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Short communication

Development and validation of highly sensitive method for determination of misoprostol free acid in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry: Application to a clinical pharmacokinetic study

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ABSTRACT

A highly sensitive, selective and evaporation free SPE extraction, ESI-LC–MS/MS method has been developed for estimation of misoprostol free acid in human plasma using misoprostol acid-d_5 as an internal standard (IS). The analyte was separated using isocratic mobile phase on reverse phase column and analyzed by MS/MS in the multiple reaction monitoring mode using the respective [M−H] anions, *m/z* 367–249 for misoprostol acid and *m/z* 372–249 for the IS. The total run time was 5.0 min and the elution of misoprostol acid and misoprostol acid-d_5 (IS) occurred at 3.6 min. The developed method was validated in human plasma with a lower limit of quantification of 2.5 pg/mL. A linear response function was established for the range of concentrations 2.5–1200 pg/mL ($r > 0.998$) for misoprostol acid in human plasma. The intra and inter-day precision values for misoprostol acid met the acceptance as per FDA guidelines. Misoprostol acid was stable in the battery of stability studies viz., bench-top, auto-sampler and freeze/thaw cycles. The developed assay method was applied to an oral pharmacokinetic study in humans.

1. Introduction

Misoprostol is a synthetic analogue of prostaglandin E_1 (PGE_1), extensively absorbed, and undergoes rapid de-esterification to its free acid (Fig. 1) (CAS no. 112137-89-0) (9-oxo-11α, 16-dihydroxy-16-methyl-prost-13E-en-1-oic acid) in the gastrointestinal tract after oral administration, which is responsible for its clinical activity and, unlike the parent compound, is detectable in plasma. The alpha side chain undergoes beta oxidation and the beta side chain undergoes omega oxidation followed by reduction of the ketone to give prostaglandin F analogs [3]. The compound is a lipophilic methyl ester prodrug and is readily metabolized to the free acid, which is the biologically active form [1–3]. It was introduced for treatment of gastric ulcer under non-steroidal anti-inflammatory drugs (NSAID) and is approved for this indication in >85 countries under the brand name of Cytotec®. Beyond this it is used worldwide for a variety of indications in obstetrics and gynecology [4–6]. It has both gastric antisecretory and mucosal protective effects.

In normal volunteers, Cytotec (misoprostol) is rapidly absorbed after oral administration with a T_{max} of misoprostol acid of 12 ± 3 min and a terminal half-life of 20–40 min. There is high variability of plasma levels of misoprostol acid between and within studies but mean values after single doses show a linear relationship with dose over the range of 200–400 mcg. Maximum plasma concentrations of misoprostol acid are diminished by 50% when the dose is taken with food and total availability of misoprostol acid is reduced. Drug interaction studies between misoprostol and several nonsteroidal anti-inflammatory drugs showed no effect on the kinetics of ibuprofen or diclofenac [3,7].

Few methods were reported earlier for the determination of misoprostol acid in human plasma or serum using radioimmunoassay [1,9–11], GC/MS/MS [11], LC/MS [12–15] and LC/MS/MS [6,16,17] method in human plasma, but several disadvantages has been come across such as lack of complete validation data, labor-intensive derivatization procedures, time-consuming sample extraction, usage of large plasma volume, provided a higher lower limit of quantification 10 pg/mL and a long chromatographic run time (>7 min). Hence to characterize clinical pharmacokinetic properties of misoprostol, a highly sensitive and rapid analytical method is required for the quantification of its active metabolite misoprostol acid in plasma samples. To achieve this purpose we are now presenting a highly sensitive, selective fully validated LC–MS/MS method which has overcome the draw backs of the previously reported methods viz., usage of single step evaporation free

