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Determination of misoprostol acid in human plasma by liquid chromatography coupled to tandem mass spectrometry

Yu Zou [a,b], Xiaoyan Chen [a], Bo Song [a], Dafang Zhong [a,*]

[a] *Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 646 Songtao Road, Shanghai 201203, PR China*
[b] *Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, PR China*

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Abstract

A rapid and sensitive liquid chromatographic/tandem mass spectrometric method for determination of misoprostol acid, the active metabolite of misoprostol, was developed and validated. Following liquid–liquid extraction, the analytes were separated using an isocratic mobile phase on a C_{18} column. An API 4000 tandem mass spectrometer equipped with Turbo IonSpray ionization source was used as detector and was operated in the negative ion mode. Multiple reaction monitoring using the precursor to product ion combinations of *m/z* 367–249 and 296–269 was performed to quantify misoprostol acid and the internal standard hydrochlorothiazide, respectively. The method was linear in the concentration range of 10.0–3000 pg mL^{-1} using 200 μL plasma. The lower limit of quantification was 10.0 pg mL^{-1}. The intra- and inter-day relative standard deviation over the entire concentration range was less than 8.3%. Accuracy determined at three concentrations (25.0, 200 and 2700 pg mL^{-1} for misoprostol acid) ranged from −0.5 to 1.2% in terms of relative error. Each plasma sample was chromatographed within 3.5 min. The method was successfully used in a pharmacokinetic study of misoprostol in human plasma after an oral administration of 0.6 mg misoprostol.

Keywords: Liquid chromatography–tandem mass spectrometry; Misoprostol; Misoprostol acid; Pharmacokinetics

1. Introduction

Misoprostol is a synthetic analogue of prostaglandin E_1 (PGE_1), which is rapidly and extensively hydrolyzed to its biologically active metabolite misoprostol acid in the gastrointestinal tract after oral administration. Unchanged misoprostol cannot be detected in the plasma even at 5 min after oral dose [1,2]. Misoprostol has been approved in more than 85 countries for the prevention and treatment of gastric ulcers and is also frequently used in the field of obstetrics and gynaecology owing to its uterotonic and cervical-ripening actions [3–5]. The therapeutic dose of misoprostol given orally is not more than 0.8 mg day^{-1}. The maximum plasma concentration of the active compound misoprostol acid in human plasma is less than 1.6 ng mL^{-1} [6–9]. Due to the low plasma concentration of misoprostol acid, a sensitive and simple analytical method is needed for its determination.

Several methods for the determination of misoprostol acid in biological matrices have been reported including radioimmunoassay [1,2,6], GC with electron capture mass spectrometry [7] or tandem mass spectrometry detection [9–11] and LC with mass [12] or tandem mass spectrometry [5,13,14]. Among them, only two [10,13] report complete validation data. The remaining methods are pharmacokinetic studies that lack complete validation data. Watzer et al. [10] reported a GC/MS/MS method for determination of misoprostol acid in human breast milk and serum. The method provided a lower limit of quantification (LLOQ) of 10 pg mL^{-1} in serum, but required complicated and labor-intensive derivatization procedures. An LC/MS/MS method for quantification of misoprostol acid in plasma was described in an early patent [13]. The method provided a LLOQ of 50 pg mL^{-1} using a 1-mL aliquot of plasma and required a long chromatographic run time (>7 min). The other reported LC/MS or LC/MS/MS methods also suffered from several disadvantages, such as time-consuming sample extraction [5] and large plasma volume used [12].

* Corresponding author at: Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 646 Songtao Road, Shanghai 201203, PR China. Tel.: +86 21 50800738; fax: +86 21 50800738.
E-mail address: zhongdf@china.com (D. Zhong).

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To better characterize the clinical pharmacokinetic properties of misoprostol, it is important to develop a highly sensitive and rapid analytical method for the quantification of its active metabolite misoprostol acid in plasma samples. To achieve this purpose, an LC/MS/MS method was developed and validated and it had been successfully applied to pharmacokinetic studies of misoprostol after a single oral of 0.6 mg misoprostol, besides, the method also has the potential of being used in the determination of PGE_1 analogues.

