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Development and validation of a selective and sensitive LC-MS/MS method for determination of misoprostol acid in human plasma: Application to bioequivalence study

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Abstract: A rapid, sensitive and specific method was developed and validated using electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS) for determination of misoprostol acid in human plasma. Misoprostol acid-d_5 was used as in internal standard (IS). The analyte and IS were extracted by simple one step solid phase extraction (SPE). Linearity in plasma was obtained over the concentration range 10~3000 pg/mL and lower limit of quantification (LLOQ) was identifiable and reproducible at 10 pg/mL. The intra- and inter-day precision values were below 9% and the accuracy was ranged from 93.81% to 102.02% at all four quality control samples. The method was has been successfully applied for routine assay to support pharmacokinetic study of misoprostol acid in human plasma after an oral administration of 0.4 mg misoprostol.

Key words: misoprostol acid, alsoben, human plasma, LC-MS/MS, pharmacokinetics

1. Introduction

Misoprostol is an analogue of prostaglandin E_1 (PGE_1), extensively absorbed, and undergoes rapid de-esterification to its free acid (*Fig.* 1). It was developed clinically in the early 1980s initially as a therapy for peptic ulcer because of its gastric acid anti-secretory properties and its various mucosal protective properties, mediated by mechanisms such as stimulation of gastric mucus and duodenal bicarbonate secretion and enhancement of gastric mucosal blood flow.[1] It was found to be equivalent to but no better than H_2-antagonists in healing gastric and duodenal peptic ulcers, and controlled the symptoms slightly more slowly, though more than one-third of patients with ulcers resistant to H_2-antagonists healed on misoprostol.[2] It was expected that the mucosal protective properties of the prostaglandin, termed 'cytoprotection' by Robert,[3] would result in ulcer healing at non-acid inhibitory doses of drug, as this occurs in the rat, an animal that requires very high doses of any anti-ulcerant to switch off gastric acid secretion.

Postpartum haemorrhage is a leading cause of maternal mortality and morbidity in developed and

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developing countries. Misoprostol appeared promising as a strong uterotonic agent, it is relatively cheap, heat stable and can be given orally. The World Health Organisation (WHO) conducted a multicentre double-blind controlled trial to evaluate the use of 600 mg oral misoprostol in the routine management of the third stage of labour.[4]

Several methods for the determination of misoprostol acid in biological matrices have been reported including radioimmunoassay,[5-7] GC mass spectrometry with electron capture[8] or or tandem mass spectrometry detection[9-11] and LC with mass[12] or tandem mass spectrometry.[13-15] Among them, only two reported complete validation data.[10,14] 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 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.[14] The method provided a LLOQ of 50 pg/mL 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[13] and large plasma volume used.[12]

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 0.4 mg misoprostol, besides, the method also has the potential of being used in the determination of PGE_1 analogues.

2. Experimental

2.1. Materials and instrument

Misoprostol acid (98% purity) and misoprostol acid-d_5 (IS, 98%) were purchased from Toronto Research Chemicals (Canada). Acetonitrile (J.T. Baker, USA) and methanol (Fisher, USA) were HPLC grade, and other chemicals were of analytical grade. All aqueous solutions including the buffer for the mobile phase were prepared with Milli Q (Millipore, Milford, MA, USA) grade water. Drug-free plasma for the preparation of calibration standard was obtained from Metro hospital blood donor service (Anyang, Korea). 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 IS.

An Agilent 1200 system consisting of G1312A quaternary pump, G1379B degasser, 1367B auto-sampler, G1316A thermostat, G1316A column oven (TTC) compartment (Agilent, Waldbronn, Germany) was used for solvent and sample delivery. An API 5000 triple-quadruple 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 1.4.2 software package.

2.2. Chromatographic and mass spectrometric conditions

Isocratic chromatographic separation was achieved on a Luna Phenyl-Hexyl column, 2.0 mm × 150 mm, i.d., 5 μm (Phenomenex, USA). The mobile phase consisted of acetonitrile -0.1% formic acid (50:50, v/v) at a flow rate of 0.3 mL/min. The column temperature was maintained at 35 ºC.

