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A rapid and most sensitive liquid chromatography/tandem mass spectrometry method for simultaneous determination of alverine and its major metabolite, para hydroxy alverine, in human plasma: application to a pharmacokinetic and bioequivalence study

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A rapid and highly sensitive method for the determination of alverine (ALV) and its metabolite, para hydroxy alverine (PHA), in human plasma using LC-MS/MS in positive ion electrospray ionization (ESI) in multiple reactions monitoring (MRM) mode was developed and validated. The procedure involves a simple solid phase extraction (SPE). Chromatographic separation was carried out on a Hypersil GOLD C_{18} column (50 mm × 4.6 mm, 5 μm) with an isocratic mobile phase and a total run time of 1.5 min. The standard calibration curves showed excellent linearity within the range of 0.060–10.051 ng/mL for ALV and 0.059–10.017 ng/mL for PHA ($r \geq 0.990$). This method was successfully applied to a pharmacokinetic study after oral administration of alverine citrate 120 mg capsule in Indian healthy male volunteers.

Keywords: alverine; para hydroxyl alverine; matrix effects; bio-equivalence; LC-MS/MS

Introduction

Alverine (ALV) citrate (Spasmonal, Norgine, Harefield, Middlesex, UK) is thought of as a smooth muscle relaxant,[1,2] and is currently used as an antispasmodic in irritable bowel syndrome and dysmenorrhoea. ALV was shown to inhibit spontaneous electrical activity and nervous control of the proximal colon of the rabbit *in vivo*.[3] It was also found to decrease the sensitivity of the intestinal mechanoreceptors in response to chemical stimulation in anaesthetized cats[4] and reduce 5-hydroxytryptamine$_{1A}$ receptor-mediated rectal hypersensitivity in the rat.[5]. ALV may increase Ca influx during action potentials due to inhibition of the inactivation of L-type Ca channels, but may also suppress evoked activity by inhibiting the sensitivity of contractile proteins to Ca^{2+}. However, the exact mechanisms of ALV's inhibitory action are still not clear, due to the lack of information on its effects on isolated smooth muscle *in vitro*.

ALV is a frequently used antispasmodic for which there was hardly any specific and sensitive LC-MS method available.[6] The published method[6] was developed in API 5000 mass spectrometry attached with ultra performance liquid chromatography (UPLC) as a solvent delivery module. In the reported method 0.100 ng/mL was the lower limit of quantification (LLOQ) value with 4.0 min of analysis time.

In the present research a method was developed and validated that can be used for quantification (as well as identification) of ALV alone or in combination with its major metabolite in human plasma using liquid chromatography-mass spectrometry (LC-MS) technique up to low picogram level. The method was developed by using API 4000 MS/MS along with high performance liquid chromatography (HPLC), having the LLOQ value of 0.060 ng/mL and 0.015 ng/mL as limit of detection (LOD). The total analysis time of single injection was 1.5 min, which was almost three times faster than that of the reported one. So the novelty of this method is that, by using the almost same extraction technique as the reported one and the comparatively less sensitive instrument than the one reported, the most sensitive method with shortest analysis time was developed and validated.

Therefore, it was worthwhile to develop a method, which can detect up to such lower limits with fast analysis at our laboratory. This bio-analytical method was validated in human plasma in multiple reactions monitoring (MRM), which can be applied to bioequivalence and pharmacokinetic studies of ALV. The principal goal of the present work was to develop an accurate, sensitive, and rapid LC-MS method with a dynamic linearity range that can cover the plasma concentrations following a single oral dose of ALV. The method had been validated by evaluating the precision, accuracy,

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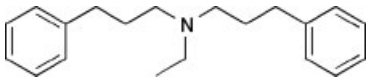

Figure 1A. Chemical structure of alverine.

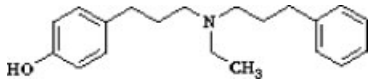

Figure 1B. Chemical structure of para hydroxy alverine.

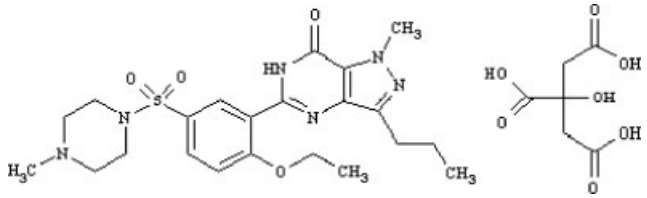

Figure 1C. Chemical structure of sildenafil citrate.

and other validation parameters for human plasma samples, as mentioned in regulatory guidelines.[7]

Experimental

Apparatus and software

The HPLC system with an auto sampler was a Shimadzu LC-10ADvp (Shimadzu, Japan) coupled with Applied Biosystem Sciex (MDS Sciex, ON, Canada) API 4000 Tandem mass spectrometry. The auto sampler was SIL-HTC (Shimadzu, Japan). The solvent delivery module was LC-10ADvp (Shimadzu, Japan). The chromatographic integration was performed by Analyst software (version: 1.4.1; Applied Biosystems). Positive pressure unit used for SPE was from Orochem Technologies Inc (Lombard, IL, USA). The Caliper turbovap LV concentration workstation used to evaporate the samples was purchased from Caliper Life Sciences (Hopkinton, MA, USA).