2. Experimental

2.1. Materials

Misoprostol acid (93.8% purity) was purchased from Everlight Chemical Industrial Co. (Taiwan). Hydrochlorothiazide (99.7% purity) for use as the internal standard (I.S.) was kindly donated by Yuanhe Pharmaceutical Co. Ltd. (Innermongolia, China). Methanol (Tedia, Fairfield, CA, USA) was of HPLC grade, and other chemicals were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study. Drug-free plasma for the preparation of calibration standards was obtained from Shanghai Blood Donor Service (Shanghai, China). Before analysis, the blank samples were analyzed by the present LC/MS/MS method. No significant peaks were observed at the retention times of the analyte and I.S.

2.2. Instrument

An Agilent 1100 system consisting of G1311A quaternary pump, G1379A degasser, G1313A autosampler, G1316A thermostatted column compartment (Agilent, Waldbronn, Germany) was used for solvent and sample delivery. An API 4000 triple-quadrupole mass spectrometer equipped with a TurboIonSpray (ESI) source was used for mass analysis and detection (Applied Biosystems, Foster City, CA, USA). Data processing was performed on Analyst 1.4.1 software package.

2.3. Chromatographic conditions

Isocratic chromatographic separation was achieved on a Zorbax SB C_{18} column, 150 mm × 4.6 mm, i.d., 5 μm (Agilent, Wilmington, DE, USA) with a 4 mm × 3.0 mm, i.d., Security-Guard C_{18} guard column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of methanol/water (55:45, v/v, 1% ammonia solution was used to adjust the pH to 6) at a flow rate of 0.5 mL min^{-1}. The column temperature was maintained at 22 °C.

2.4. Mass spectrometric conditions

The mass spectrometer was operated in negative ionization mode. The tuning parameters were optimized for misoprostol acid and the I.S. by infusing a solution containing 200 ng mL^{-1} of both analytes at a flow rate of 10 μL min^{-1} into the mobile phase (0.2 mL min^{-1}) using a post-column 'T' connection. The nebulizer, TurboIonSpray and curtain gases (nitrogen) were set at 50, 60 and 10 psi, respectively. The optimized TurboIonSpray voltage and temperature were set at −4500 V and 450 °C, respectively. For collision-induced dissociation (CID), nitrogen was used as the collision gas at a back-pressure of approximately 3 psi. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions m/z 367 → 249 for misoprostol acid and m/z 296 → 269 for the I.S., respectively, with a dwell time of 200 ms per transition. The optimized collision energy of 25 eV was used for the analyte and 27 eV for the I.S. The mass spectrometer was operated at unit mass resolution (peak width at half-height set at 0.7 Da) for both Q1 and Q3. The value of DP (declustering potential) was set at −58 V.

2.5. Preparation of calibration standards and QC samples

A stock solution of misoprostol acid with a concentration of 938 μg mL^{-1} (calculated from purity) was prepared by dissolving 10.0 mg of misoprostol acid in 10 mL of methanol. Seven standard working solutions of 50.0, 125, 400, 1000, 2500, 7500 and 15,000 pg mL^{-1} of misoprostol acid were made by further dilution of the stock solution with methanol–water (50:50, v/v). The quality control (QC) samples were similarly prepared at concentrations of 125, 1000 and 13,500 pg mL^{-1}, by a separate weighing of the pure standard. A 50 ng mL^{-1} solution of the I.S. was also prepared by diluting the 400 μg mL^{-1} stock solution of hydrochlorothiazide with methanol–water (50:50, v/v).

Matrix-matched calibration standards and QC samples of misoprostol acid were prepared by spiking 40 μL of the working solutions into 200 μL of drug-free plasma. The calibration standards were prepared at concentrations of 10.0, 25.0, 80.0, 200, 500, 1500 and 3000 pg mL^{-1} of misoprostol acid in plasma, while the corresponding QC samples were prepared at 25.0, 200 and 2700 pg mL^{-1}.

These standard-spiked plasma calibration solutions and QC samples were stored at −20 °C. For each batch of unknown samples to be analyzed, the appropriate standard and QC solutions were brought to room temperature, and processed through the plasma sample preparation procedure in parallel with the unknown samples.

2.6. Sample preparation

A 40-μL aliquot of the I.S. solution (hydrochlorothiazide, 50 ng mL^{-1}), 40 μL of methanol–water (50:50, v/v) and 100 μL of 3 M phosphoric acid solution were added to 200 μL of plasma samples. The sample was vortex-mixed and extracted with 3 mL of diethyl ether–dichloromethane (3:2, v/v) by shaking for 10 min; the organic and aqueous phases were separated by centrifugation at 2000 × g (4 °C) for 5 min. The upper organic phase was transferred to another tube and evaporated to dryness at 30 °C under a stream of nitrogen in the TurboVap evaporator (Zymark, Hopkinton, MA, USA). The residue was reconstituted in 150 μL of the mobile phase and vortex-mixed for 1 min. A 20-μL aliquot of the reconstituted extract was injected for the LC/MS/MS analysis.