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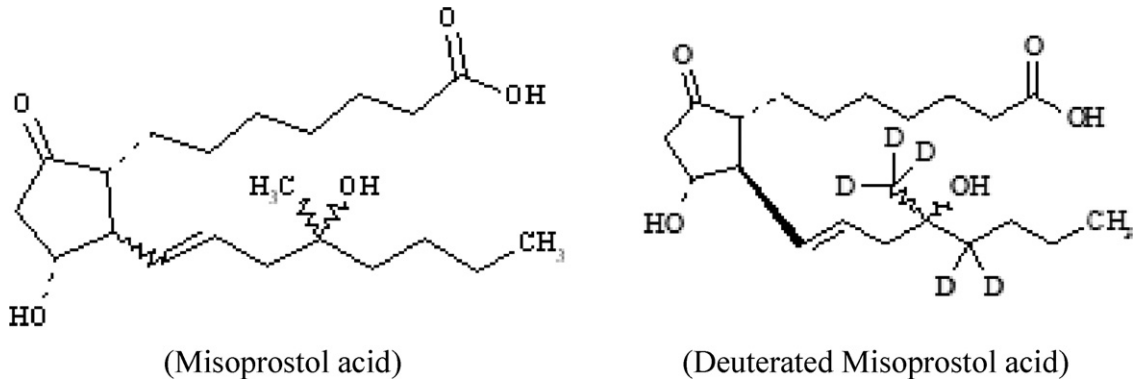

Fig. 1. Structural representation of misoprostol acid and deuterated misoprostol acid (d_5).

extraction method which has enabled sensitive and reproducible lower LLOQ of 2.5 pg/mL which is 4-fold less than the previously reported LLOQ for misoprostol acid. In the present method misoprostol acid-d_5 was used as IS to track the analyte response which is most suitable IS when compared to hydrochlorothiazide (IS) [18]. Sample extraction has become much simpler and rapid since it does not have evaporation and reconstitution steps. The current method is sensitive enough to characterize single dose of 200 μg dose as per FDA guidance on misoprostol [8]. The method was successfully applied to pharmacokinetic studies following the oral administration of single dose of misoprostol 0.2 mg tablets vs. previously published methods which has used oral dose of 0.6 mg misoprostol [18]. Hence, in all facets the current method is superior to previously reported methods [1,6,9–18].

2. Experimental

2.1. Chemicals and reagents

Misoprostol free acid and Misoprostol free acid-d_5 was obtained from Sigma–Aldrich, Germany. All the compounds were found to be >98.5% purity determined by chromatographic (HPLC, LC–MS/MS) analysis. Chemical structures are presented in Fig. 1. HPLC grade of acetonitrile; analytical grade ammonium acetate and formic acid was purchased from Merck Specialties Pvt. Ltd, Mumbai, India. All aqueous solutions including the buffer for the mobile phase were prepared with Milli Q (Millipore, Milford, MA, USA) grade water. The control K2 EDTA human plasma was purchased from registered blood bank, Secunderabad, India.

2.2. Instrumentation and chromatographic conditions

An Agilent (Agilent Technologies, Waldbronn, Germany) 1100 series LC system equipped with degasser (G1322A), isopump (G1310A) along with auto-sampler (G13167B) was used to inject 10 μL aliquots of the processed samples on a Zodiacsil 120-5-C_{18} H column (150 mm × 4.6 mm, 5 μm, Zodiac Life Sciences, USA), which was kept at room temperature (24 ± 2 °C). The isocratic mobile phase, a mixture of 0.2% formic acid buffer and acetonitrile mixture (20:80, v/v) was delivered at 0.50 mL/min into the mass spectrometer's electrospray ionization chamber.

Quantitation was achieved by MS–MS detection in negative ion mode for both misoprostol acid and IS, using a MDS Sciex (Foster City, CA, USA) API-4000 mass spectrometer, equipped with a Turboionspray™ interface at 400 °C. The ion spray voltage was set at 4000 V. The common parameters viz., nebulizer gas, curtain gas, auxillary gas and collision gas were set at 35 psi, 25 psi, 40 psi and 5 psi, respectively. The compound and IS parameters viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were −45 V, −22 V, 10 V, −6 V. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, the transition pairs of misoprostol acid at the *m*/*z* 367.1 amu precursor ion to the *m*/*z* 249.0 amu, 372.1 amu precursor ion to the *m*/*z* 249.0 amu product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (version 1.4.2).

2.3. Standard solutions

Primary stock solutions of calibration curve (CC) standards and quality control (QC) samples were prepared by weighing separately. The primary stock solution (1.00 mg/mL) of misoprostol acid and IS were prepared in acetonitrile and stored at −20 °C, which were found to be stable for one month (data not shown). Appropriate dilutions were made in acetonitrile to produce working stock solutions of 117.65, 105.89, 88.21, 58.83, 17.65, 5.88, 1.25, 0.49 and 0.24 ng/mL on the day of analysis and these stocks were used to prepare CC standards. Another set of working stock solutions were made in acetonitrile (from primary stock) at 100.98, 53.46, 0.72 and 0.248 ng/mL for preparation of QC samples accordingly. Working stock solutions were stored at approximately 5 °C for a week (data not shown). A working IS solution (50 ng/mL) was also prepared in acetonitrile. Calibration curve standards were prepared by spiking 490 μL of control human plasma with the appropriate amount of analytes (10 μL) and IS (50 μL) on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control human plasma in bulk at appropriate concentrations and 200 μL volumes were aliquoted into different tubes and depending on the nature of experiment samples were stored at −80 ± 10 °C until analysis.