Chemicals and reagents

ALV (99.5%) (Figure 1A), PHA (98.40%) (Figure 1B) and the internal standard (IS), (Figure 1C), were procured from Astron Research Limited (Ahmedabad, Gujarat, India); Chiron AS (Trondheim Norway) and Quality Control Department of Cadila Pharmaceuticals Limited (Ahmedabad, Gujarat, India), respectively. All reagents were of analytical reagent grade unless stated otherwise. Water used for the preparation of mobile phase and other solutions was collected from Milli Q_{PS} (Billerica, Massachusetts Milli Pore, USA) installed in the Laboratory. Phillipsburg, NJ HPLC-grade methanol, acetonitrile, and formic acid were supplied by J. T. Baker, USA and Finar Chemicals Limited (Ahmedabad, India), respectively. Ammonium formate was purchased from Qualigens Fine Chemicals (Mumbai, India), whereas SPE cartridges (30 mg, 1cc) used for SPE were supplied by Waters Corporation (Milford, Massachusetts USA). Drug free human K_2EDTA plasma was used during validation and study sample analysis was supplied by Prathma Blood Centre (Ahmedabad, India). Plasma was stored at $-20 \pm 5\,^{\circ}$C before sample preparation and analysis.

Standards and working solutions

An individual stock standard solution of ALV, PHA, and IS containing 1 mg/mL was prepared by dissolving pure compound in methanol. Intermediate and working solutions were prepared from corresponding stock solutions by diluting with a mixture of water : methanol 50:50 *v*/*v*. Calibration standards were prepared in the range of 0.060 to 10.051 ng/mL for ALV and 0.059 to 10.017 ng/mL for PHA using eight concentration levels each. Quality control samples of three different levels low (0.241 ng/mL), medium (5.056 ng/mL) and high (8.847 ng/mL) for ALV and low (0.242 ng/mL), medium (5.008 ng/mL) and high (8.838 ng/mL) for PHA were also prepared. All these stock solutions, calibration standards and quality control samples were stored at $6 \pm 2\,^{\circ}$C.

Characterization of the product ions using tandem mass spectrometry

1 μM ALV, PHA and IS solutions were separately infused into the mass spectrometer at a flow rate of 10 μL/min, to characterize the product ions of each compound. The precursor ions $[M+H]^+$ and the pattern of fragmentation were monitored using the positive ion mode. The major peaks observed in the MS/MS scan were used to quantify ALV, PHA, and IS.

Analytical System

The plasma ALV and PHA concentrations were quantified using SCIEX API 4000 LC-MS/MS system (MDS Sciex, ON, Canada), equipped with an electrospray ionization (ESI) interface used to generate positive ions $[M+H]^+$. The compounds were separated on a reversed phase column (Hypersil GOLD C18, 50 × 4.6 mm ID, particle size 5 μ, Auchtermuchty Fife Thermo Electron Corporation, UK), with an isocratic mobile phase consisting of 10 mM ammonium formate (pH: 3.50 ± 0.05) in water and acetonitrile at a ratio of 10:90 *v*/*v*. The mobile phase was eluted at 0.80 mL/min. Total analysis time of single injection was 1.5 min. Auto sampler rinsing volume was 500 μL. The column and auto sampler temperature were maintained at 35° and 4 °C, respectively.

The ion spray voltage and temperature were set at 5500 V and 400 °C. The typical ion source parameters, viz., declustering potential, collision energy, entrance potential and collision cell exit potential were 70, 50, 10 and 9 V for ALV; 70, 60, 10 and 9 V for PHA; 75, 60, 10, and 9 V for IS, respectively. Nitrogen gas was used for the nebulizer gas, curtain gas, and collision-activated dissociation gas, which were set at 40, 20, and 9 psi, respectively. Quantification was performed by MRM of the protonated precursor ion and the related product ion for ALV and PHA using the IS method with a peak area ratios and a linear least-squares regression curve with weighting factor of $1/x^2$. The mass transitions used for ALV, PHA, and IS were m/z 282.30 → 91.11, m/z 298.30 → 106.90 and m/z 475.30 → 58.40, respectively, with a dwell time of 300 ms per transition. Quadrupoles Q1 and Q3 were set on a unit resolution. The analytical data were processed by Analyst software (Version 1.4.1; Applied Biosystems).