2.7. Method validation

Plasma samples were quantified using the ratio of the peak area of analyte to I.S. as the assay response. To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on three consecutive days over the range of 10.0–3000 pg mL^{-1}. Least-squares linear regression was used for curve fitting with $1/x^2$ as the weighting factor.

QC samples at three concentration levels (25.0, 200 and 2700 pg mL^{-1}) were analyzed to assess the accuracy and precision of the method. Again, the assays were performed on three separate days, and on each day six replicates of QC samples at each concentration level were analyzed. The accuracy and precision were calculated using one-way ANOVA. The accuracy was expressed by relative error (R.E.) and the precision by relative standard deviation (R.S.D.). The intra- and inter-day precisions were required to be below 15%, and the accuracy to be within ±15%.

The LLOQ defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was evaluated by analyzing samples which were prepared in six replicates.

The extraction recoveries of misoprostol acid at three QC levels ($n = 6$) were determined by comparing peak-area ratios of the analyte to I.S. in samples that were spiked with the analyte prior to extraction with samples to which the analyte was added post-extraction. The I.S. was added to both of these sets of samples post-extraction. The extraction recovery of the I.S. was determined in a similar way using the QC samples at medium concentration as a reference.

To evaluate the matrix effect, i.e., the potential ion suppression or enhancement due to co-eluting plasma components, six different lots of blank plasma were extracted and then spiked with the analyte at 80.0 and 2700 pg mL^{-1}. The corresponding peak areas of the analyte in spiked plasma post-extraction (A) were then compared to those of the solution standards in mobile phase (B) at equivalent concentrations. The ratio (A/B × 100) is defined as the absolute matrix effect (ME). A value of 100% indicates that the responses for misoprostol acid in the mobile phase and in the plasma extracts were the same and that no absolute ME was observed. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression. The assessment of the relative ME was made by a direct comparison of the analyte peak-area values between different lots of plasma. The variability in the values, expressed as R.S.D. (%), is a measure of the relative ME for the target analyte. The same evaluation was performed for the I.S.

The stabilities of misoprostol acid in human plasma were evaluated by analyzing replicates ($n = 3$) of plasma samples at the concentrations of 80.0 and 2700 pg mL^{-1}, which were exposed to different conditions (time and temperature). These results were compared with those obtained for freshly prepared plasma samples. The analytes were considered stable in the biological matrix when 85–115% of the initial concentrations were found. The short-term stability was determined after the exposure of the spiked samples at 22 °C for 2 h, and the ready-to-inject samples (after extraction) to the autosampler rack (22 °C) for 4 h. The long-term stability was assessed after storage of the standard-spiked plasma samples at −20 °C for 30 days. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (−20 to 22 °C) on consecutive days. The stability of standard solutions was also tested at 22 °C for 6 h and at 4 °C for 20 days.

2.8. Pharmacokinetic study

The validated method was used to determine the plasma concentrations of misoprostol acid from a clinical trial in which 20 healthy and nonpregnant female volunteers received a single oral dosage of 0.6 mg misoprostol. The pharmacokinetic study was approved by the Ethics Committee. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Blood samples were collected into heparinized glass tubes before and 7.5, 15, 30, 45, 60, 90, 120, 180, 240 and 360 min post-dosing, and centrifuged at 2000 × g (4 °C) for 10 min to separate the plasma fractions. The collected plasma samples were stored at −20 °C until analysis.

Determination of the pharmacokinetic parameters was performed by non-compartmental assessment of data using the computer program WinNonlin (WinNonlin V5.0.1, Pharsight Corporation, California, USA). Mean and individual concentration–time profiles were generated and used to determine the maximum plasma concentration (C_{max}) and the time to attain these maximum concentrations (T_{max}). The area under the plasma concentration–time curve from time zero to the time of the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The terminal elimination rate constant (k_e) was estimated by log-linear regression of concentrations observed during the terminal phase of elimination, and the corresponding elimination half-life ($T_{1/2}$) was then calculated as $0.693/k_e$.

