients suffering from IBS show visceral hypersensitivity and hyper-reactive intestinal motility [3]. Voltage-gated calcium channels are the main transducers of membrane potential changes into intracellular Ca²⁺ transients and they mediate smooth muscle contraction and endocrine secretion [4]. Alverine (ALV) belongs to the class of antispasmodic drugs which reduce the sensitivity of smooth muscle contractile proteins to calcium. ALV selectively binds with 5-HT_{1A} receptors and thereby acts as an antagonist that reduces the visceral pronociceptive effect of 5-HT. This mode of action can account for its antinociceptive effects in post-inflammatory visceral hypersensitivity [3,5]. ALV in combination with simethicone has demonstrated considerable efficiency in the treatment of abdominal

pain in IBS [3]. It is commercially available under the brand name Spasmonal[®] Forte, alverine citrate 60/120 mg hard capsules and also as 60 mg alverine citrate/300 mg simethicone combination (Laboratoires Galeniques Vernin, France) in a soft capsule. After oral administration, ALV is rapidly converted to its primary active metabolite, para hydroxy alverine (PHA), which is then further metabolized to two secondary metabolites [6].

Methods to determine ALV as a single analyte have been reported using UV spectrophotometry [7] and potentiometry [8] in pharmaceutical preparations. However, literature on the quantitative analysis of ALV and PHA presents only four bioanalytical methods for their simultaneous determination using high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) technique [9–12]. Due to very low plasma levels of the drug and its metabolite, it is essential to develop more sensitive and selective methods especially for better assessment of their pharmacokinetic parameters. Thus, the present work was intended to achieve enhanced sensitivity using API

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5500 MS/MS, employing minimum plasma sample for processing. Additionally, the developed method utilizes a deuterated internal standard for better accuracy and precision of the data. Though the chromatographic analysis time is identical with one report [9] and slightly longer than other methods, the sensitivity of the developed method for ALV is higher than that of all existing reports [9–12]. Moreover, the plasma volume for processing (150 μ L) is also much less in comparison with the available methods. The developed method is extensively validated as per the current regulatory guidelines and is successfully applied for a bioequivalence study in 52 healthy Indian subjects after oral administration of 120 mg ALV capsule formulation.

2. Experimental

2.1. Chemicals and materials

The reference standards of ALV (purity 98.04%), PHA (purity 98.30%) and the internal standard (IS) alverine-d5 (ALV-d5, purity 99.48%) were procured from Toronto Research Chemicals (Ontario, Canada). HPLC grade methanol and acetonitrile were the products of J.T. Baker Inc. (Phillipsburg, NJ, USA), AR grade ammonium formate (AF) and formic acid (FA) were purchased from Qualigens Fine Chemicals (Mumbai, India). Phenomenex Strata-X (30 mg/1 mL) solid phase extraction cartridges were purchased from Phenomenex (Bangalore, India). Water was purified using Milli-Q water purification system from Millipore (Bangalore, India). Blank human blood was collected with Na heparin as anticoagulant from healthy and drug free volunteers. Plasma was separated by centrifugation at 2061 g at 10 °C and stored at –70 °C.

2.2. Liquid chromatographic and mass spectrometric conditions

The HPLC system from Shimadzu (Kyoto, Japan) consisted of binary LC-20 CE prominence pump, autosampler (SIL-HTc), a solvent degasser (DGU-20A₃ prominence) and temperature-controlled compartment for column (CTO-10AS_{VP}). The chromatographic separation of the analytes and IS was carried out under reversed-phase conditions at 40 °C using Symmetry Shield RP₁₈ (150 mm \times 3.9 mm, 5 μ m) analytical column from Waters (Bangalore, India). The mobile phase consisted of acetonitrile and 10 mM ammonium formate, pH 6.2 (65:35, v/v). For isocratic elution, the flow rate of the mobile phase was maintained at 0.9 mL/min and the injection volume was kept at 5.0 μ L. The autosampler temperature was held at 5 °C and the pressure of the system was 1400 psi. A triple quadrupole mass spectrometer MDS SCIEX API-5500 (Toronto, Canada) equipped with electro-spray ionization and operated in positive ionization mode was used for detection and quantification of the analytes and IS. The multiple reaction monitoring (MRM) transitions used for quantitation of the analytes and IS were m/z 282.3/91.0, m/z 298.2/107.0 and m/z 287.3/91.0 for ALV, PHA and ALV-d5, respectively. The optimized source parameters like ion spray voltage, turbo heater temperature, curtain gas, Gas1, Gas2, and collision activation dissociation were kept at 2200 V, 500 °C, 30 psi, 40 psi, 60 psi and 7 psi, respectively. The compound dependent parameters, namely declustering potential, entrance potential, collision energy and collision cell exit potential, were set at 100.0 V, 10.0 V, 36.0 eV and 12 V for ALV, 70.0 V, 10.0 V, 36.0 V and 13.0 V for PHA and 80.0 V, 10.0 V, 36.0 V and 12.0 V for ALV-d5, respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 200 ms. Analyst classic software version 1.5.2 was used to control all parameters of LC and MS/MS.