Sample treatment

SPE technique was used to extract ALV and PHA. 500 μL plasma sample was transferred to a ria vial for analysis. 30 μL of IS (1 μg/mL) was added into it, the sample was vortexed for 15 s. In solid phase extraction, the cartridge was conditioned with 1 mL of methanol

followed by 1 mL of water; the plasma samples were then loaded on the cartridges; the cartridges were washed twice with 1 mL of water, followed by air drying and elution with 1 ml of methanol. Methanol was collected and evaporated to dryness at 50 °C under the stream of nitrogen. The residue was then reconstituted with 300 μL of mobile phase and injected to LC-MS/MS.

Method Validation

The validation parameters were specifically, linearity, sensitivity, accuracy, precision, and matrix effects of the assay and the recovery and stability in human plasma, according to the US Food and Drug Administration (FDA) guidance for the validation of Bioanalytical methods.[7]

Selectivity was studied by comparing the chromatograms of six different lots of plasma obtained from six subjects; the plasma samples were spiked with ALV, PHA, and IS. Calibration curves were prepared by assaying standard plasma samples at ALV and PHA concentrations, ranging from 0.061 to 10.051 ng/mL for ALV and 0.059 to 10.017 ng/mL for PHA.

The linearity of each method's matched calibration curve was determined by plotting the peak area ratio (y) of ALV or PHA to IS versus the nominal concentration (x) of ALV or PHA, respectively. The calibration curves were constructed by weighing ($1/x^2$) least-squares linear regression.

The LLOQ for ALV or PHA in human plasma was defined as the lowest concentration with acceptable accuracy (80–120%), and sufficient precision (within 20%); this was verified by analysis of six replicates.

Intra- and inter-day accuracy and precision for this method were determined at three different concentration levels on three different days, and on each day, six replicates were analyzed with independently prepared calibration curves. The percentage accuracy was expressed as (mean observed concentration)/(nominal concentration) × 100, and the precision was the relative standard deviation (RSD, %).

To evaluate the matrix effects of ALV, PHA and IS on the ionization of the analyte, low quality control (LQC) samples from six different plasma batches in triplicate were processed. For recovery calculation, the peak areas obtained by direct injection of solvent (or neat) standard solutions spiked after extraction into plasma extracts as A and the peak areas for solvent (or neat) standard solutions spiked before plasma extraction as B, the extraction recovery value can be calculated as follows:

$$\text{Extraction recovery (\%)} = \text{B/A} \times 100 \quad (1)$$

The stability of ALV or PHA in human plasma was assessed by analyzing six replicate samples spiked with 0.241 and 8.847 ng/mL for ALV and 0.242 and 8.838 ng/mL for PHA, respectively, under six conditions: after short-term storage for 14 h at room temperature; after long-term storage of 6 months at −30 °C; after 4 freeze-thaw cycles; after 6 h on bench top; after 30 h within the auto sampler; and 25 h at dry extract stage in 4 °C. The concentrations obtained were compared with the nominal values of the QC samples.

Clinical Protocol

The bioequivalence study protocol and bioanalytical method presented in this paper were approved by the Independent Medical Ethics Committee of Cadila Contract Research Organization, Ahmedabad, Gujarat, India. In a randomized, single-dose, two-treatment, two-sequence, replicate, four-period crossover bioequivalence study, subjects were administered a single dose of alverine citrate 120 mg capsule along with 200 mL of drinking water after an overnight fasting of at least 10 h in each period with at least 10 days of washout period between each administration. All subjects were healthy, adult, male, human volunteers of Indian origin. In each period, a total of 17 blood samples were collected including a pre-dose sample prior to drug administration and after drug administration at 0.333, 0.667, 1.0, 1.333, 1.667, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0, 72.0, and 96.0 h. The blood samples were immediately centrifuged at 2000 × g for 10 min at 4 °C, and the plasma samples were stored at −30 °C until LC-MS/MS analysis.

Results and Discussions

Optimization of chromatographic condition and sample clean-up

In order to have an optimal selectivity and sensitivity different types of column and mobile phase were used. Length of the columns varied from 50 cm to 150 cm, and the particle size varied from 3.5 μ to 5.0 μ. Columns of different types of stationary phase like C_8, C_{18} and cyano were used which showed considerable matrix effects on peak shape and intensity. Finally, Hypersil GOLD C_{18} column of 50 × 4.6 mm, 5 μ was selected for analysis based on good peak shape and no matrix effects.

The influence of buffer molarity, pH, and types of organic modifier on the signal intensities was also studied at the optimized declustering potential. Based on the peak intensity of the ALV, PHA, and IS, 10 mM ammonium formate buffer (pH: 3.50 ± 0.05) in water and acetonitrile, were selected as the organic phase, at a flow rate of 0.80 mL/min for further studies. Higher proportions of organic modifier in the mobile phase were found to improve the signal intensity. Initially, 95 : 5 (*v*/*v*) acetonitrile: buffer at a flow rate of 0.30 mL/min was tried. On the other hand, very high proportions of organic phase led to improper elution leading to peak deformation. Therefore, the 90 : 10 (*v*/*v*) organic phases to buffer were selected as optimum at a flow rate of 0.80 mL/min. Moreover, reconstitution of the extracted dry residue in mobile phase improved the peak symmetry.