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 100 ng/mL of both analytes at a

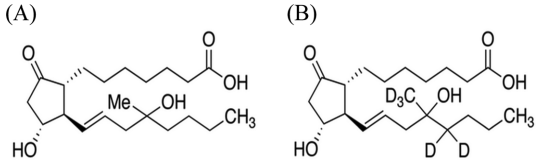

Fig. 1. Structural representation of misoprostol acid (A) and deuterated misoprostol acid-d_5 (B).

flow rate of 10 μL/min into the mobile phase (0.20 mL/min) using a analyte column 'T' connection. Optimized instrument settings specific misoprostol acid and IS were as follows: curtain gas was 20 psi, ion source gas 1 was 50 psi, ion source gas 2 was 50 psi, ionspray voltage was 4500 V, turbo heater temperature was 500 °C. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions m/z 367.1→249.1 for misoprostol acid and m/z 372.2→249.0 for the IS, respectively, with a dwell time of 300 ms per transition. The precursor ions of misoprostol acid and IS were formed using declustering potentials of 80 and 85 V, respectively, and their precursor ions were fragmented at collision energies of 24 and -28 eV by collision-activated dissociation with nitrogen at a pressure setting of 5 (arbitrary units). Both quadrupoles were maintained at unit resolution.

2.3. Preparation of calibration standard and QC samples

A stock solution 10 mg of misoprostol acid in 10 ml of acetonitrile seven standard working solutions of 100, 500, 1000, 5000, 10000, 20000 and 30000 pg/mL of misoprostol acid were made by further dilution of the stock solution with acetonitrile–water (50:50, v/v). The quality control (QC) samples were similarly prepared at concentrations of 300, 15000 and 24000 pg/mL, by a separate weighing of the pure standard. The I.S. working solution (20 ng/mL) was prepared by diluting its stock solution (20 μg/mL) with acetonitreil-water (50:50, v/v). Matrix-matched calibration standard and QC samples of misoprostol acid were prepared by spiking 30 μL of the working solutions into 270 μL of drug-free plasma. The calibration standards were prepared at concentrations of 10, 50, 100, 500, 1000, 2000 and 3000 pg/mL of misoprostol acid in plasma, while the corresponding QC samples were prepared at 30, 1500 and 2400 pg/mL.

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.

2.4. Sample preparation

A 30 μL aliquot of the I.S. solution (misoprostol acid-d_5, 20 ng/mL) was added to 300 μL of plasma samples and vortex mixed for 30 sec. This sample was loaded on pre-conditioned (1 mL methanol followed by 1 mL water) Oasis HLB cartridges (1 cc, 30 mg) and washed with 2 mL water. The cartridges were then dried under full pressure for 2 min and eluted 1 mL of methanol into new glass tubes. The eluent was evaporated under nitrogen gas at 50 °C and the dry contents reconstituted with 100 μL of 60% acetonitrile and vortex mixed for 1 min. The contents were finally transferred into appropriate auto-sampler vials and an aliquot (5 μL) was injected onto the LC-MS/MS for analysis.

2.5. Method validation

Plasma samples were quantified using the ratio of the peak area of analyte to IS as the assay response. The specificity 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 calibration curve (CC) standards and QC samples (n=4 at each concentration) were prepared and analyzed on five different occasions to evaluate linearity, precision and accuracy. To evaluate linearity, plasma calibration curves were prepared and assayed on five consecutive days over the range of 10~3000 pg/mL. Least-squares linear regression was used for curve fitting with $1/x^2$ as the weighting factor. For determining the intra-day precision and accuracy, a replicate analysis of plasma samples of misoprostol acid in human plasma was performed on the same day. The run consisted of a CC and five replicates of each the lower limit of quantification (LLOQ), low, mid and high concentration QC samples. The inter-day precision and accuracy were assessed by analysis of five batches on different days. The precision was expressed as the coefficient of variation (CV%) and