2.3. Calibrators and quality control samples

Separate stock solutions (200 μ g/mL) of ALV and PHA were prepared by dissolving accurately weighed amounts in methanol. The plasma calibration standards (CSs) and quality control (QC) samples were made by spiking blank plasma with appropriate volumes of

working solutions prepared from intermediate stock solutions for both the analytes. The final CS concentrations were 15.0, 30.0, 210, 1050, 3000, 6000, 9000, 12750, and 15000 pg/mL for ALV; 30.0, 60.0, 210, 1050, 3000, 6000, 9000, 12,750, and 15,000 pg/mL for PHA. The QC samples were prepared at five concentration levels, lower limit of quantification quality control (LLOQ QC): 15.0/30.0 pg/mL; low quality control (LQC): 45.0/90.0 pg/mL; medium quality control-1 (MQC-1): 420/1200 pg/mL; medium quality control-2 (MQC-2): 4800/4800 pg/mL; high quality control (HQC): 12000/12000 pg/mL for ALV/PHA, respectively. Stock solution of ALV-d5 (100 μ g/mL) was prepared by dissolving accurately weighed amount in methanol. Working solution of ALV-d5 (8.00 ng/mL) was prepared from stock solution in methanol: water (50:50, v/v). Standard stock and working solutions used for spiking were stored at 2–8 °C, while CSs and QC samples in plasma were kept at –70 °C until use.

2.4. Sample preparation procedure

Prior to extraction, all frozen subject samples, CSs and QC samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 150 μ L of spiked CSs/QC/subject sample, 25 μ L of working solution of IS was added and vortexed for 10 s. Further 200 μ L of 2.0 mM ammonium formate buffer (pH 3.0, adjusted with formic acid) was added, vortexed for another 30 s and centrifuged at 13148 g for 5.0 min at 10 °C. After centrifugation, the plasma samples were applied to Phenomenex Strata-X (30 mg/1 mL) cartridges which were pre-treated with 1.0 mL methanol followed by 1.0 mL of 2.0 mM ammonium formate buffer. The plasma matrix was drained out from the extraction cartridges by applying reduced nitrogen pressure (positive pressure). The extraction cartridges were washed sequentially with 1.0 mL of 2.0 mM ammonium formate buffer and thereafter with 1.0 mL of 20% (v/v) methanol in water twice. The analytes and IS were eluted with 500 μ L of elution solution (acetonitrile and 2.0 mM ammonium formate buffer, pH 3.0) in 80:20 (v/v) ratio into pre-labeled vials, briefly vortexed and 5.0 μ L of the eluant was used for injection in the chromatographic system.

2.5. Procedures for method validation

The method was validated as per the current regulatory guidance [13,14]. The procedures were similar to those of our previous report [15] and are briefly described. System suitability experiment was performed by injecting six consecutive injections, using extracted sample of ALV/PHA (15,000 pg/mL) and IS (8.00 ng/mL) at the beginning of each batch. Selectivity of the method was assessed for potential matrix interferences in ten different sources (6 Na-heparinized, 2 haemolysed and 2 lipemic) of blank human plasma by extraction and inspection of the resulting chromatograms for interfering peaks. Additionally, the selectivity of the method towards commonly used medications by human volunteers was also checked. This included paracetamol, ranitidine, diclofenac, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (200 μ g/mL) were prepared by dissolving requisite amount in methanol. Further, their working solutions were prepared in methanol: water (50:50, v/v) and 5 μ L was injected to check for any possible interference at the retention time of the analytes and IS. Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle and port is able to avoid any carry forward of injected sample in subsequent runs. Autosampler carryover was evaluated by sequentially injecting extracted blank plasma, LLOQ sample in duplicate, ULOQ sample in duplicate followed by duplicate injection of previously injected blank plasma.

Cross selectivity test was performed to check the conversion of ALV to PHA and vice versa during successive steps of analysis. This test was performed at LLOQ and HQC levels for both the analytes in duplicate and processed along with two blank plasma samples. Cross contribution of the drug and its metabolite into one another was evaluated by monitoring any

interfering peak at their respective retention time and MRM window.

Five calibration lines containing nine non-zero concentrations were used to determine linearity. A linear, $1/x^2$, least-squares regression algorithm was used to plot the peak area ratio (analyte/IS) from MRM versus concentration. The linear equations were then used to calculate the predicted concentrations in all samples within the analytical runs. The correlation coefficient for each calibration curve was expected to be ≥ 0.99 for both the analytes. Re-injection reproducibility for extracted samples was also checked by reinjection of an entire analytical run after storage at 5 °C.