2.4. Sample preparation

To an aliquot of 200 μL human plasma sample, IS solution (50 μL) was added; 500 μL of 10 mM of ammonium acetate buffer, and vortex mixed for 30 s on a cyclomixer (Remi Instruments, Mumbai, India). This sample mixture was loaded on pre-conditioned (1 mL acetonitrile followed by 1 mL water) Oasis MAX cartridges (1 cc, 30 mg) and washed with 1 mL 10 mM ammonium acetate buffer, 1 mL water followed by 1 mL 20% acetonitrile in water and finally eluted with 500 μL of mobile phase. From the eluate 10 μL was directly injected onto LC–MS/MS system.

2.5. Method validation

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [19].

The selectivity of the method was determined by analyzing six different batches of human plasma as is, to demonstrate the lack of chromatographic interference from endogenous plasma components. Sets of spiked CC standards and QC samples ($n = 6$ at each concentration) were prepared and analyzed on four different occasions to evaluate linearity, precision and accuracy. Precision and accuracy was also assessed at the lowest concentration of the standards (2.5 pg/mL), representing the lower limit of quantification (LLOQ) for the assay.

The recovery of misoprostol acid and IS was determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 6$) with the response of analytes from neat samples at equivalent concentrations. Recovery was determined at low, medium and high quality control concentrations, whereas the recovery of the IS was determined at a single concentration of 50 ng/mL. The effect of plasma constituents over the ionization of analytes and IS was determined by comparing the responses of the post extracted plasma standard QC samples ($n = 6$) with the response of analytes from neat samples at equivalent concentrations [20,21].

The stability of analytes and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 72 h (in auto-sampler) after the initial injection. The peak-areas of the analytes and IS obtained at initial cycle were used as the reference to determine the relative stability of the analytes at subsequent points. Stability of analytes in the biomatrix after 8 h exposure (bench top) was determined at two concentrations in six replicates. Long term stability of the analytes in biomatrix was assessed by analyzing the QC samples stored at -80 ± 10 °C for at least 90 days. The stability of analytes in biomatrix following repeated three freeze/thaw cycles (stored at −80 ± 10 °C between cycles) was assessed using QC samples spiked with analytes. Samples were processed as described under Section 2.4. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., 15% R.S.D.) and precision (i.e., 15% R.S.D.) [19].

2.6. Pharmacokinetic study

A pharmacokinetic study was performed in healthy male volunteers. The ethics committee approved the protocol and the volunteers provided with informed written consent. The volunteers participated in the study were healthy, adult humans between 18 and 45 years of age and body mass index was between 18.5 and 24.9 kg/height in m^2. In each period volunteers received single dose of 0.2 mg misoprostol tablet while in the sitting posture with about 240 mL of drinking water at ambient temperature according to the randomization schedule in presence of principal investigator. About, 2 mL of blood was collected into polypropylene tubes containing K_2 EDTA solution as anti-coagulant at pre-dose 0.17, 0.25, 0.33, 0.42, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, and 4.0 h. Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 1760 × *g* for 5 min and stored frozen at −80 ± 10 °C until analysis.

An aliquot of 200 μL of thawed plasma samples were spiked with IS and processed as mentioned in sample preparation Section 2.4. Along with study samples, QC samples at low, medium and high concentration were assayed in duplicate and were distributed among unknown samples in the analytical run. The criteria for acceptance of the analytical runs encompassed the following: (i) not more than 33% of the QC samples were greater than ±15% of the nominal concentration (ii) not less than 50% at each QC concentration level must meet the acceptance criteria. Plasma concentration–time data of misoprostol acid was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA).