the accuracy as the relative error (RE%). The extraction recovery of the analytes from the plasma was evaluated by comparing the mean detector responses of three replicates of processed QC samples at low and high concentration to the detector responses of standard solutions of same concentration. Endogenous matrix components may change the efficiency of droplet formation or droplet evaporation, which in turn affects the amount of charged ion in the gas phase that ultimately reaches the detector. Three set of samples were prepared by directly spiking the analytes into reconstitution solution and without the presence of residue extracted from blank plasma. Post-preparative stability, three aliquots each of low and high QC samples were stored at 10 ºC in an auto-sampler for 26 hr, analyzed and the concentrations were compared with the actual values. Three aliquots of each low and high QC samples were kept in deep freezer at -70 ºC for 24 day. The samples were processed and analyzed and the concentrations obtained were compared with the actual value of QC samples to determine the long term stability of analyte in human plasma. Three aliquots each of low and high unprocessed QC samples were kept at ambient temperature (25 ºC) for 23 hr in order to establish the short term stability of the analytes. The stability of the analytes after three freeze and thaw cycles was determined at low and high QC samples. The samples were stored at -70 ºC for 24 hr and thawed unassisted at room temperature. After completely thawing, the samples were refrozen for 12~24 hr. After three freeze-thaw cycles, the concentration of the samples were analyzed. Separate standard working solutions containing 300 pg/mL, 2400 pg/mL of misoprostol acid and 20 ng/mL of IS were prepared and stored at 2~8 ºC for 15 day. The response obtained from the two drugs was calculated and compared with that of the freshly prepared solutions of the same concentration.

2.6. Pharmacokinetic study

The validated method was used to determine the plasma concentrations of misoprostol acid from a clinical trial in which 45 healthy male volunteers received a single oral dosage of 0.4 mg misoprostol. Eligible volunteers were Korean men aged 20 to 36 years (25.72±3.80) and the average body weight was 69.90±7.43 kg. The study protocol was approved by the Human Investigation Ethics Committee of Metro hospital, Anyang-ci, Korea. Blood samples were collected into heparinized glass tubes before and 0.08, 0.17, 0.25, 0.33, 0.5, 0.75, 1, 1.5, 2, 3 and 4 hr post-dosing, and centrifuged at 4000 rpm (4 ºC) for 10 min to separate the plasma fractions. The collected plasma samples were stored at 70 ºC 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 (ke) was estimated by log-linear regression of concentrations observed during the terminal phase of elimination.

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.

By negative ESI mode, The detector was operated at unit resolution in the multiple-reaction monitoring (MRM) mode using the transitions of the protonated molecular ions of misoprostol acid at m/z 367.1→249.1 and IS at m/z 372.2→249.0 (*Fig.* 2). Optimized parameters were as follows: curtain gas, gas 1 and gas 2 (nitrogen) 20, 50 and 50 units, respectively;