Intra-day accuracy and precision were evaluated by replicate analysis of plasma samples on the same day. The analytical run consisted of a calibration curve and six replicates of LLOQ QC, LQC, MQC-1, MQC-2 and HQC samples. The inter-day accuracy and precision were assessed by analysis of three precision and accuracy batches on three different days. The precision (% CV) at each concentration level should not be greater than 15%. Similarly, the mean accuracy should be within 85%–115%, except for the LLOQ where it can within 80%–120% of the nominal concentration.

Extraction recovery of the analytes and IS from human plasma was evaluated in six replicates by comparing the mean peak area responses of pre-extraction fortified samples to those of post-extraction fortified samples. Absolute matrix effect (expressed as matrix factors) was assessed by comparing the mean area responses of post-extraction fortified samples to those of neat samples prepared in elution solution. To evaluate the relative matrix effect in different plasma lots, post-extraction fortified samples were prepared in duplicate at LQC and HQC concentrations and the precision (% CV) in the measurement was assessed. Ion suppression/enhancement effects on the MRM LC–MS/MS sensitivity were evaluated by post column analyte infusion experiment [16]. Briefly, a standard solution containing ALV, PHA and ALV-d5 (at ULOQ level) was infused post column into the mobile phase at 5 $\mu\text{L}/\text{min}$ employing infusion pump. Aliquots of 5 μL of extracted control blank plasma sample were then injected into the column and chromatograms were acquired for the analytes.

Stock solutions of analytes and IS were checked for short-term stability at room temperature (25 °C) and long-term stability at 5 °C. Stability results were evaluated by measuring the area response (analyte/IS) of stability samples against freshly prepared comparison samples with identical concentration. The % change should be within $\pm 10\%$ for short-term and long-term stock solution stability. Bench top stability at room temperature, freeze-thaw stability at -70 °C, auto sampler stability (wet extract) at 5 °C, processed sample stability at 25 °C and long term stability at -70 °C were performed at LQC and HQC levels using six replicates. The stability samples were quantified against freshly prepared calibration curve standards.

To prove method ruggedness for the determination of the analytes, two batches were studied for accuracy (%) and precision (% CV). The first batch was evaluated on two Symmetry Shield RP₁₈ (150 mm \times 3.9 mm, 5 μm) analytical columns with different batch numbers, while the second batch was analyzed by two different analysts. The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. It was performed at 1.6 times the ULOQ concentration (24000 pg/mL for ALV and PHA). Six replicates samples of $1/2$ (12,000 pg/mL for ALV and PHA) and $1/4$ th (6000 pg/mL for ALV and PHA) concentration were prepared and their concentrations were calculated by applying the dilution factor of 2 and 4 respectively against the freshly prepared calibration curve standards.

2.6. Bioequivalence study and incurred sample reanalysis

The aim of the study was to determine the bioequivalence of a test dose of 120 mg alverine citrate capsules (Generic Company, UK) and the corresponding reference product, Spasmonal Forte, 120 mg capsule

from Meda Pharmaceuticals Ltd., UK. The design was an open label, balanced, two-treatment, two-period, two-sequence, single dose, fully replicated crossover, bioequivalence study in 52 healthy adult Indian subjects under fasting condition. All subjects gave their written consent to participate in the study after they were informed about the objectives and possible risks involved. The health of the subjects was checked through medical history, physical examination and routine laboratory tests. The work was subject to review by an Independent Ethics Committee constituted as per Indian Council of Medical Research (ICMR), India, which approved the study protocol. The study was conducted as per International Conference on Harmonization, E6 Good Clinical Practice Guidelines [17]. The subjects were orally administered with a single dose of test and reference formulations with 240 mL of water after recommended wash out period of at least 7 days. Blood samples were collected at 0.00 (pre-dose), 0.16, 0.33, 0.50, 0.67, 0.83, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.00, 3.50, 4.00, 6.00, 8.00, 10.0, 16.0, 24.0, 36.0, 48.0, 72.0 and 96.0 h after oral administration of test and reference formulations in labeled Na heparin-vacuettes. Plasma was separated by centrifugation and kept frozen at -70 °C until analysis. During study, subjects had a standard diet while water intake was unmonitored. The pharmacokinetic parameters of ALV and PHA were estimated by using SAS software version 9.2.

Assay reproducibility was checked by reanalysis of 175 samples near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug. The results were compared with initial pharmacokinetic study using the same procedure. As per the acceptance criterion at least two-thirds of the original results and repeat results should be within 20% of each other [18].