3. Results and discussion

3.1. Optimization of the mass spectrometric condition

Misoprostol acid is a polar compound, containing a carboxyl group in its structure. Therefore, it could only be ionized in the negative ionization mode and the signal intensity obtained under ESI source was much higher than that under APCI. For I.S., hydrochlorothiazide has a sulfonamide group in its structure, which presents strong electronegativity, therefore, the mass spectrometric response obtained in negative mode was much higher than that in positive mode as well.

By negative ESI mode, the analyte and internal standard formed predominantly deprotonated molecules $[M-H]^-$ at m/z 367 and 296 in Q1 full scan mass spectra, respectively. No adduct ions were detected. The most suitable collision energy (CE) for the analyte and I.S. was determinded by observing the response of the obtained fragment ion peaks. Fig. 1 displays the product ions spectra of $[M-H]^-$ ions from the analyte and I.S. A predominant fragment ion at m/z 249 was formed when the collision energy was 25 eV and the intensity of $[M-H]^-$ ion for misoprostol acid was reduced by more than 80%. The fragment

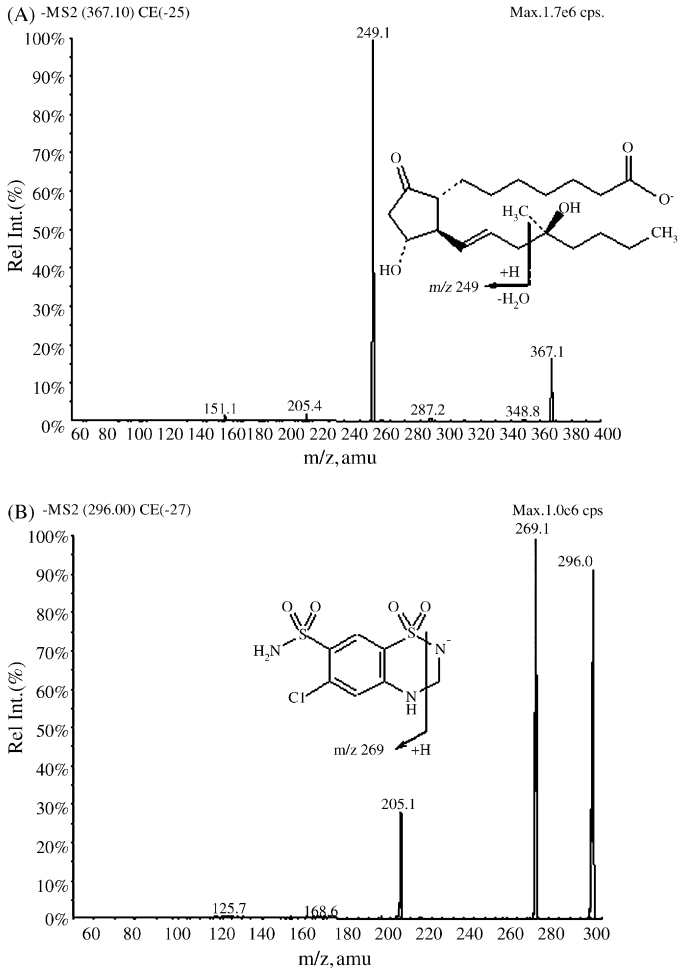

Fig. 1. Product ion spectra of $[M-H]^-$ of misoprostol acid (A) and hydrochlorothiazide (B).

ion at *m/z* 249 was formed by cleavage of one branched chain and H_2O from the $[M-H]^-$ ion. Other fragment ions showed relative intensities below 10% and the sensitivity was not likely to be improved when used in MRM mode. As a result, the transition *m/z* 367 → 249 at CE 25 eV was chosen for use as in MRM. Additional tuning of the ESI source parameters for the transition of *m/z* 367 → 249 further improved the sensitivity. For I.S., the product ion spectrum of the $[M-H]^-$ ion showed a major fragment ion at *m/z* 269 $[M-HCN-H]^-$. The optimum collision energy (27 eV) was determined by observing the maximum response obtained for *m/z* 269.

