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Short communication

LC–MS determination and bioavailability study of loperamide hydrochloride after oral administration of loperamide capsule in human volunteers

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Abstract

The purpose of the present study was to develop a standard protocol for loperamide hydrochloride bioequivalence testing. For this purpose, a simple rapid and selective LC–MS method utilizing a single quadrupole mass spectrometer was developed and validated for the determination of loperamide hydrochloride in human plasma, and we followed this with a bioavailability study. Methyl *tert*-butylether (MTBE) was used to extract loperamide hydrochloride and ketoconazole (internal standard (IS)) from an alkaline plasma sample. LC separation was performed on a Zorbax RX C_{18} column (5 μm, 2.1 mm × 150 mm) using acetonitrile–water–formic acid (50:50:0.1 (v/v)) as a mobile phase. The retention times of loperamide hydrochloride and IS were 1.2 and 0.8 min, respectively. Quadrupole MS detection was by monitoring at *m*/*z* 477 (*M* + 1) corresponding to loperamide hydrochloride and at *m*/*z* 531 (*M* + 1) for IS. The described assay method showed acceptable precision, accuracy, linearity, stability, and specificity. The bioavailability of loperamide hydrochloride was evaluated in eight healthy male volunteers. The following pharmacokinetic parameters were elucidated after administering a single dose of four 2 mg capsules of loperamide: the area under the plasma concentration versus time curve from time 0 to 72 h ($AUC_{72\,h}$) 19.26 ± 7.79 ng h/ml; peak plasma concentration (C_{max}) 1.18 ± 0.37 ng/ml; time to C_{max} (T_{max}) 5.38 ± 0.74 h; and elimination half-life ($t_{1/2}$) 11.35 ± 2.06 h. The developed method was successfully used to study the bioavailability of a low dose (8 mg) of loperamide hydrochloride.

Keywords: Loperamide hydrochloride; Quadrupole mass spectrometry; Bioavailability

1. Introduction

Loperamide, 4-(*p*-chlorophenyl)-4-hydroxy-*N*,*N*,-dimethyl-α,α-diphenyl-1-piperidine butyramide monohydrochloride (Fig. 1), an opiate analogue, has been widely used for the control and symptomatic relief of acute non-specific diarrhea and chronic diarrhea associated with inflammatory bowel diseases since it was introduced in 1973 [1,2]. The determination of loperamide in pharmaceutical formulations and biological fluids has been described and reviewed [3]. Many methods can be used for determining loperamide, i.e., radioimmunoassay (RIA) [4,5], high-performance liquid chromatography (HPLC) [6,7], spectrofluorimetry and spectrophotometry [8,9], and LC–MS [10].

The pharmacokinetics of loperamide in human has been described [1,4,5]. The average daily maintenance dosage in therapeutic trials has been determined to be two to four capsules (4–8 mg) and a dose of eight capsules (16 mg) has rarely been exceeded [1], although clinical studies with loperamide single doses of up to 54 mg have been tested [4]. About 70% of administered loperamide is absorbed in the gastrointestinal tract, but due to a large 'first pass' effect its systemic bioavailability is low. Following the oral administration of four 2 mg

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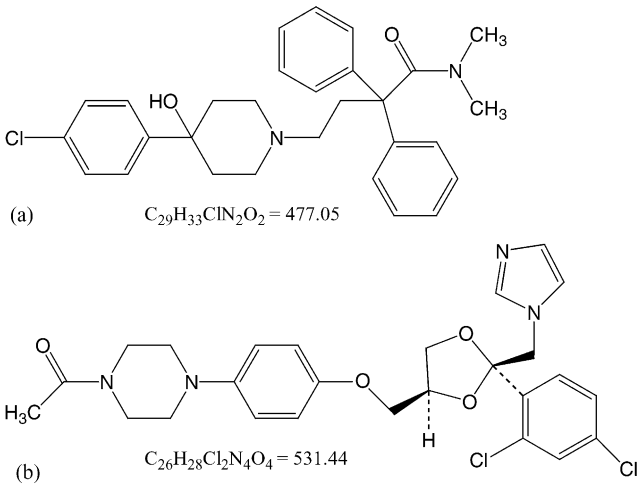

Fig. 1. Chemical structure of (a) loperamide (monoisotopic exact mass, 476) and (b) ketoconazole (monoisotopic exact mass, 530).

capsules, mean peak plasma levels of 2.24 ± 0.42 ng/ml were obtained after about 5 h.