3. Results

3.1. Method development

3.1.1. Sample pre-treatment

Different methods of sample pre-treatment were investigated. Protein precipitation using acidified acetonitrile or methanol gave strong interferences. Liquid–liquid extraction with various organic solvents such as hexane, methyl tert-butyl ether, diethyl ether and ethyl acetate and their mixtures resulted in non-reproducible recoveries and interferences from the sample matrix with the chromatography of the analytes (data not shown). Subsequently, SPE was investigated as samples pre-treatment technique. Hydrophilic–lipophilic balance, cation exchange and anion exchange cartridges were used for optimizing the extraction procedure. Hydrophilic–lipophilic balance cartridges were investigated as per Oasis® SPE protocol and also with several dilution, conditioning, washing and elution reagents and it resulted in good recovery but had strong matrix interferences, whereas anion exchange cartridges, Oasis MAX cartridges (1 cc, 30 mg) with several dilution, conditioning, washing and elution reagents gave consistent results in terms of recovery of misoprostol acid and its IS and also gave cleaner plasma blank samples. The SPEs were pre-conditioned (1 mL acetonitrile followed by 1 mL water) and sample mixture was loaded and were washed with 1 mL 10 mM ammonium acetate buffer, 1 mL water followed by 1 mL 20% acetonitrile in water and finally eluted with 500 μL of mobile phase. From this eluate was directly injected into the LC–MS/MS system.

3.1.2. Liquid chromatography

In pursuit of symmetric peak shape and retention time of ~2.8 min, feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid with variable pH range of 4.0–7.0, along with altered flow-rates (in the range of 0.3–1.0 mL/min) were tested for complete chromatographic resolution of misoprostol acid and IS (data not shown). The resolution of peaks was achieved with 0.2% formic acid and acetonitrile mixture (20:80, v/v) with a flow rate of 0.5 mL/min, on a Zodiacsil C_{18} column and was found to be suitable for the determination of electrospray response for misoprostol acid and IS.

3.1.3. Mass spectrometry

In order to optimize ESI conditions for misoprostol acid and IS, quadruple full scans were carried out in negative ion detection mode. During a direct infusion experiment, the mass spectra for misoprostol acid and IS revealed peaks at *m/z* 367.1 amu and 372.1 amu, respectively as anion, [M−H]. Following detailed optimization of mass spectrometry conditions (provided in Instrumentation and chromatographic conditions section) *m/z* 367.1 amu precursor ion to the *m/z* 249.0 amu was used for quantification for misoprostol acid. Similarly, for IS *m/z* 372.1 amu precursor ion to the *m/z* 249.0 amu was used for quantification purpose.

3.2. Selectivity

A typical chromatogram for the control human plasma (free of analyte and IS) and human plasma spiked with misoprostol acid at LLOQ are shown in Fig. 2, respectively. No interfering peaks from endogenous compounds are observed at the retention times of analytes and IS. The retention time of misoprostol acid and IS was 3.6 min. The total chromatographic run time was 5 min.