3. Results and discussion

3.1. Method development

The present method was intended to improve upon the existing methods in order to achieve higher sensitivity, lower sample volume requirement for extraction and use of deuterated internal standard for better accuracy and precision data. Moreover, none of the reported method afforded baseline chromatographic separation of ALV and PHV under the established chromatographic conditions except one report [9]. As both ALV and PHA are basic in nature ($\text{pK}_a > 10$) electrospray ionization was carried out in the positive mode as reported previously [6]. Initially, the precursor and product ions of the analytes and IS were optimized by infusing 100 ng/mL solutions in the mass range of 50–500 Da. The Q1 MS full scan spectra for both the analytes and IS primarily contained protonated precursor $[M+H]^+$ ions at m/z 282.3, 298.2 and 287.3 for ALV, PHA and ALV-d5, respectively. The corresponding stable and abundant product ions in Q3 MS were observed at m/z 91.0, 107.0 and 91.0, respectively by applying 36 eV collision energy. The product ions formed for the analytes and IS can be ascribed to the breaking up of phenyl propyl chain as shown in Fig. 1. As ALV and ALV-d5 have identical product ions (m/z 91.0), cross talk experiment was also performed. The results showed no memory effects in the collision cell due to identical mass in their protonated precursor-product ion channels.

Except for one report [9] which employed a C₈ column, all existing chromatographic methods developed for ALV and PHA, have used reversed-phase Hypersil C₁₈ columns with acetonitrile and ammonium formate as the mobile phase. Though these methods have chromatographic run time ranging from 1.5 to 3.5 min, the analytes were not baseline resolved [10–12]. During trials it was possible to separate both the analytes on Kromasil C₈ column using acetonitrile and 10 mM ammonium formate as reported earlier [9]. However, some endogenous matrix interference was found in some subject samples, which prompted us to redevelop our method for better performance. Thus, the chromatographic conditions were suitably optimized to maximize response (peak area), minimize interference of endogenous peaks,

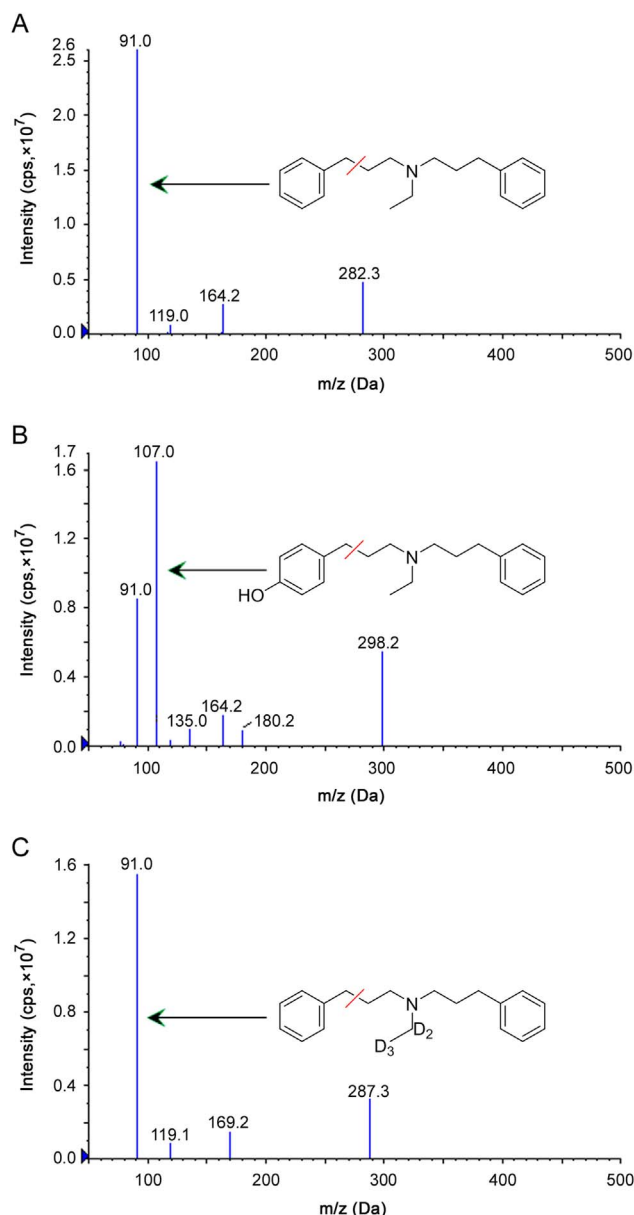


Fig. 1. Product ion mass spectra of (A) alverine (m/z 282.3 → 91.0, scan range 50–500 Da), (B) para hydroxy alverine (m/z 298.2 → 107.0, scan range 50–500 Da) and (C) alverine- d_5 , IS (m/z 287.3 → 91.0, scan range 50–500 Da) in the positive ionization mode.

along with adequate retention of the analytes within a short analysis time. In order to achieve this, several reversed-phase columns like Hypersil Gold (150 mm×4.6 mm, 5 μ m), Hypurity Advance (150 mm×4.6 mm, 5 μ m), BDS Hypersil C_{18} (150 mm×4.6 mm, 5 μ m) and Symmetry Shield RP_{18} (150 mm×3.9 mm, 5 μ m) were tested using the same mobile phase as used previously [9]. All four columns afforded chromatographic separation of the analytes but the response was not adequate especially at the LLOQ levels of ALV and PHA. However, the signal intensity of the analytes was fairly high on Symmetry Shield RP_{18} column compared to other columns, with comparatively less peak tailing in PHA. Thus, subsequent optimization was done by changing the organic: aqueous composition of the mobile phase on this column. Increasing the organic diluent (> 70%) resulted in unacceptable peak shapes, while at 50:50 (v/v) ratio the analysis time was greater than 5.0 min. Nevertheless, symmetric peak shapes, proper retention and adequate response with minimal matrix effect were found using acetonitrile and 10 mM ammonium formate, pH 6.2