3.2. Optimization of the chromatographic condition

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve high sensitivity and symmetric peak shapes for the analyte and I.S., as well as a short chromatographic time. In our experiment, we found that methanol can increase the signal (2–3 times) in (−) ESI condition in contrast to acetonitrile employed in literature [12,13]. The similar phenomena was observed in another LC/MS/MS method [15] which determined two PGE_1 analogues of PGE_2 and PGD_2. In that literature, the best signal intensity was obtained with a methanolic mobile phase, however, in order to separate the two isomers of PGE_2 and PGD_2 by liquid chromatography, acetonitrile was used at last. The ammonia solution was found to be necessary in order to improve sensitivity by promoting ionization of the analytes, shorten the chromatographic cycle time and achieve good peak shape. When we added 1% ammonia solution in the mobile phase to adjust the pH to 6 and varied the percentage of organic phase to 55%, the retention time of misoprostol acid was shorten to 2.90 min, which was close to the retention time of I.S. The developed liquid chromatography method was also employed to assay PGE_1 in human plasma (data not shown), and the LLOQ was as low as 10 pg mL^{-1}. Therefore, the developed chromatographic condition is suitable for the determination of PGE_1 analogues.

3.3. Sample preparation

Sample preparation is a critical step for accurate and reliable LC/MS/MS assays. The most widely employed biological sample preparation methodologies currently are liquid–liquid extraction (LLE), protein precipitation (PPT), and solid-phase extraction (SPE). Fiala et al. [14] extracted misoprostol acid from plasma samples using a monolithic reversed phase cartridge. LLE can be helpful in producing a relative clean sample and avoiding the introduction of highly polar materials onto the column and MS system. Misoprostol acid has carboxyl group in its structure, which makes the extraction of the compound from acidified biological fluids possible using different organic solvents. Danielsson et al. [12] used toluene–ethyl acetate (1:1, v/v) and 10% aqueous solution of phosphoric acid to extract misoprostol acid from plasma twice. Andolina et al. [5] introduced a one-step extraction using ethyl acetate–cyclohexane mixture to extract misoprostol acid from acidified plasma by formic acid. In our experiment, we investigated several organic extraction solvents including ethyl acetate and the mixed solvent of diethyl ether–dichloromethane (3:2, v/v). It was found that diethyl ether–dichloromethane (3:2, v/v) could yield the highest recovery (>80%) and it can significantly decrease evaporation time compared with ethyl acetate used in literature [5,12,15]. The acidic modifier was also evaluated in the early method development stage, it was found that 3 M phosphoric acid solution could yield high and reproducible recovery for misoprostol acid instead of 0.5 M phosphoric acid solution.

3.4. Method validation

3.4.1. Assay selectivity and matrix effect

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Fig. 2 shows the typical chromatograms of a blank plasma sample, blank plasma sample spiked with misoprostol acid at the LLOQ and I.S. and a plasma sample obtained at 3 h after an oral administration of 0.6 mg misoprostol to a volunteer. No significant interference from endogenous substances observed at the retention times of the analyte and I.S. Typical retention times for misoprostol acid and hydrochlorothiazide were 2.9 and 3.0 min, respectively.