Until recently, chromatographic methods like HPLC [6,11] and GC–MS [12] were insufficiently sensitive for use in pharmacokinetic studies. However, HPLC with electrochemical detection was used to evaluate the bioequivalence of capsule and tablet formulations of loperamide at a dosage of 16 mg [11].

LC–MS has also been used to quantify loperamide and its major metabolite *N*-demethyl-loperamide in human plasma [10]. In this method, an acetonitrile–water gradient containing 29 mM ammonium acetate was used as the mobile phase and analytes were quantified by positive electrospray ionization in a triple quadrupole mass spectrometer operating in MS–MS mode. Selected reaction monitoring was used to quantify loperamide (*m*/*z* 477 → 266) due to the loss of the 4-(*p*-chlorophenyl)-4-hydroxy-piperidyl group upon low energy collision-induced dissociation. This assay achieved higher sensitivity and better specificity than other methods developed to analyze loperamide and its metabolite in human plasma [10].

During the last few years, tandem mass spectrometry has repeatedly been proven to be a powerful technique for the rapid, quantitative determination of drugs and metabolites in physiologic fluids. In the present study, a simple, rapid, selective LC–MS method was developed and validated for the determination of loperamide in human plasma, and a standard protocol for bioequivalence testing of loperamide hydrochloride was devised.

2. Experimental

2.1. Materials and reagents

Loperamide hydrochloride and ketoconazole (Fig. 1) standards were both purchased from Sigma (St. Louis, MO, USA); Loperin® 2 mg capsules (lot no. 6814) from Janssen Korea Co. Ltd.; methyl *tert*-butylether (MTBE) (HPLC grade) from Aldrich (Milwaukee, WI, USA); acetonitrile and water (both HPLC grade) from Burdick & Jackson (Muskegon, MI, USA). All other reagents were of analytical grade.

2.2. Instrumentation

Many operational conditions in the present assay were as previously reported [10,13]. The LC–MS system used was an Agilent 1100 HPLC system equipped with a degasser, binary pump, autosampler, thermostatic column compartment, and 1946D mass selective detector (Agilent, USA), fitted with a Zorbax RX C_{18} column (5 μm, 150 mm × 2.1 mm, Agilent, USA). Electrospray ionisation–mass spectrometry (ESI–MS) was performed in the positive mode using the following operating parameters: nebulizer pressure, 35 psig; nebulizer nitrogen gas flow-rate, 12 l/min; drying gas temperature, 350 °C; and a capillary voltage of 3 kV. System control and data evaluation were carried out using Agilent Chemstation (USA). The gas blowing concentrator used was a dry thermo bath MG-2100 (Eyela, Japan). Compounds were eluted up to a total retention time 2 min using an isocratic mobile phase consisting of acetonitrile–water–formic acid (50:50:0.1 (v/v)) at a flow-rate of 0.5 ml/min. The gas used was high purity. Selected ion monitoring was used to quantify loperamide (*m*/*z* 477) and ketoconazole (*m*/*z* 531) [10,13]. Mass spectra were obtained in positive ion SIM mode by single quadrupole mass spectrometry with electrospray ionization.