The sample clean-up technique was also optimized in order to get minimum interference of endogenous compounds or matrix effects and good analyte recovery. Different techniques, such as protein precipitation, liquid-liquid extraction, and SPE were used for sample extraction. Based on non-interference and optimal recovery, SPE was found to be the best suitable for sample preparation.

Full-scan spectra of ALV, PHA and IS showed the presence of parent ions only. The product ion scan in flow injection mode showed significant and stable daughter ions. So the detection was made in multiple ions monitoring mode with Q1 and Q3 set at *m/z* 282.30 → 91.11 for ALV, 298.30 → 106.90 for PHA and for IS, the transition monitored was m/z 475.30 → 58.40.

To the best of our knowledge, there are not many reported LC-MS/MS methods[6] were available. Even among the reported methods, there is not one with a sensitivity which could detect up to 0.015 ng/mL as LOD for both ALV and PHA and 0.060 ng/mL as LLOQ for ALV and 0.059 ng/mL as LLOQ for PHA with acceptable accuracy and precision and 1.5 min run time in ESI MRM mode.

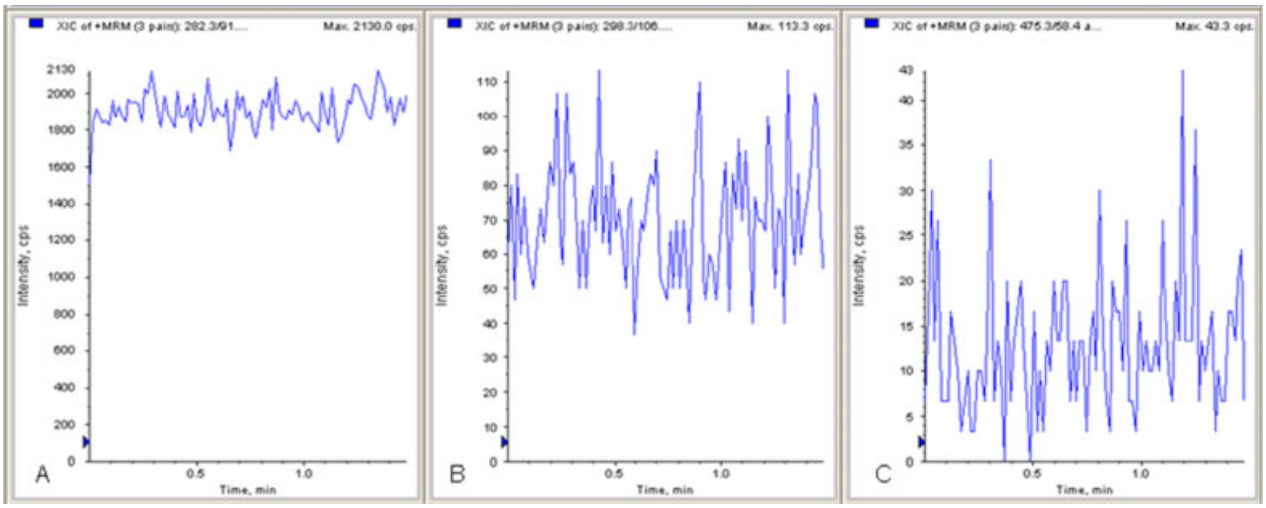

Figure 2. Representative chromatogram of Plasma blank.