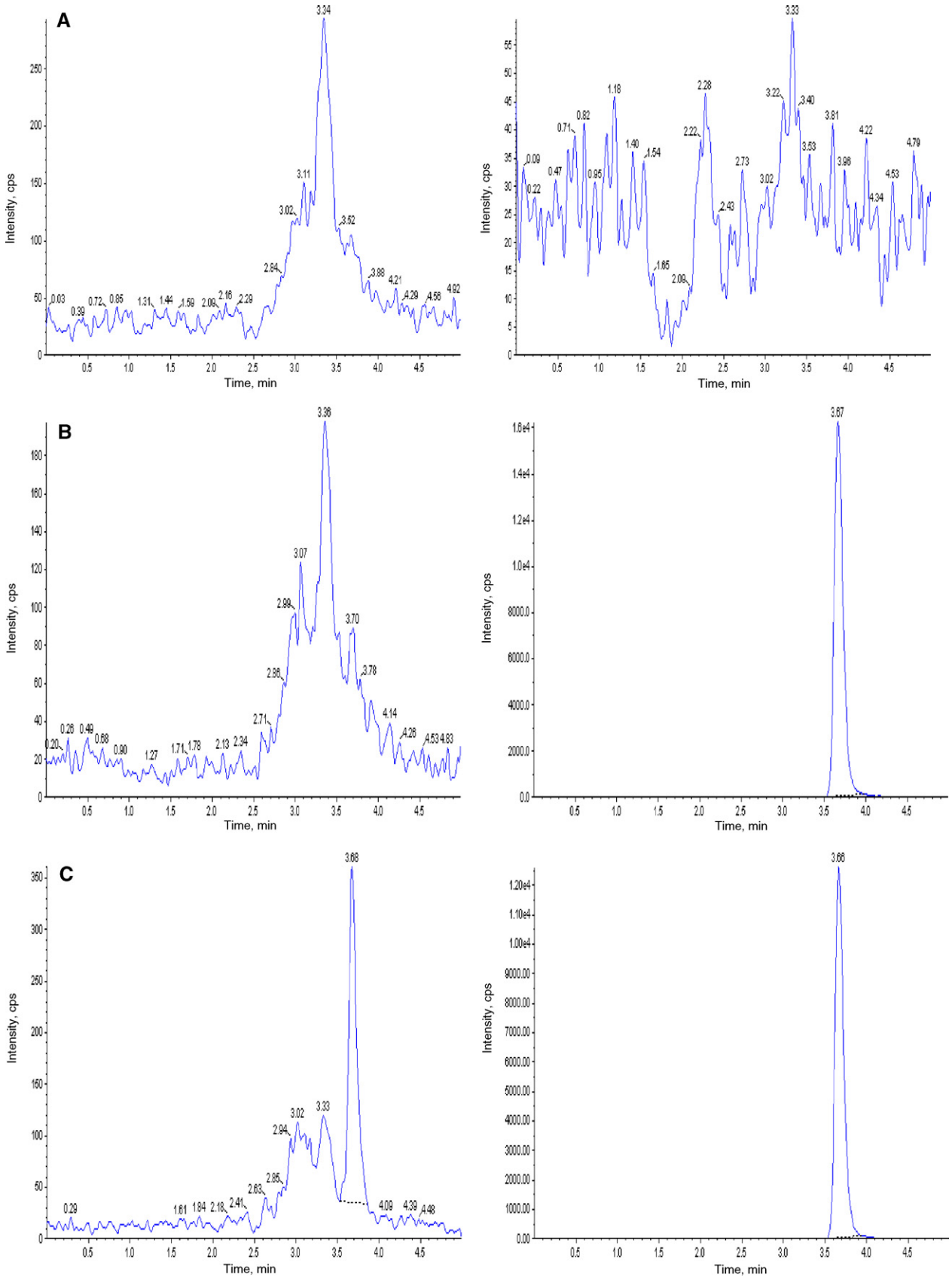

Fig. 2. Typical MRM chromatograms of misoprostol acid (left panel) and IS (right panel) in (A) human blank plasma, (B) human blank plasma spiked with IS, (C) human plasma spiked with misoprostol at LLOQ (2.5 pg/mL) and IS (D) a 0.33 h (672.42 pg/mL) plasma sample showing misoprostol acid peak obtained following oral dose of misoprostol tablet to healthy volunteer along with IS under fasted conditions (E) a 0.42 h (428.27 pg/mL) plasma sample showing misoprostol acid peak obtained following oral dose misoprostol tablet to healthy volunteer along with IS under fed conditions.