(65:35, v/v) as the mobile phase. It was necessary to have the chromatographic analysis time of 4.0 min for higher sensitivity and better ionization efficiency. The representative chromatograms in Fig. 2A–C showed no interference of endogenous compounds at the retention time of ALV and PHA for double blank plasma, blank plasma, and samples spiked at LLOQ concentration. Noticeably, despite our best efforts, two endogenous peaks were observed around 0.9–1.2 min in the MRM chromatograms of PHA in subject samples (Fig. 2D). However, as they eluted much ahead of PHA there was no interference in the quantitation and no further attempt was made towards their identification. The resolution factor between the analytes under the established conditions was 3.8, while the capacity factors for ALV and PHV were 2.01 and 0.93, respectively. Further, there was no interference of commonly used medications by subjects at the retention time of the analytes. Post column infusion chromatograms proved the absence of matrix effects with no signal enhancement or suppression at the retention time of ALV and PHA (Fig. S1).

Apart from one report [12], all other methods have used solid phase extraction (SPE) to optimize conditions for simultaneous extraction of ALV and PHA from human plasma. Protein precipitation with acetonitrile/methanol and liquid-liquid extraction with diethyl ether, methyl *tert*-butyl ether, dichloromethane and their combinations were unsuccessful in providing efficient and precise recovery of the analytes as reported previously [10]. As a result, SPE was tested on different extraction cartridges, namely Bond Elut C_{18} , Phenomenex Strata-X, Oasis HLB and Lichrosep DVB HL. Under the established conditions of pre-treatment of cartridges, washing and elution, Phenomenex Strata-X gave quantitative and highly precise recovery for both the analytes as compared to other cartridges investigated. It was found that addition of 2.0 mM ammonium formate buffer (pH 3.0) was essential during all stages of work-up to obtain adequate response and consistency in the recovery with minimal matrix interference. Moreover, the extracts obtained using Strata-X were much cleaner than other cartridges and were used directly for injection without additional steps of drying and reconstitution unlike previous reports [9,10].

The salient features of all the LC–MS/MS methods developed for the simultaneous determination of ALV and PHA are summarized in Table S1. The highlights of the present method include high sensitive, especially for ALV, and low sample processing volume (150 μ L), compared to all available methods for these analytes. Additionally, the developed method employed a deuterated analog, ALV- d_5 as IS to maintain the overall efficiency of the analysis and thereby the accuracy of the data.

3.2. Method validation results

The precision (% CV) for system suitability test ranged from 0.09% to 0.31% for the retention time and 0.52% to 1.57% for the area response of both the analytes and IS. For selectivity assessment, the apparent response for ten different sources of blank plasma at the retention time of the analytes was compared with the response at LLOQ. None of the blank plasma sources showed any obvious interference ($\leq 0.52\%$ of LLOQ sample for ALV and PHA) at their retention times. The column and autosampler carryover found in blank plasma ($\leq 0.64\%$ of LLOQ sample for ALV and PHA) was minimal after subsequent injection of ULOQ sample of the analytes. Peak area ratios between the analyte and the IS were plotted against the concentrations and a linear regression was performed. The calibration curves were linear over the range of 15.0–15,000 pg/mL for ALV and 30.0–15,000 pg/mL for PHA. The corresponding regression equations for ALV and PHA were $y = (0.00093 \pm 0.00003)x + (0.00062 \pm 0.00103)$ and $y = (0.00051 \pm 0.00001)x + (0.00048 \pm 0.00027)$, respectively, with a correlation coefficient (r^2) of ≥ 0.9994 . For both the analytes, the bias of back-calculated concentrations in the calibration curves was within 7.6% of the nominal values. The signal-to-noise ratio at the LLOQ concentration was ≥ 15 and ≥ 29 for ALV and PHA, respectively.