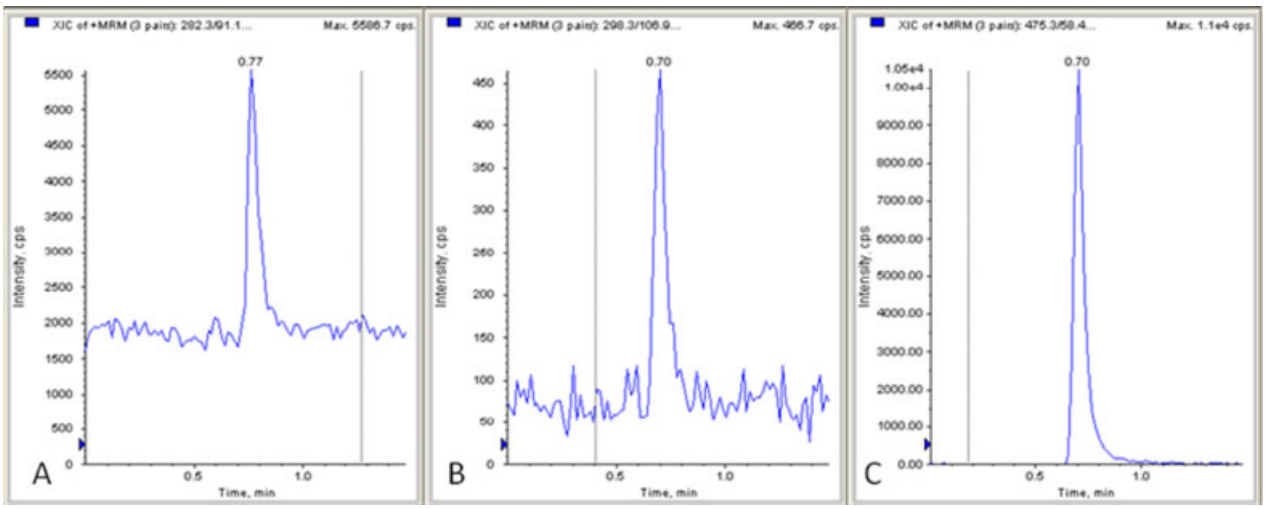

Figure 3. Representative chromatogram of LLOQ.

Method validation

Linearity

Linearity of calibration standards was assessed by subjecting the spiked concentrations and the respective peak areas using $1/X^2$ linear least-squares regression analysis. Linearity ranged from 0.060 to 10.051 ng/mL for ALV ($r > 0.990$), 0.059 to 10.017 ng/mL for PHA ($r > 0.990$). In aqueous solution, accuracy of all calibration standards was within 85–115%, except LLOQ where it was 80–120%.

Specificity and selectivity

Six different lots of plasma (Prathma Blood Centre, Ahmedabad) were analyzed to ensure that no endogenous interference took place with the mass transitions chosen for ALV, PHA and IS. Six LLOQ level samples along with plasma blank from the respective plasma lot were prepared from six different lots of plasma and analyzed. In all six plasma blanks, the response at the retention time of ALV and PHA was <20% of LLOQ response and at the retention time of IS, the response was <5% of mean IS response in LLOQ. Figure 2 represents the chromatogram of respective plasma blank.

LOD and LLOQ

The LOD for ALV and PHA was defined as the signal in the plasma after the sample clean-up method that corresponds to three times the baseline noise (signal to noise ratio $\geq$3) and for LLOQ it was five times the baseline noise (signal to noise ratio $\geq$5). The LOD was found to be 0.015 ng/mL for ALV and PHA. The LLOQ was found to be 0.060 ng/mL for ALV and 0.059 ng/mL for PHA (Figure 3) in the plasma sample for calibration.

Accuracy and precision

For the validation of the assay, QC samples were prepared at three concentration levels of low, medium, and high. Six replicates of each QC samples were analyzed together with a set of calibration standard. The accuracy of each sample preparation was determined by injection of calibration samples and three QC samples in six replicates for three days. Obtained accuracy and precision (inter- and intra-day) are presented in Table 1A for ALV and Table 1B for PHA. The results show that the analytical method is accurate, as the accuracy is within the acceptance limits of 100 $\pm$ 20% of the theoretical value

Table 1A . Inter and Intra-day accuracy and precision of ALV

	QC levels	Mean accuracy	Mean precision (% RSD)
Day 1	LQC	103.32	3.43
	MQC	101.61	7.22
	HQC	107.84	4.21
Day 2	LQC	101.11	4.13
	MQC	105.52	6.42
	HQC	106.94	5.44
Day 3	LQC	94.47	6.20
	MQC	101.32	4.81
	HQC	104.40	7.91
Inter-day	LQC	99.63	5.84
	MQC	102.72	6.03
	HQC	106.27	5.92

Each mean and %CV of intra-day accuracy and precision represent six observations (n=6). The inter-day accuracy and precision are averages and %CV of three intra-day observations.

Table 1B . Inter and Intra-day accuracy and precision of PHA

	QC levels	Mean accuracy	Mean precision (% RSD)
Day 1	LQC	104.98	1.87
	MQC	98.33	2.91
	HQC	96.42	3.22
Day 2	LQC	105.88	1.89
	MQC	103.22	3.00
	HQC	99.36	1.57
Day 3	LQC	105.74	1.18
	MQC	100.47	2.62
	HQC	100.13	1.73
Inter-day	LQC	105.10	1.62
	MQC	101.64	3.37
	HQC	98.74	2.71

Each mean and %CV of intra-day accuracy and precision represent six observations (n=6). The inter-day accuracy and precision are averages and %CV of three intra-day observations.

at LLOQ and 100 ± 15% at all other concentration levels. The precision around the mean value was never greater than 15% at any of the concentration studied. Figure 4 represents the representative chromatogram of upper limits of quantification (ULOQ).

Recovery study

A recovery study was performed by comparing processed QC samples of three different levels in six replicates with aqueous samples of same level. The recovery of ALV at low quality control (LQC) level was 75.26%, medium quality control (MQC) level was 72.35% and for high quality control (HQC) level was 75.57%. The mean recovery of ALV was 74.39%; % coefficients of variation (%CV) of mean recovery of all three QCs were 2.39.