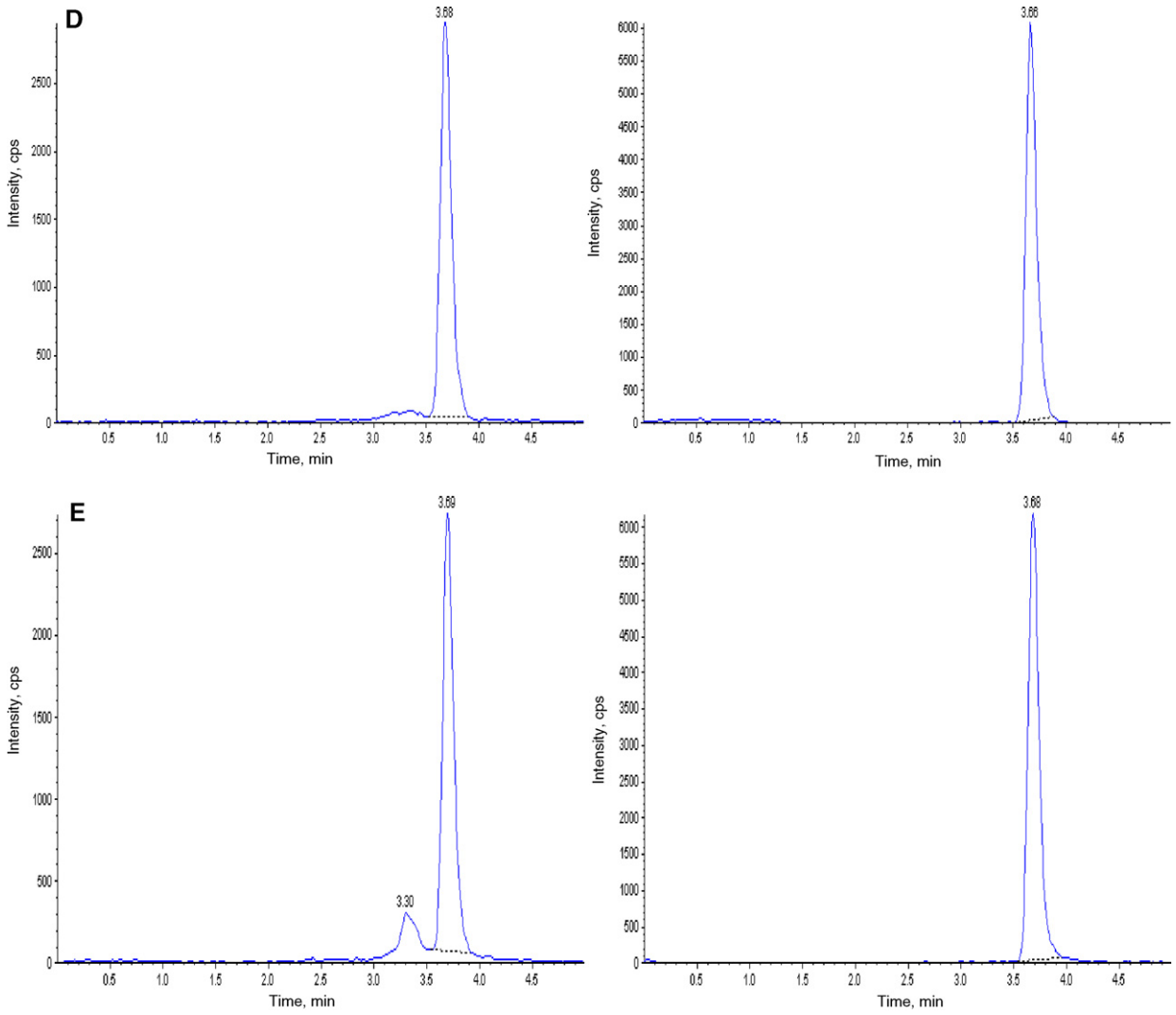

Fig. 2. *Continued*

Table 1
Intra- and inter-day precision of determination of misoprostol acid in human plasma.

Theoretical concentration (pg/mL)	Run	Measured concentration (pg/mL)			
		Mean	SD	RSD	Accuracy (%)
Intra day variation (six replicates at each concentration)					
2.475	1	2.587	0.262	10.15	104.5
	2	2.667	0.190	7.14	107.7
	3	2.503	0.288	11.50	101.1
	4	2.390	0.217	9.10	96.6
7.362	1	7.653	0.429	5.61	104.0
	2	7.523	0.541	7.19	102.2
	3	7.323	0.594	8.11	99.5
	4	7.213	0.753	10.43	98.0
534.620	1	527.752	32.332	6.13	98.7
	2	521.057	33.280	6.39	97.5
	3	554.218	43.228	7.80	103.7
	4	539.990	60.951	11.29	101.0
1009.850	1	1083.437	88.670	8.18	107.3
	2	1008.000	68.923	6.84	99.8
	3	1054.220	101.348	9.61	104.4
	4	1089.838	94.543	8.67	107.9
Inter day variation (eighteen replicates at each concentration)					
2.475		2.54	7.43	535.75	1058.87
7.362		0.25	0.58	42.99	89.48
534.620		9.82	7.78	8.03	8.45
1009.850		102.5	100.9	100.2	104.9

Table 2
Stability data – quality controls in human plasma.