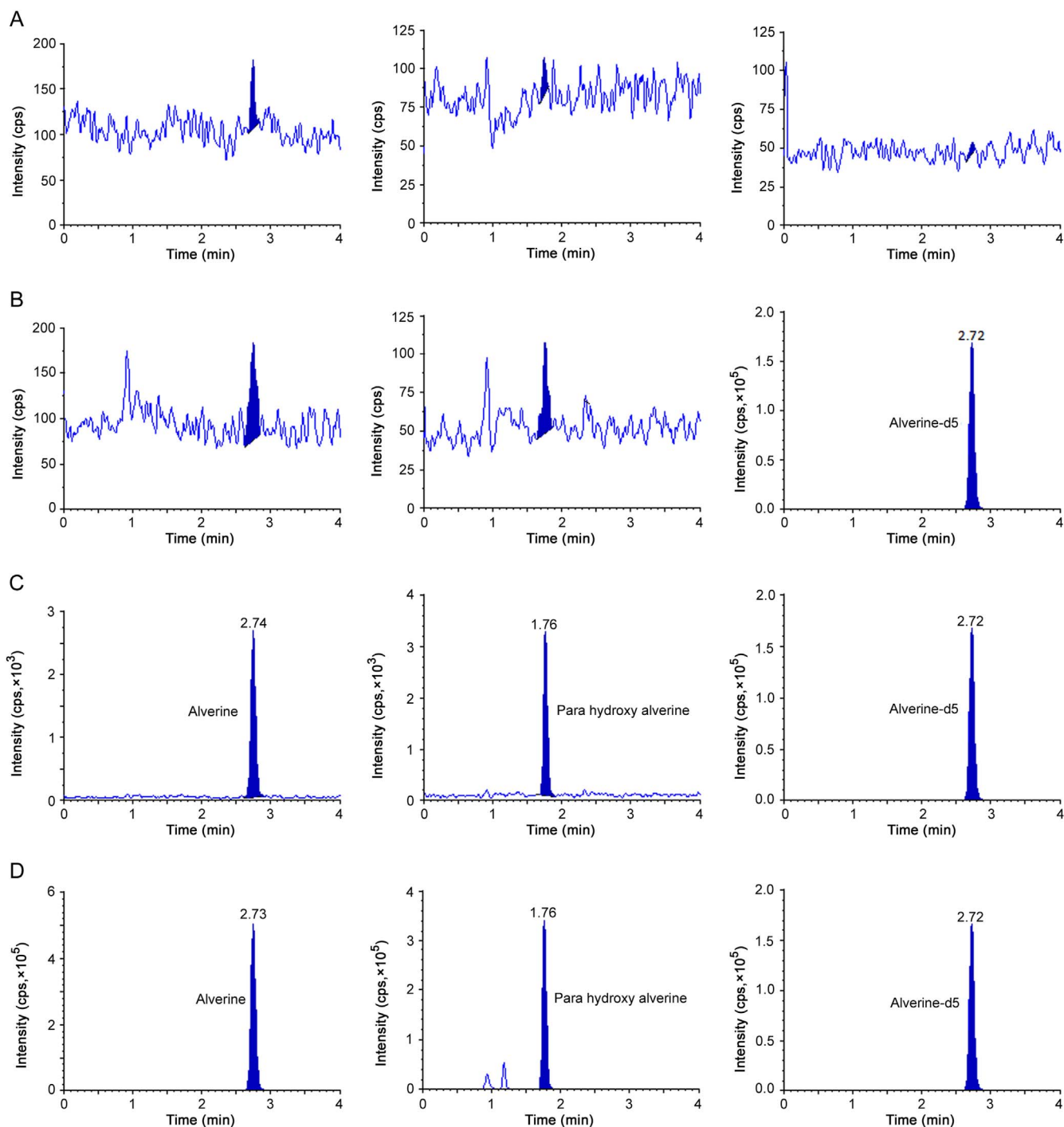


Fig. 2. Representative MRM chromatograms of alverine, para hydroxy alverine and alverine-d5 (IS) in (A) double blank plasma (without analytes and IS), (B) blank plasma with working solution of IS, (C) alverine, para hydroxy alverine at LLOQ and IS, and (D) alverine and para hydroxy alverine in subject sample at C_{max} and IS after oral administration of 120 mg alverine capsules.

The intra-day and inter-day accuracy and precision results are presented in Table 1. The relative standard deviations were $\leq 4.15\%$ and the overall mean accuracy ranged from 94.00% to 96.00% for both the analytes. The mean extraction recovery of ALV and PHA varied from 80.09% to 81.03% and 80.15% to 81.95%, respectively (Table 2). The mean recovery of IS was 87.86%. These results indicate that the developed method is unlikely to be modified by intra- or inter-individual changes in the bio-matrix and that the method has good accuracy and reproducibility. The effects of matrix ion suppression or enhancement, expressed as IS-normalized matrix factors, ranged from

0.982 to 1.009 for both the analytes, indicating that the responses in the elution solution and plasma extract were almost identical (Table 3). The precision (% CV) results for relative matrix effect in Na-heparin plasma, lipemic and haemolysed plasma sources also indicate that there was no ion suppression or enhancement of the intensity in any of the samples (Table S2).

The stock solutions of the analytes and IS in methanol were found to be stable at room temperature and under refrigerated conditions for 23 h and 2 weeks, respectively. The bench top stability of the analytes in plasma was established up to 8 h. Both the analytes were stable

Table 1

Intra-day and inter-day precision and accuracy for alverine and para hydroxy alverine.