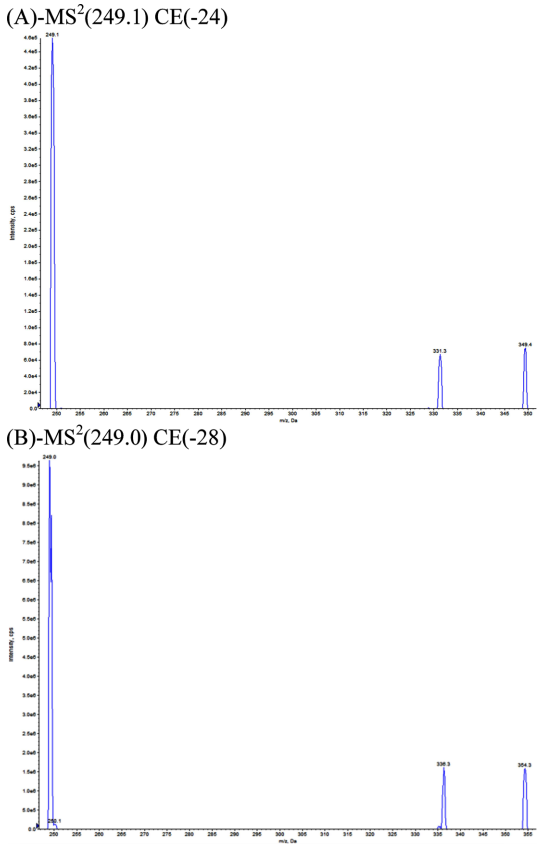

Fig. 2. Product ion spectra of $[M-M]^-$ of misoprostol acid (A) and misoprostol acid-d_5 (B).

dwell time 300 ms; source temperature 500 °C; ion spray voltage -4500 V. Declustering potential and collision energy were -80 V and -24 eV for misoprostol acid and -85 V and -28 eV for IS, respectively.

3.2. Optimization of the chromatographic condition

In pursuit of symmetric peak shape and retention time of ~2.10 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 3~7, along with altered flow-rates (in the range of 0.2~0.5 mL/min) were tested for complete chromatographic resolution of misoprostol acid and IS (data not shown). The resolution of peaks was achieved with 0.1% formic acid and acetonitrile mixture (50:50, v/v) with a flow rate of 0.3 mL/min, on a Luna phenyl-Hexyl column and was found to be suitable for the determination of electrospray response for misoprostol acid and IS.

3.3. 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 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 HLB cartridges (1 cc, 30 mg) with several dilution, conditioning, washing and elution reagents gave consistent results in terms of recovery of misoprostol acid and IS and also gave cleaner plasma blank samples. The SPEs were pre-conditioned (1 mL methanol followed by 1 mL water) and sample mixture was loaded and were washed with 2 mL of water and finally eluted with 1 mL of methanol. The eluent was evaporated under nitrogen gas at 50 °C and the dry contents reconstituted with 100 μL of 60% acetonitrile.

3.4. Assay specificity

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.* 3, 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 2.10 and 2.09 min. The total chromatographic run time was 4 min.

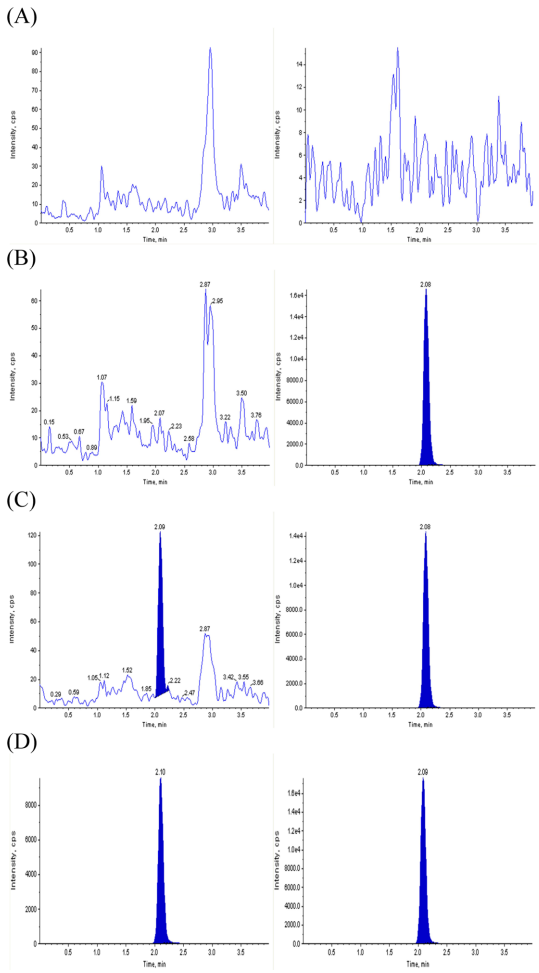

Fig. 3. Typical MRM chromatograms of misoprostol acid (left panel) and IS (right panel) in (A) human blank plasma, (B) human blank plasma spiked with IS, (C) human plasma spiked with misoprostol acid at LLOQ (10 pg/mL) and IS, (D) a representative chromatogram (0.33 hr) of extracted a male volunteer.