Nominal conc. (pg/mL)	Stability	Mean ± S.D.[a] $n = 6$ (pg/mL)	Accuracy (%)[b]	Precision (% CV)
2.475	0 h (for all)	7.413 ± 0.650	100.7	8.77
	3rd freeze–thaw	7.402 ± 0.610	100.5	8.25
	8 h (bench-top)	7.558 ± 0.562	102.7	7.44
	72 h (in-injector)	7.193 ± 0.660	97.7	9.18
	90 days at −80 °C	7.735 ± 0.406	105.1	5.25
1009.850	0 h (for all)	1040.683 ± 95.227	103.1	9.15
	3rd freeze–thaw	1023.530 ± 89.042	101.4	8.70
	8 h (bench-top)	1063.877 ± 85.110	105.3	8.00
	72 h (in-injector)	1062.697 ± 73.954	105.2	6.96
	90 days at −80 °C	1100.392 ± 93.800	109	8.52

[a]Back-calculated plasma concentrations; [b](Mean assayed concentration/mean assayed concentration at 0 h) × 100.

3.3. Recovery

Recovery was found to be 85.35 (CV = 3.78%), 82.53 (CV = 1.89%) and 80.29 (CV = 2.87%) at LQC, MQC and HQC, respectively for misoprostol acid. The mean recovery for misoprostol acid was found to be 82.72 (CV = 3.07%). The recovery of IS was 86.58 (CV = 4.90%).

3.4. Matrix effect

In this study, the matrix effect was evaluated by analyzing LLOQ sample. Average matrix factor values (matrix factor = response of plasma post spiked concentrations/response of neat concentrations) obtained for misoprostol acid was +0.96 (CV: 6.98%, $n = 6$), at LLOQ level, whereas on IS it was found to be +0.98 (CV: 3.83%, $n = 6$) at tested concentration of 50 ng/mL.

3.5. Calibration curve

The plasma calibration curve was constructed using calibration standards of 2.50–1200.00 pg/mL. Calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) vs. concentration, and fitted to the $y = mx + c$ using weighing factor ($1/X^2$). The average regression ($n = 4$) was found to be ≥0.998 respectively. The lowest concentration with the R.S.D. < 20% was taken as LLOQ [18] and was found to be 2.5 pg/mL. The % accuracy observed for the mean of back-calculated concentration for four linearities was within 94.17–105.02. The precision (% CV) values ranged from 0.98 to 4.68.

3.6. Precision and accuracy

The accuracy, intra and inter-assay precision which were determined by analyzing six replicates of QC samples at four concentrations on two different days are shown in Table 1.