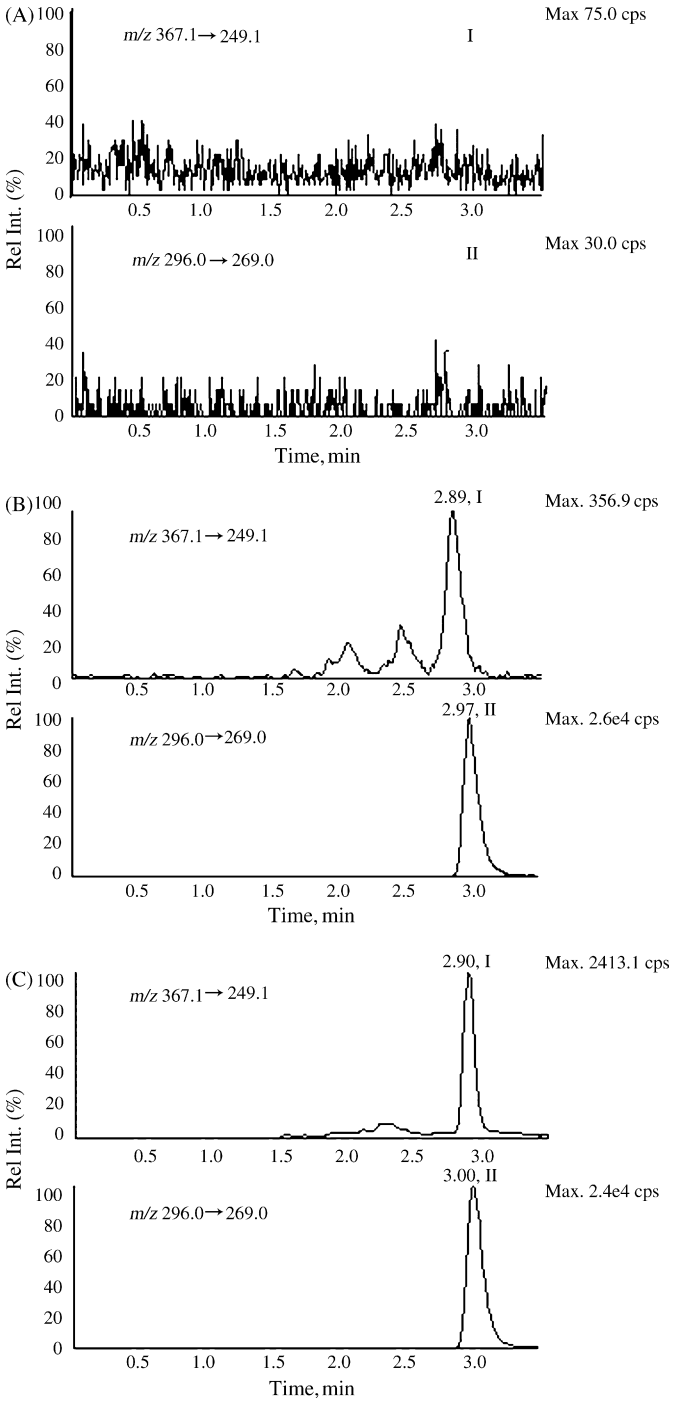

Fig. 2. Representative MRM chromatograms of misoprostol acid (I) and I.S. (hydrochlorothiazide, II) in human plasma: (A) blank plasma sample; (B) plasma spiked with 10.0 pg mL^{-1} misoprostol acid and 10 ng mL^{-1} I.S.; (C) plasma sample 3 h after an oral dose of 0.6 mg misoprostol to a female volunteer.

The absolute matrix effects for misoprostol acid at concentrations of 80.0 and 2700 pg mL^{-1} were 95.7 ± 7.7 and 97.0 ± 5.3%, respectively. The relative matrix effect for misoprostol acid at concentrations of 80.0 and 2700 pg mL^{-1} were 8.0 and 5.4%, respectively. The absolute and relative matrix effects for I.S. (10.0 ng mL^{-1}) were 96.9 ± 5.5 and 5.7%, respectively. As a result, ion suppression from plasma matrix was negligible in the present condition.

Table 1
Precision and accuracy data for the analysis of misoprostol acid in human plasma (in prestudy validation, 3 days, six replicates per day)

Concentration (pg mL^{-1})		R.S.D. (%)		Relative error (%)
Added	Found	Intra-day	Inter-day	
10.0	9.95	7.4	–	−0.5
25.0	25.3	7.1	3.5	1.2
200.0	200.9	6.7	1.7	0.4
2700	2686	5.3	8.3	−0.5

3.4.2. Linearity and lower limit of quantification

The linear regression of the peak-area ratios versus concentrations was fitted over the concentration range of 10.0–3000 pg mL^{-1} in human plasma. A typical equation of the calibration curves was as follows: $y = 3.76 \times 10^{-3} + 1.59 \times 10^{-4}x$ ($r = 0.9964$), where y represents the peak-area ratio of analyte to I.S. and x represents the plasma concentration of misoprostol acid. Good linearity was seen in this concentration range.

The lower limit of quantification was 10.0 pg mL^{-1} for determination of misoprostol acid in plasma. The precision and accuracy at the concentration of LLOQ are shown in Table 1. Under the present LLOQ, the misoprostol acid concentration could be determined in plasma samples up to 6.0 h after a single oral dose of 0.6 mg misoprostol, which is sensitive enough to investigate the pharmacokinetic behavior of the drug.

3.4.3. Precision and accuracy

The method showed good precision and accuracy. Table 1 summarizes the intra- and inter-assay precision and accuracy for misoprostol acid from QC samples. The intra- and inter-assay precisions were measured to be below 7.1 and 8.3%, respectively, with relative errors from −0.5 to 1.2%.

3.4.4. Extraction recovery and stability

Mean extraction recoveries of misoprostol acid at 25.0, 200 and 2700 pg mL^{-1} were 86.6 ± 4.5, 86.7 ± 4.3 and 80.3 ± 4.5%, respectively ($n = 6$). Mean recovery of the internal standard (10.0 ng mL^{-1}) was 76.8 ± 2.2% ($n = 6$).