3.5. Linearity and lower limit of quantification

The linear regression of the peak-area ratios versus concentrations was fitted over the concentration range of 10~3000 pg/mL in human plasma. A typical equation of the calibration curves was as follows: $y = 0.0007x + 0.0047$ (r^2=1.000), where y represents the peak-area ratio of analyte to IS and x represents the plasma concentration of misoprostol acid. Good linearity was seen in this concentration range. The lower limit of quantification was 10 pg/mL for determination of misoprostol acid in plasma. The precision and accuracy at the concentration of LLOQ are shown in *Table* 1.

3.6. Precision and accuracy

The method showed good precision and accuracy. Table 1 summarizes the intra- and inter-day precision and accuracy for misoprostol acid from QC samples. The intra-day precision (CV %) for QC samples (10, 30, 1500, 2400 pg/mL) were 3.54%, 1.55%, of 1.05% and 0.38%, respectively and that of inter-day analysis were 8.78%, 4.46%, 1.26%, 1.42% with a relative errors (RE %) within -6.19% to 2.02%.

3.7. Recovery and matrix effect

The extraction recoveries of misoprostol acid from human plasma were 100.04% (CV=2.56%) and 100.06% (CV=0.57%) at concentration levels of 30 pg/mL and 2400 pg/mL, respectively, and the mean extraction recovery of IS was 95.91% (CV=1.59%).

The endogenous components are mainly the cause of ion suppression effects during electospray ionization.

The extent of this effect is mainly dependent on sample extraction procedure and is also compound dependent. The result indicated that the matrix components did not alter or deteriorate the performance of the proposed method as the % coefficient of variation (CV) of two QC samples was less than 71.69% and 82.48% for misoprostol acid and IS

Table 1. Precision and accuracy data for the analysis of misoprostol acid in human plasma (n=5)

Added (pg/mL)	Intra-day			Inter-day		
	Found (pg/mL)	CV(%)	RE (%)	Found	CV(%)	RE (%)
10	10.03	3.54	0.30	9.54	8.78	-4.64
30	28.14	1.55	-6.19	30.49	4.46	1.63
1500	1520.74	1.05	1.38	1491.10	1.26	-0.59
2400	2448.56	0.38	2.02	2399.55	1.42	-0.02

Table 2. Recovery and matrix effect of misoprostol acid and misoprostol acid-d_5 (IS)

Analyte	Concentration (pg/mL)	CV (%)	Recovery (%)	Matrix effect (%)
Misoprostol acid	30	2.56	100.04	91.41
	2400	0.57	100.06	71.69
Misoprostol acid-d_5	2000	1.59	95.91	82.48

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

Storage conditions	Concentration (pg/mL)			CV (%)	Variation (%)
	Added	Initial	After		
Post preparative (26 hr at 10 °C)	30	27.20	30.09	3.47	10.63
	2400	2453.45	2417.28	2.85	-1.47
Short-term (23 hr at 25 °C)	30	27.20	29.51	3.43	8.49
	2400	2453.45	2391.19	3.09	-2.54
Long-term (24 day at -70 °C)	30	27.20	28.56	3.65	5.00
	2400	2453.45	2371.18	0.51	-3.35
Three freeze/thaw (3 cycles)	30	27.20	26.65	3.55	-2.01
	2400	2453.45	2398.44	1.00	-2.24
Stock solution (15 day at 2~8 °C)*	300	9289	9591	3.22	3.25
	24000	728916	761704	0.37	4.50

*Stability of stock solution was evaluated peak area.

respectively indicates the reproducibility of peak area as well as the extracts were 'clean' and no unseen component interfere with the ionization of the analytes. The matrix effect on the estimation of the analytes was shown in *Table* 2.