2.3. Preparation of stock solutions and calibration standards

Primary stock solutions of loperamide hydrochloride (1 μg/ml) were prepared in acetonitrile, and spiking standard solutions of 0.5, 1.0, 2.5, 5, 10, 25, and 50 ng/ml were prepared by diluting the stock solution with acetonitrile. The ketoconazole (IS) working stock solution was made up to 90 ng/ml in acetonitrile. All stock solutions were stored at 4 °C when not in use. Calibration standards of loperamide hydrochloride and ketoconazole were prepared by spiking the appropriate amount of the stock solutions into the blank plasma obtained from healthy, non-smoking volunteers who were not drinking coffee. Calibration standards were prepared by adding 50 μl of stock solution (0.5, 1.0, 2.5, 5, 10, 25, and 50 ng/ml, respectively) into 450 μl of the human plasma and well mixed. Prepared calibration curves covered the range 0.05–5 ng/ml in plasma.

2.4. Sample preparation and extraction procedures

A 100 μl aliquot of ketoconazole (90 ng/ml) stock solution was added to 500 μl of each plasma sample and vortex-mixed. The plasma was then made alkaline by addition of

50 μl of 0.5 M sodium carbonate, Loperamide and ketoconazole were extracted with 6 ml of MTBE for 5 min, vortex-mixed for 5 min, and centrifuged at 3000 × *g* for 10 min. The organic layer (5 ml) was then transferred to another clean glass tube and evaporated under a stream of nitrogen gas at 55 °C until completely dry. The residue was then reconstituted in 100 μl of water and acetonitrile (50:50 (v/v)) containing 0.1% formic acid, vortex-mixed for 1 min and centrifuged at 3000 × *g* for 10 min. A 10 μl aliquot of the reconstituted sample was then automatically injected onto the LC–MSD.

2.5. Assay validation

2.5.1. Assay specificity

Specificity was assessed by extracting samples of six different batches of blank plasma, zero sample (spiked with ketoconazole only), and then comparing the results for plasma samples spiked with ketoconazole (IS) and loperamide that were the lowest (0.05 ng/ml) or the highest concentration (5 ng/ml) of loperamide hydrochloride in calibration standard. The chromatograms were also inspected visually for interfering chromatographic peaks from endogenous substances.

2.5.2. Linearity

Calibration standards at seven loperamide hydrochloride concentrations (range, 0.05–5.0 ng/ml) were extracted and assayed. Least-squares linear regression was used to determine the plasma concentration from the peak area ratios (loperamide hydrochloride versus ketoconazole).

2.5.3. Precision and accuracy

The precision of the assay was determined from plasma samples of seven concentrations of loperamide hydrochloride (0.05–5.0 ng/ml). Intra-day precision was determined by repeating the analysis of standards five times in a single day, and inter-day precision and accuracy were determined by repeating analysis on five consecutive days. Sample concentrations were determined using calibration standards prepared on the same day. Assay precision was defined as the relative standard deviation (S.D.) from the mean (*M*), as calculated using the equation R.S.D.% = (S.D./*M*) × 100. Accuracy was defined as the ratio of the mean computed value (*E*) to the true value (*T*) expressed as a percentage (accuracy (%)).

2.5.4. Recovery

The absolute recovery of loperamide hydrochloride and ketoconazole by extraction was determined by comparing peak areas in plasma sample and in acetonitrile solution samples, spiked with loperamide hydrochloride at 0.5 ng/ml and ketoconazole at 18 ng/ml (n = 5). Recovery was defined as the ratio of the peak areas in plasma samples to those in acetonitrile solution sample for loperamide and ketoconazole, respectively (recovery (%)).

2.5.5. Stability

The (1) freeze/thaw; (2) short-term room temperature; (3) long-term storage; (4) stock solution; and (5) post-preparative stabilities were tested. No significant degradation of loperamide and ketoconazole were observed. To test the stability of loperamide hydrochloride in plasma, two sets of samples with low and high concentration (i.e., 0.5 and 5 ng/ml) were stored under different conditions. The freeze–thaw stability test was performed by freeze–thawing three times; specifically, freezing was performed at −20 °C for 24 h and thawing at room temperature. During each cycle, triplicate 10 μl aliquots were processed, analyzed, and the results averaged. Short-term stability testing was performed at room temperature over 6 h, and long-term stability was examined at −20 °C over 2 weeks. The results of the freeze–thaw, and short and long-term stability tests were compared to average of intra-day. To test the stock solution stability of loperamide hydrochloride and IS, stock standard (loperamide, 1000 ng/ml) and IS (ketoconazole, 90 ng/ml) solutions were left at room temperature for 6 h. Post-preparative stability testing was performed by comparing after-intra-day analysis to the first intra-day analysis.