Analytes and QC level (pg/mL)	Intra-day (n = 6; single batch)			Inter-day (n = 18; 6 from each batch)		
	Mean conc. found (pg/mL)	CV (%)	Accuracy (%)	Mean conc. found (pg/mL)	CV (%)	Accuracy (%)
Alverine						
LLOQ QC (15.0)	14.4	1.63	96.00	14.2	2.64	94.67
LQC (45.0)	42.6	1.53	94.67	42.8	1.55	95.11
MQC-1 (420.0)	400.1	0.48	95.26	400.1	0.62	95.26
MQC-2 (4800.0)	4540.6	0.68	94.60	4534.9	0.64	94.48
HQC (12,000.0)	11431.9	0.80	95.27	11449.9	0.79	95.42
Para hydroxy alverine						
LLOQ QC (30.0)	28.2	3.07	94.00	28.6	4.15	95.33
LQC (90.0)	84.8	2.14	94.22	85.4	2.32	94.89
MQC-1 (1200.0)	1149.5	1.06	95.79	1150.1	1.14	95.84
MQC-2 (4800.0)	4542.5	1.28	94.64	4562.5	1.26	95.05
HQC (12,000.0)	11466.5	1.38	95.55	11515.6	1.00	95.96

CV: coefficient of variation; LQC: low quality control; MQC: medium quality control; HQC: high quality control; LLOQ QC: lower limit of quantitation quality control.

Table 2

Extraction recovery for alverine and para hydroxy alverine.

Analyte and QC level	Area response (n = 6)		Extraction recovery, A/B (%)
	Pre-extraction spiking (A)	Post-extraction spiking (B)	
Alverine			
LQC	37414	46715	80.09
MQC-1	364885	450642	80.97
MQC-2	4220020	5207972	81.03
HQC	10003896	12465914	80.25
Para hydroxy alverine			
LQC	38845	47401	81.95
MQC-1	554556	678190	81.77
MQC-2	2206982	2718628	81.18
HQC	5198955	6486531	80.15
Alverine-d5			
LQC	919795	1056264	87.08
MQC-1	902371	1014128	88.98
MQC-2	883607	1003529	88.05
HQC	886249	1015060	87.31

LQC: low quality control; MQC: medium quality control; HQC: high quality control.

during five freeze-thaw cycles and for at least 74 h in the autosampler. Processed sample stability of the analytes was established up to 16 h at 25 °C with no obvious change in the concentration of ALV and PHA. Spiked plasma samples stored at -70 °C for long-term stability were found stable for a minimum period of 116 days. The detailed stability results are presented in Table 4.

The precision (% CV) and accuracy values obtained in order to establish the ruggedness of the method with different columns and analysts ranged from 0.83% to 4.25% and 97.60% to 98.37%, respectively for ALV and PHA. Likewise, the % CV and accuracy for dilution

Table 3

Matrix effect on alverine and para hydroxy alverine in human plasma.

Analytes/IS	Mean area response (n = 6)				Matrix factor (A/B)		IS-normalized matrix factor	
	Post-extraction spiking (A)		Neat samples in elution solution (B)					
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
Alverine	46715	12465914	50503	13393095	0.925	0.931	0.986	1.009
Para hydroxy alverine	47401	6486531	51450	6977496	0.921	0.930	0.982	1.008
Alverine-d5	1056264	1015060	1125608	1099541	0.938	0.923	–	–

LQC: low quality control; HQC: high quality control.

Table 4

Stability results of alverine and para hydroxy alverine under different conditions (n = 6).

Storage condition	QC level	Accuracy (%)		Precision (% CV)	
		Alverine	Para hydroxy alverine	Alverine	Para hydroxy alverine
Bench-top stability (8 h, 25 °C)	LQC	95.07	97.32	1.57	2.13
	HQC	94.81	96.10	0.30	1.51
Freeze-thaw stability (5 cycles, -70 °C)	LQC	94.90	96.79	2.08	1.71
	HQC	94.59	97.43	0.86	0.98
Auto-sampler stability (74 h, 5 °C)	LQC	94.37	95.22	0.79	1.01
	HQC	95.37	96.38	0.62	0.44
Processed sample stability (16 h, 25 °C)	LQC	94.05	96.76	1.81	1.29
	HQC	94.26	95.96	0.77	0.28
Long-term stability in plasma (116 days, -70 °C)	LQC	98.32	96.70	3.93	2.60
	HQC	96.03	97.26	0.80	1.80

LQC: low quality control; HQC: high quality control; CV: coefficient of variation.

reliability of 1/2 and 1/4th dilution varied from 1.67% to 3.27% and 95.6% to 99.25%, respectively for both the analytes.

3.3. Application of the method and assay reproducibility

The suitability of the developed method for clinical use was demonstrated by analyzing human plasma samples for a bioequivalence study of ALV and PHA in 52 healthy Indian subjects. The mean concentration-time profiles of ALV and PHA after a single oral dosage of 120 mg ALV capsules is shown in Fig. 3. The plasma concentration

Table 5

Pharmacokinetic parameters after oral administration of alverine capsule formulation to 52 healthy Indian subjects under fasting condition (mean±SD).