3.8. Stability

The result of stability experiments showed that no significant degradation occurred during the chromatography, extraction and sample storage of misoprostol acid plasma samples. Stability data are shown in *Table* 3.

3.9. 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 0.4 mg misoprostol. Profile of the mean plasma concentration of misoprostol acid versus time is shown in *Fig.* 4. The main pharmacokinetic parameters of misoprostol acid in 45 volunteers were calculated. Oral administration of 0.4 mg misoprostol, the time of maximum concentration (T_{max}) and the maximum drug concentration (C_{max}) of misoprostol acid were found to be 0.25±0.08 hr and 777.74±259.80 pg/mL, respectively. Plasma concentration declined with the terminal elimination half life ($t_{1/2}$) of 0.55±0.34 hr. The area under the plasma concentration-time curve from zero hours to time (AUC_{0-t}) and the area under the plasma concentration-time curve from zero hours to infinity ($AUC_{0-\infty}$) values obtained were 445.75±158.06 and 457.31±159.98 pg·hr/mL, respectively. Application in pharmacokinetic study data are shown in *Table* 4.

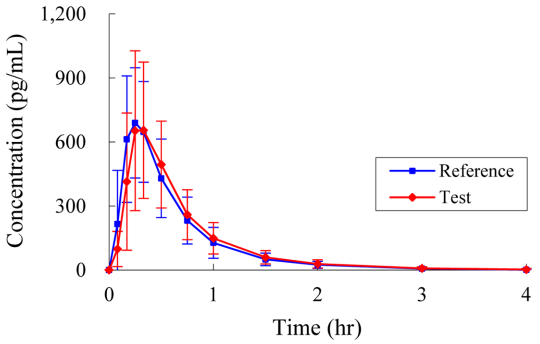

Fig. 4. Mean plasma concentration–time curve of misoprostol acid after a single oral dose of 0.4 mg misoprostol to 45 healthy male volunteers. Each point represents the mean±S.D.

Table 4. Pharmacokinetic parameters of misoprostol acid after a single oral dose 0.4 mg misoprostol tablets of the test and reference

Parameters	Test*	Reference**
C_{max}, pg/mL	777.74±259.80	790.63±348.55
T_{max}, hr	0.25±0.08	0.34±0.12
AUC_{0-t}, pg·hr/mL	445.75±158.06	454.97±165.99
$AUC_{0-\infty}$, pg·hr/mL	457.31±159.98	467.49±166.15
$t_{1/2}$	0.55±0.34	0.56±0.29

*Alsoben Tab., 200 μg (Unimed Pharm. Inc.)
**Cytotec Tab., 200 μg (Pfizer Inc.)

Misoprostol has been associated with clinical benefit and used worldwide for the prevention and treatment of obstetrics and gynaecology or duodenal ulcer, gastric ulcer and peptic ulcers induced by non-steroidal antiinflammatory drugs (NSAIDs). Misoprostol is quickly metabolized to misoprostol acid after oral administration and the metabolite can be used to evaluate the bioequivalence of misoprostol tablets.

The present study used a rapid and sensitive LC-MS/MS method for determination of misoprostol acid concentration in human plasma. The LLOQ (10 pg/mL) of the assay was sufficient to characterize the absorption kinetics of misoprostol acid, with a linear performance at concentrations between 10~3000 pg/mL, and intraday and interday precision of less than 9%. The C_{max} and T_{max} values (777.74±259.80 pg/mL and 0.25±0.08 hr, respectively) for a 0.4 mg dose of misoprostol under fasting conditions in this study were comparable to those reported by Yu *et al.* for a 0.6 mg dose under fasting conditions (857±600 pg/mL and 0.48±0.21 hr, respectively).[14] 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.

4. Conclusions

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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