2.6. Bioavailability study design

The Korean Food and Drug Administration (KFDA) and the Institutional Review Board (IRB) on human studies at Chungnam National University, approved the present bioavailability study according to Bioequivalence Test Regulation. Healthy male volunteers aged 19–28 years and weighing from 60 to 81 kg were enrolled in the study. The study was performed at Daejeon Sungsim Hospital (Daejeon, Korea). Based on medical history, clinical examinations, and laboratory tests, which included hematology, blood biochemistry, and urine analysis, no subject had a history or evidence of hepatic, renal, gastrointestinal or hematological deviations, or any acute or chronic diseases or drug allergy. Subjects were instructed to abstain from taking any medication or xanthin containing foods for at least 2 weeks prior to and during the study period. No milk or dairy products were allowed during the study. Informed consent was obtained from all subjects the nature and purpose of the study had been explained. Data concerning the physical examination of eight healthy male volunteers can be found in Table 1.

After an overnight fast (more than 10 h), subjects were given a single dose of four 2 mg capsules (8 mg loperamide) with 240 ml of water. During the 24 h period after drug administration, no strenuous physical or mental activity was permitted. Food and drinks were not allowed until 4 h after capsule administration. Lunch and dinner were served at 5 and 12 h after administration. Approximately, 10 ml blood samples were drawn into Vacutainer™ tubes containing heparin from a forearm vein using an indwelling catheter or by direct veinpuncture before dosing (0 h) and then at 1, 2, 3, 4, 5, 6, 7, 9, 12, 24, 36, 48, and 72 h after dosing. The blood samples were immediately stored at 4 °C, and centrifuged within

Table 1
Physical examination and laboratory results of the eight healthy male volunteers

No.	Age	Weight (kg)	Height (cm)	Hematocrit (%)	Hb (g/dl)	GPT (IU/L)	GOT (IU/L)	Glucose (mg/dl)	Cholesterol (mg/dl)	Total protein (g/dl)	Albumin (g/dl)	BUN (mg/dl)
				39–52*	13–17*	0–40*	5–40*	70–120*	128–250*	6.5–8.3*	3.5–5.0*	8.0–20.0*
1	23	60	175	43	14.6	28	36	83	147	6.95	4.92	10.9
2	22	61	172	45.5	15	10	17	77	161	7.52	4.92	13.4
3	22	63	175	43.7	14.8	11	17	78	158	7.5	4.9	11.1
4	26	65	173	45.5	15	18	21	95	175	7.76	5.02**	15.2
5	22	68	177	45.6	15.4	18	17	81	146	7.36	4.76	14.6
6	21	78	174	46.9	15.6	15	14	72	170	7.37	4.61	14.3
7	24	78	178	44.1	14.5	37	33	94	165	7.33	4.65	14
8	19	81	185	43.4	14.6	8	17	87	157	7.36	4.66	13.7

* Normal range.
** Although albumin level of volunteer no. 4 was over 5, he was judged normal by a doctor.

1 h at 3000 rpm for 10 min. Separated plasma was immediately divided into two 2.5 ml aliquots, transferred into coded test tubes and stored at −20 °C until required for analysis.

2.7. Pharmacokinetic analysis

Plasma loperamide concentrations were analyzed using the developed LC–MS method. The lower limit of quantitation (LOQ) of the present assay was 0.05 ng/ml. Pharmacokinetic parameters including $AUC_{72\,h}$ (the area under the plasma concentration versus time curve from time 0 to 72 h), C_{max} (peak plasma concentration), T_{max} (time to C_{max}), K_e (terminal rate constant) and $t_{1/2}$ (elimination half-life) were calculated using K-BE TEST 2002 [14].