The recovery of PHA at LQC level was 77.26%, MQC level was 70.35% and for HQC level was 72.87%. The mean recovery of PHA was 73.49%. % coefficients of variation (%CV) of mean recovery of all three QCs were 4.76. Recovery of IS was 64.19%.

Matrix effects

Matrix effect was determined to examine whether there was co-elution of any phospholipids, analytes or IS with any matrix or its components. It showed that the method was free from any matrix interferences. This was evaluated by comparing with unextracted samples. Along with that, 18 LQC samples, 3 each from 6 different plasma lots were processed and analyzed. For ALV the average % accuracy for all LQC level was 102.27 and %CV of all LQC samples was 5.48. Whereas, for PHA the average % accuracy for all LQC level was 103.63 and %CV of all LQC samples were 2.51.

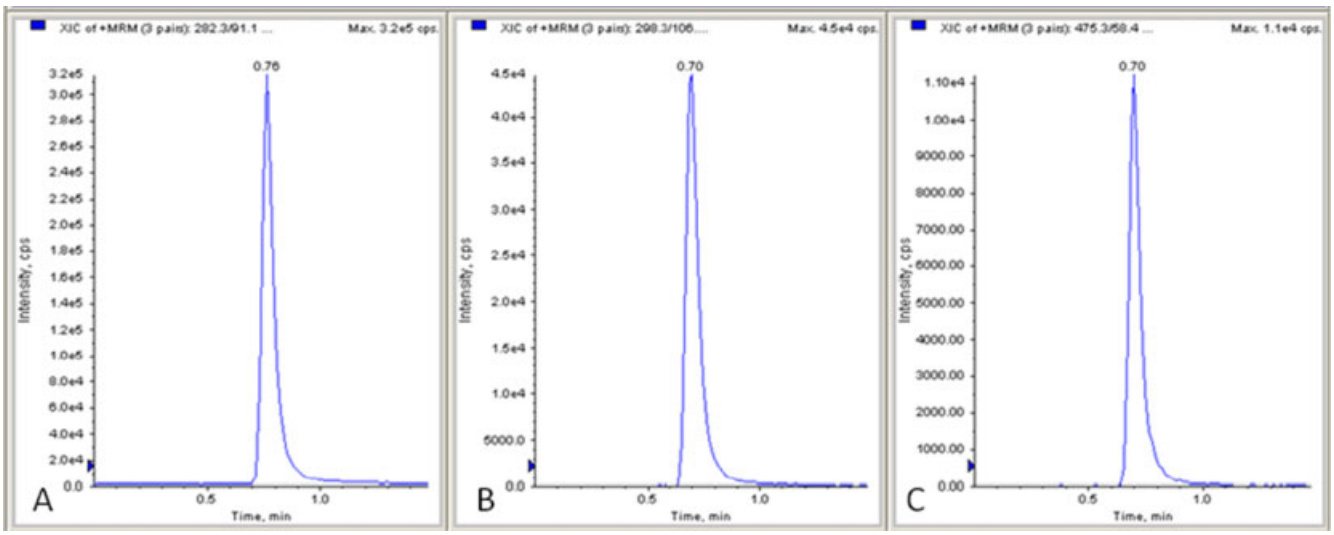

Figure 4. Representative chromatogram of ULOQ.

Haemolysis affects

To determine haemolysis affects, six haemolysed plasma blank and QC samples were prepared in haemolysed plasma with two concentration levels of low and high. Six replicates of each QC samples were analyzed together with a set of calibration standards prepared in normal plasma. The accuracy of each sample preparation was determined by injection of calibration samples and two QC samples in six replicates. The observed interference in haemolysed plasma blank at the retention time of ALV and PHA was less than 20% of LLOQ level response and less than 5% of IS response at the retention time of IS. For ALV the average % accuracy for LQC level was 98.62 and for HQC level was 104.60. The %CV of LQC was 1.18 and for HQC was 3.66.

For PHA the average % accuracy for LQC level was 100.83 and for HQC level was 105.38. The %CV of LQC was 5.84 and for HQC was 2.82.

Stability studies

The stability of ALV, PHA, and IS were investigated in the stock and working solutions, in plasma during storage, during processing, after four freeze-thaw cycles, and in the final extract. Stability samples were compared with freshly processed calibration standards and QC samples. Analyte and IS were considered stable when the change of concentration is ±10% with respect to their initial concentration.

The %CV of ALV at LQC and HQC levels for freeze thaw stability, dry extract stability, bench-top stability and auto-sampler stability were 3.55 & 6.77; 2.53 & 7.13; 5.93 & 3.95; and 3.77 & 4.85; respectively, where as, the %CV of PHA at LQC and HQC levels for freeze thaw stability, dry extract stability, bench-top stability and auto-sampler stability were 4.83 & 3.44; 5.44 & 2.34; 2.70 & 2.47; and 2.82 & 2.02; respectively.

A summary of stability data is presented in Table 2.