Parameter	Alverine		Para hydroxy alverine	
	Test	Reference	Test	Reference
C_{max} (pg/mL)	2574.65±539.73	2628.13±525.87	2841.78±458.45	2743.49±461.16
T_{max} (h)	1.31±0.53	1.34±0.55	1.74±0.85	1.77±0.80
$t_{1/2}$ (h)	11.37±4.52	12.24±4.50	9.84±2.59	9.74±2.36
AUC _{0–96 h} (h·pg/mL)	14690.3±3653.2	14473.3±3385.1	18970.4±2615.7	18714.9±2640.4
AUC _{0–inf} (h·pg/mL)	15562.3±3928.5	15531.8±3723.9	19755.5±2639.5	19486.3±2660.9
K_{el} (1/h)	0.08±0.04	0.07±0.03	0.07±0.02	0.07±0.02

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_{0–96 h}: Area under the plasma concentration vs time curve from 0 h to 96 h; AUC_{0–inf}: Area under the plasma concentration-time curve from zero hour to infinity; K_{el} : Elimination rate constant; SD: Standard deviation.

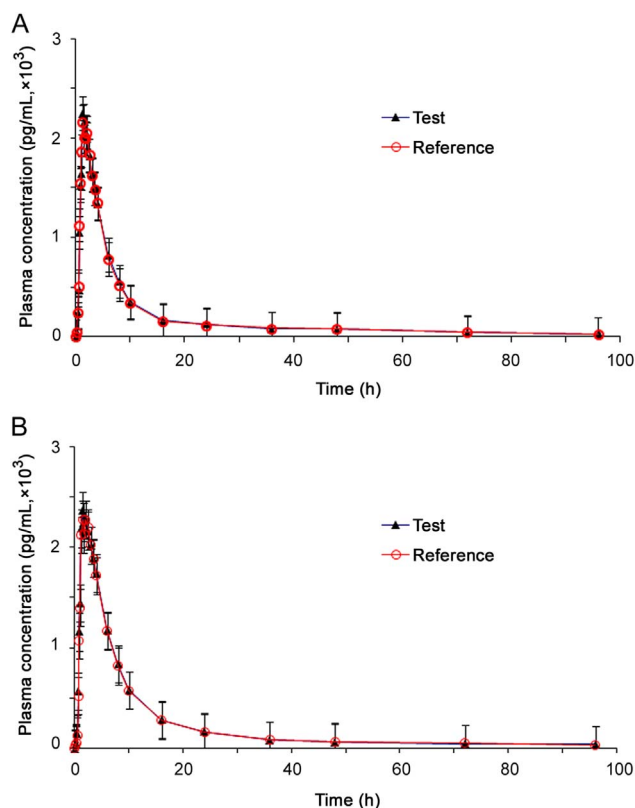


Fig. 3. Mean plasma concentration-time profiles of (A) alverine and (B) para hydroxy alverine after oral administration of test and reference (Spasmonal Forte, Meda Pharmaceuticals Ltd., UK) 120 mg alverine capsules to 52 healthy Indian subjects.

of ALV and PHA was detectable from 15 and 30 min onwards, respectively and up to 96 h after oral administration of ALV, further it was below the LLOQ concentration. The mean values of pharmacokinetic parameters for ALV and PHA are given in Table 5. Incidentally, all published reports on the pharmacokinetics of ALV have involved healthy Indian subjects and only two reports have presented detailed information on the pharmacokinetic parameters of ALV and PHA. The mean peak plasma concentration (C_{max}) and area under the plasma concentration-time curve (AUC_{0–t}) values obtained in the present work were close to one of the reports with 12 subjects [12]. However, these values were much higher than those of the study performed with 24 healthy subjects [10]. Conversely, the time for maximum plasma concentration (T_{max}), elimination half life ($t_{1/2}$) and elimination rate constant (K_{el}) values for ALV and PHA were in good agreement with the work of Ghosh et al. [10]. Nevertheless, there was no significant difference between the two formulations in any parameter for both the

analytes. Further, the method reproducibility was verified by reanalysis of 175 incurred samples. The % change in the concentration of both the analytes was within ±14% from the initial results.

4. Conclusions

An improved LC–MS/MS method was developed and validated for the sensitive and specific determination of ALV and PHA in human plasma using a deuterated analog as an internal standard. There was no interference or matrix effect from endogenous substances in the quantitative analysis. The calibration range established for ALV and PHA was adequate for clinical pharmacokinetic studies using small sample volume for analysis. The optimized SPE protocol gave highly consistent and precise recovery for both the analytes with no additional steps of drying and reconstitution. The method was successfully applied to a bioequivalence study with 120 mg ALV capsules in 52 healthy Indian subjects. Finally, the reproducibility was suitably confirmed by reanalysis of incurred subject samples.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jpha.2016.11.003>.

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