3. Results and discussion

3.1. Chromatography and specificity

To develop this LC–MS-based method for quantifying loperamide hydrochloride in human plasma, electrospray ionization (ESI) sources were evaluated in positive ion mode. In general, ESI produced greater sensitivity and exhibited less interference than an atmospheric pressure chemical ionization (APCI) source, and thus was selected as previously described [10]. ESI positive MS spectra for loperamide and ketoconazole were dominated by the $[M + H]^+$ ions, i.e., *m*/*z* 477 for loperamide and *m*/*z* 531 for ketoconazole.

Mobile phase composition was found to be critical factor for achieving good chromatographic peak shape and resolution. In the present study, formic acid was selected as a buffer, and acetonitrile–water–formic acid (50:50:0.1 (v/v)) was selected as an isocratic mobile phase, which is more straightforward that the gradient elution method [10]. The retention times of loperamide and ketoconazole were less than 2 min. And ketoconazole was selected as an internal standard (IS) because of its similarity solubility characteristics in alkalinized pH and in organic phase, its similar hydrophobicity, its appropriate *m*/*z* value, and its similar recovery efficiency (ca. 85%) as compared to loperamide.

A plasma blank (free of analyte and IS), a zero sample (plasma blank spiked with IS only), and plasma sample (0.05–5 ng/ml) were used to check interference. A typical chromatogram is shown in Fig. 2. For all plasma sample, the regions of the analyte and the IS were found to be free of interference. The result obtained showed that the method exhibited good specificity. The retention times of loperamide and ketoconazole (IS) using our system were 1.2 and 0.8 min, respectively (Fig. 2).

3.2. Linearity and lower limit of quantitation

The standard curve range used was 0.05–5 ng/ml for loperamide hydrochloride calculated based on 0.5 ml plasma. The linearity of the response/concentration curve was established in human plasma over the concentration range 0.05–5 ng/ml by using the goodness-of-fit test. The lower detection limit (LOD), defined at a S/N > 3, was 10 pg/ml and the lower limit of quantification (LOQ) of loperamide, defined at a S/N > 10, was 0.05 ng/ml. In a previous paper [10], the LOD and LOQ of loperamide were 5.2 fmol (equivalent to ca. 2.48 pg/ml) and 0.25 pmol (equivalent to ca. 0.119 ng/ml), respectively.

3.3. Precision, accuracy, and recovery

The intra- and inter-day (n = 5) precision and accuracy (Table 2) were satisfactory for our purposes. Intra-day precision (R.S.D.%) was less than 15% with results ranging from 1.97 to 14.02%, intra-day limit of quantitation (LOQ) precision was 12.50%. Inter-day precision was also less than 15% (range, 3.89–15.27%), and inter-day LOQ precision was 14.07%. Accuracy was within 85–115% at all concentrations investigated, and LOQ accuracy was 110.5%. These results show that the developed method has good precision and accuracy. The recovery (%) (mean ± S.D.) of loperamide and ketoconazole were 88.4 ± 1.21% and 85.6 ± 0.72%, respectively.