Calibration curve parameters

The summary of calibration curve parameters was as follows. For ALV the mean slope and y-intercepts were 1.5760 (Range: 1.3300 to 2.1100) and −0.01682 (Range: −0.0262 to −0.0015) respectively.

Table 2A . Summary of stability data of ALV

Stability	QC level	Mean precision (%CV)	Mean accuracy	Percent change	Stability duration
Bench top	LQC	5.93	100.84	−0.27	6 h
	HQC	3.95	109.36	2.26	
Freeze thaw	LQC	3.55	98.16	3.88	4 cycles
	HQC	6.77	103.23	−1.12	
Dry extract	LQC	2.53	96.64	2.27	69 h
	HQC	7.13	101.17	−3.09	
Auto sampler	LQC	3.77	102.74	1.64	49 h
	HQC	4.85	109.21	2.12	

Table 2B . Summary of stability data of PHA

Stability	QC level	Mean precision (%CV)	Mean accuracy	Percent change	Stability duration
Bench top	LQC	2.70	107.21	1.70	6 h
	HQC	2.47	102.64	3.22	
Freeze thaw	LQC	4.83	102.11	−3.07	4 cycles
	HQC	3.44	99.87	−0.36	
Dry extract	LQC	5.44	101.02	−3.99	69 h
	HQC	2.34	99.61	−0.63	
Auto sampler	LQC	2.82	108.30	2.68	49 h
	HQC	2.02	101.62	2.17	

The mean correlation coefficient, r was 0.9981 (Range: 0.9976 to 0.9989).

For PHA the mean slope and y-intercepts were 0.5254 (Range: 0.4460 to 0.6730) and −0.00081 (Range: −0.0010 to 0.0018) respectively. The mean correlation coefficient, r was 0.9988 (Range: 0.9972 to 0.9996).

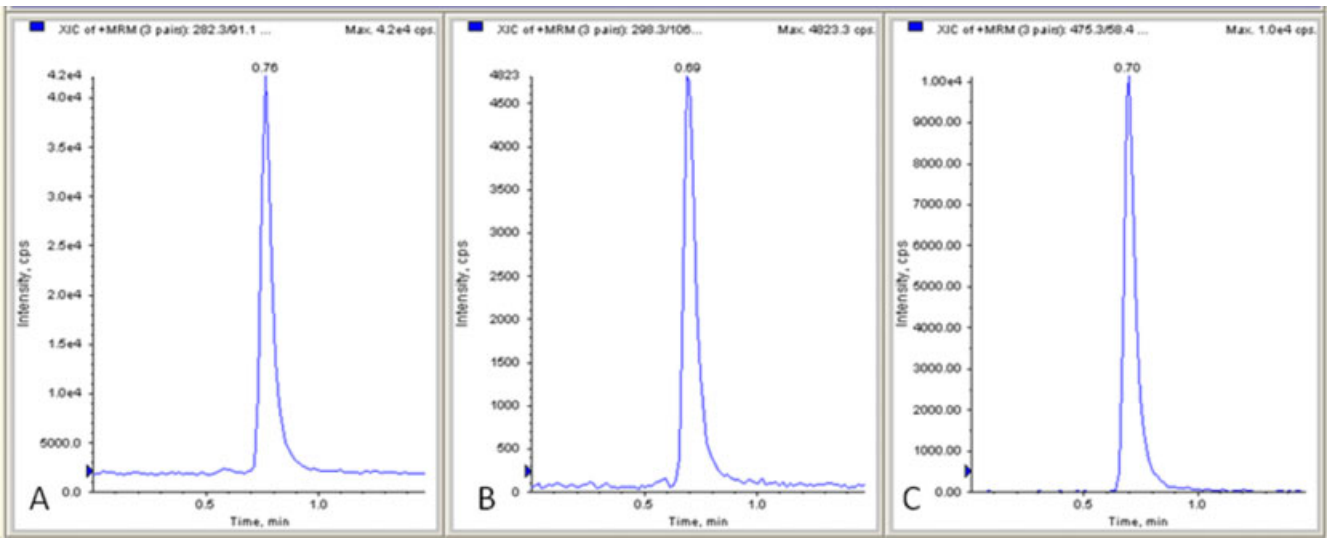

Figure 5. Representative chromatogram of a real study sample.