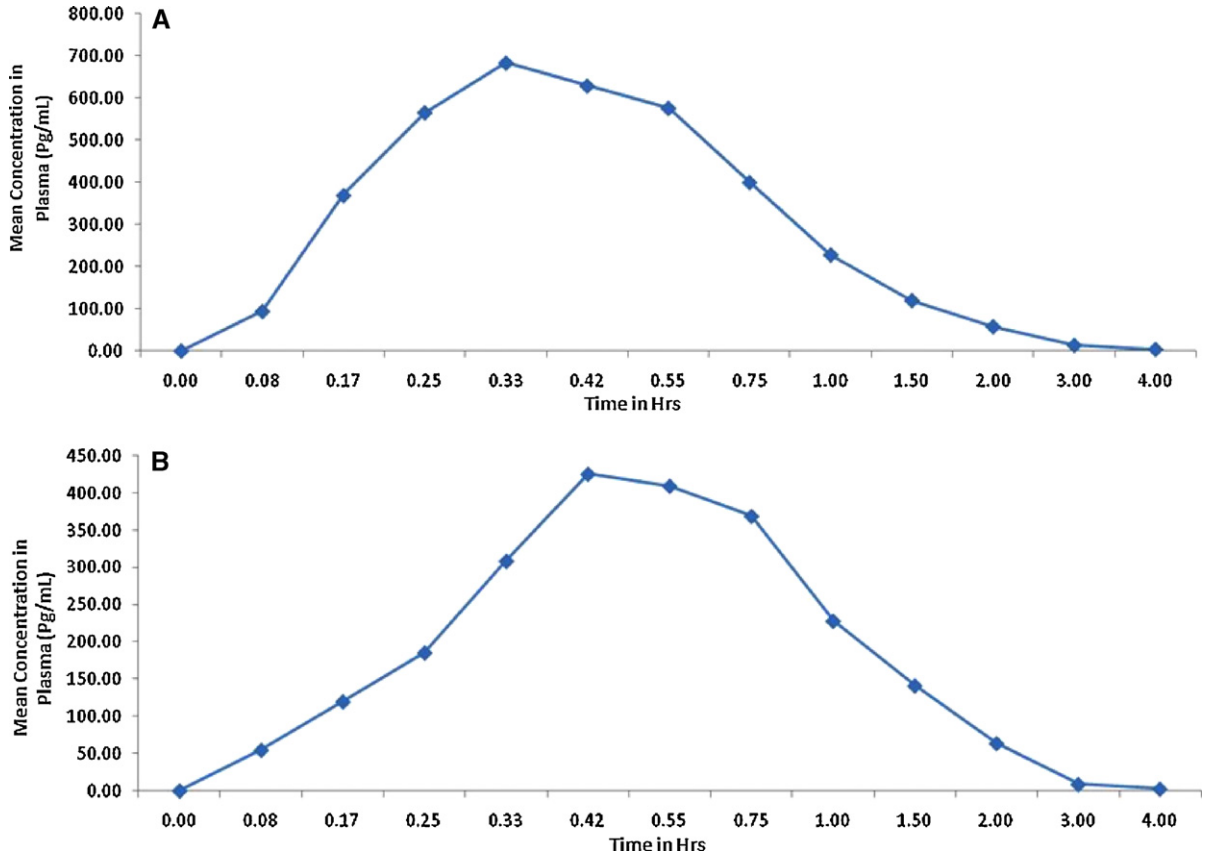

Fig. 3. Mean plasma concentration–time profile of misoprostol acid in human plasma following oral dosing of 0.2 mg misoprostol tablet to 18 subjects under (A) fasting and (B) fed conditions.

3.7. Stability

The predicted concentrations for each analyte at LQC and HQC samples deviated within ±15% of the nominal concentrations in a batter of stability tests viz., in-injector (72 h), bench-top (8 h), repeated three freeze/thaw cycles and at long term at −80 ± 10 °C for at least for 90 days (Table 2). The results were found to be within the assay variability limits during the entire process.

3.8. Pharmacokinetic study

The present method was applied to the analysis of plasma samples obtained from 24 healthy human volunteers following oral administration of 0.2 mg of misoprostol tablets manufactured by Dr. Reddy's Laboratories Limited as a part of pharmacokinetic study. The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of misoprostol acid in humans. Fig. 3A depicts the mean plasma concentration vs. time profile of misoprostol acid in these volunteers under fasted conditions. Following the oral administration of 0.2 mg of misoprostol tablets to volunteers under fasting conditions, the mean maximum plasma concentrations (C_{max}), 672.42 pg/mL, were attained at ~0.33 h (T_{max}), while the $AUC_{(0-\infty)}$ was 366 pg * h/mL.

Fig. 3B depicts the mean plasma concentration vs. time profile of misoprostol acid in these volunteers under fed conditions. Following the oral administration of 0.2 mg of misoprostol tablets to volunteers, the mean maximum plasma concentrations (C_{max}), 428.27 pg/mL, were attained at ~0.42 h (T_{max}), while the $AUC_{(0-\infty)}$ was found to be 226.09 pg * h/mL.

4. Discussion

To the best of our knowledge, we have developed for the first time fully validated LC–MS/MS method for the determination of misoprostol acid, which provides the highest sensitivity (2.5 pg/mL) using a simple SPE extraction procedure which did not involve reconstitution or drying step to achieve the desired sensitivity. Usually drying and reconstitution step is used to obtain lower sensitivity but in the present method directly SPE eluate gives sensitivity as low as 2.5 pg/mL, further we can still go lower by adding drying and reconstitution step to the current method. A good internal standard should track the analyte during extraction and any inconsistent response due to matrix effect. This is also established with almost the same recovery of IS compared to the analyte. The most appropriate IS for typical anions are none other than deuterated compounds and hence misoprostol acid-d_5 was used as IS. Results obtained by usage of d_5 internal standard were consistent and reproducible which was evident by incurred sample analysis conducted on this study. The use of only 10 μL of the final eluate gave an on-column loading of 0.10 pg/injection for misoprostol acid. This minimizes matrix interference and suppression of analyte peak and helps to extend the life of the column. Hence the sensitivity further can be brought down at least four times by using the present method.

5. Conclusions

In summary, we have developed and validated a highly sensitive, specific and reproducible LC–MS/MS assay to quantify misoprostol acid in human plasma. From the results of all the validation parameters, we can conclude that the present method can be useful for pharmacokinetic studies with desired precision and accuracy.

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