Acceptable analyte stability was demonstrated for all phases of storage and processing. In order to prevent any decomposition during sample handling, the samples were centrifuged at 4 °C and evaporated to dryness at 30 °C under a stream of nitrogen, which is similar to those reported in the patent [13]. In addition, it was found that misoprostol acid was stable at room temperature for 4 h after the reconstitution of plasma extract residue with mobile phase, but it degraded more than 20% at room temperature after 12 h, suggesting that sample should be analyzed within 4 h after extraction if left at room temperature. The results of stability experiments (Table 2) showed that no significant degradation occurred during chromatography, extraction and sample storage processes for misoprostol acid in plasma samples. In addition, standard stock solutions of misoprostol acid were shown to be stable for at least 6 h at 22 °C and 20 days at 4 °C.

Table 2
Summary of stability of misoprostol acid in human plasma under various storage conditions ($n = 3$)

Storage conditions	Concentration (pg mL^{-1})		R.S.D. (%)	R.E. (%)
	Added	Found		
Three freeze/thaw cycles	80.0	81.7	5.6	2.1
	2700	2693	2.0	−0.2
Freezing for 30 days	80.0	81.7	7.1	2.2
	2700	2667	6.7	−1.2
Autosampler for 4 h (22 °C)	80.0	82.5	8.5	3.2
	2700	2720	4.8	0.7
Short-term (2 h at 22 °C)	80.0	80.5	11.0	0.7
	2700	2740	7.1	1.5

3.5. Application in pharmacokinetic study

This validated analytical method was applied to investigate the pharmacokinetic profiles of misoprostol acid in human plasma after an oral administration of 0.6 mg misoprostol. Profile of the mean plasma concentration of misoprostol acid versus time is shown in Fig. 3. The main pharmacokinetic parameters of misoprostol acid in 20 volunteers were calculated. After oral administration of 0.6 mg misoprostol, T_{max} and C_{max} of misoprostol acid were found to be 28.5 ± 12.8 min and 857 ± 600 pg mL^{-1}, respectively. Plasma concentration declined with $T_{1/2}$ of 1.12 ± 0.33 h. The AUC_{0-t} and $AUC_{0-\infty}$ values obtained were 930 ± 575 and 961 ± 589 pg h mL^{-1}, respectively. Compared with the pharmacokinetic results reported previously [6], it was observed that the value of $T_{1/2}$ in this study is a little longer, but that T_{max} did not differ between them, which might be resulted from the different sensitivities of the analytical methods. Significant inter-individual variabilities in C_{max} and AUC were observed, which are in agreement with previous published pharmacokinetic studies [6,7,9,11]. The value of C_{max} that we obtained is similar to that described by Foote et al. [6], but higher than that reported in pregnant or postpartum subjects [8,11]. For example, Abdel-Aleem et al. [11] reported the C_{max} of 344.6 ± 268.9 pg mL^{-1} after a single oral dose of 0.6 mg misoprostol in postpartum subjects.

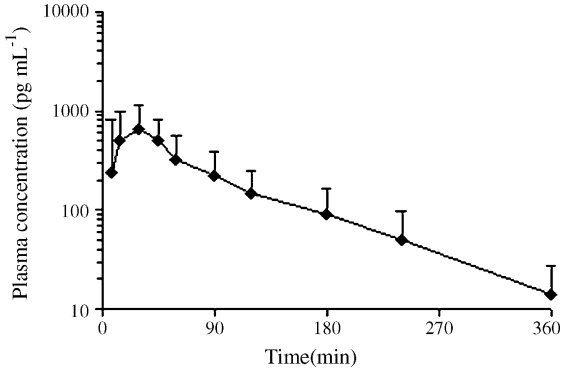

Fig. 3. Mean plasma concentration–time curve of misoprostol acid after a single oral dose of 0.6 mg misoprostol to 20 healthy female volunteers. Each point represents the mean ± S.D.

4. Conclusion

An LC/MS/MS assay for misoprostol acid in human plasma was developed and validated with respect to linearity, precision and accuracy, and analysis of real samples was demonstrated. It was proved to be superior in sensitivity, sample pretreatment and speed of analysis in comparison to the previously reported analytical methods and it had the potential of employing in determination of PGE_1 analogues. This method was successfully applied to pharmacokinetic studies for misoprostol and was found to be sensitive and reliable.

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