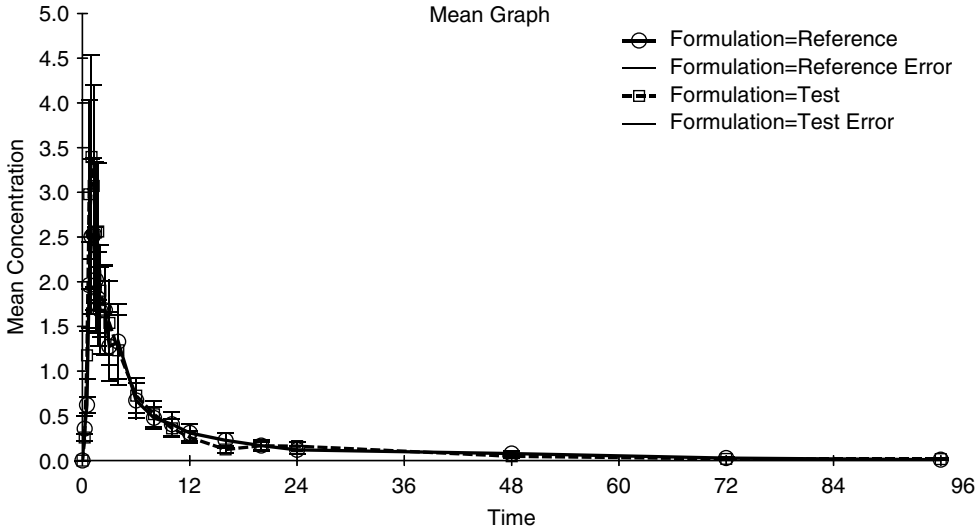

Figure 6A. Concentration versus time profile of ALV in human plasma from 24 subjects receiving a single oral dose of 120 mg ALV capsule as test and reference.

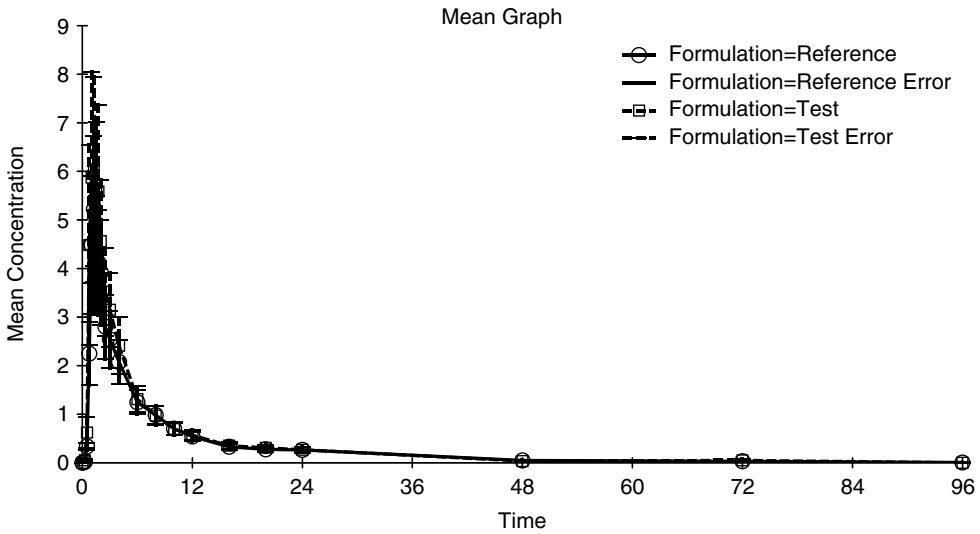

Figure 6B. Concentration versus time profile of PHA in human plasma from 24 subjects receiving a single oral dose of 120 mg ALV capsule as test and reference.

Application

This fully validated method was applied to determine the concentration time profile following single-dose administration of ALV in healthy human volunteers. The chromatograms obtained from analysis of real samples are presented in Figure 5 for ALV and PHA. After LC-MS/MS analysis the plasma concentration of ALV and PHA for all volunteers at times 0.00, 0.333, 0.667, 1.0, 1.333, 1.667, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0, 72.0, and 96.0 h for the test and reference products were measured. The C_{max} for both test and reference products were 8.293 ± 2.640 ng/mL and 5.519 ± 1.345 ng/mL, AUC_{0-t} for both test and reference products were 30.752 ± 7.136 ng × h/mL and 27.350 ± 6.030 ng × h/mL, $AUC_{0-\infty}$ for both test and reference products were 34.546 ± 7.205 ng × h/mL and 30.198 ± 5.993 ng × h/mL, T_{max} for both test and reference products were 1.406 ± 0.070 h and 1.521 ± 0.135 h, $t_{1/2}$ for both test and reference products were 13.303 ± 1.948 h and 12.502 ± 1.536 h and K_{el} for both test and reference products were 0.073 ± 0.008 h^{-1} and 0.070 ± 0.006 h^{-1}. The concentration versus time profile for both ALV and PHA is presented in Figures 6A and 6B.

Conclusion

A simple, sensitive, selective, precise, accurate, and fast LC-MS\MS method for simultaneous determination of ALV and its hydroxy metabolite (PHA) in human plasma, over a range of 0.060–10.051 ng/mL for ALV and 0.059–10.017 ng/mL for PHA, was developed and validated. This method requires only 0.500 mL of biological samples, owing to simple sample preparation and shortest run time (1.5 min); it allows high sample throughput with almost 800 samples per day. The method was successfully applied to a single dose 120 mg capsule bio equivalence study of ALV and its major metabolite, PHA.

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