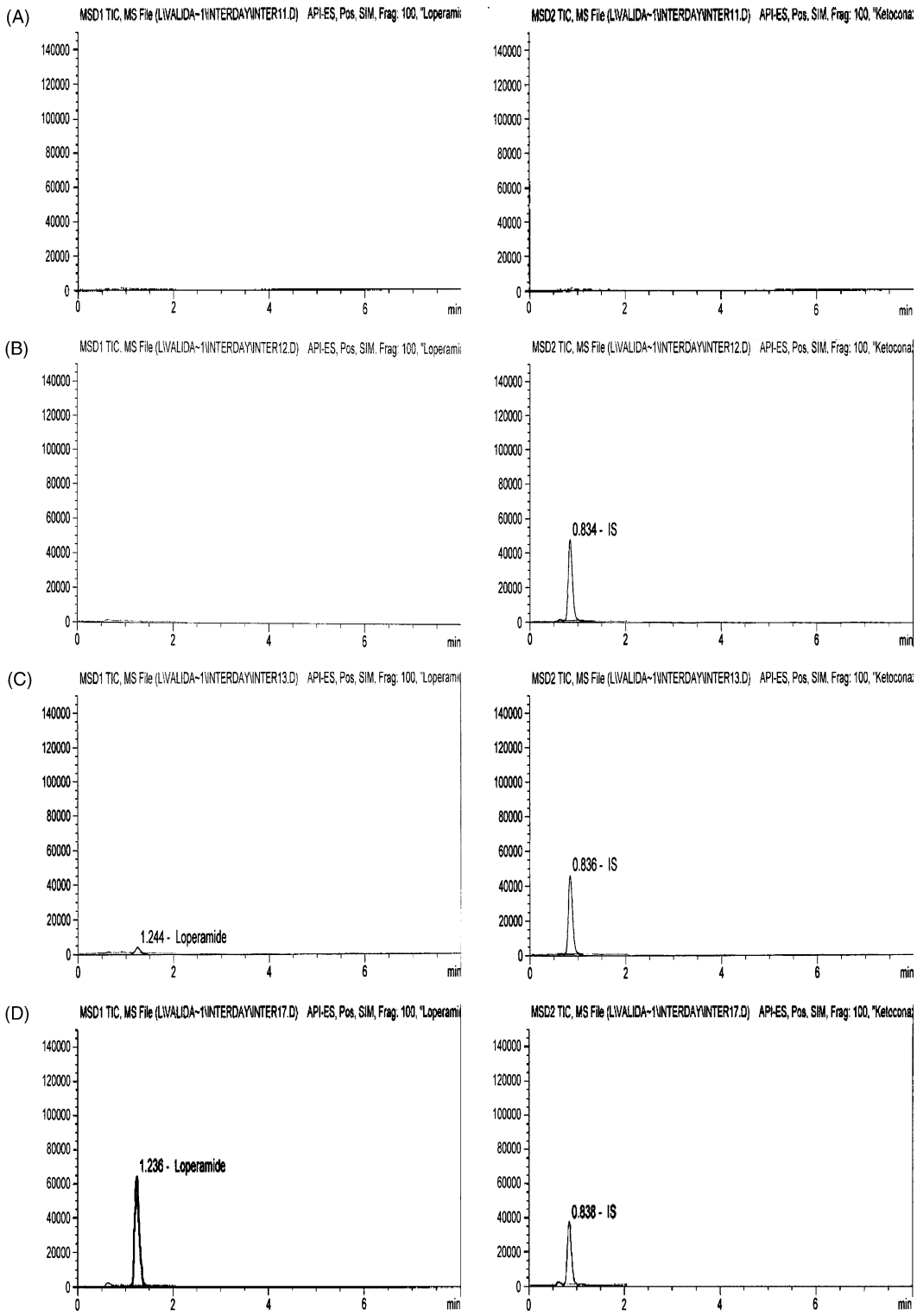

Fig. 2. Chromatograms of (A) blank plasma sample; (B) zero plasma sample spiked with ketoconazole 18 ng/ml only; (C) plasma spiked with loperamide hydrochloride 0.05 ng/ml and ketoconazole 18 ng/ml; and (D) plasma spiked with loperamide hydrochloride, 5 ng/ml and ketoconazole, 18 ng/ml (left panel: loperamide and right panel: ketoconazole).

Table 2
Precision and accuracy

Concentration (ng/ml)	Precision (R.S.D., %)		Accuracy (%, n = 9)
	Intra-day (n = 5, day 1)	Inter-day (n = 5)	
0.05	12.50	14.07	110.5
1	14.02	15.27	106.4
0.25	4.42	10.20	112.6
0.5	3.30	3.94	102.5
1	1.97	5.02	99.8
2.5	2.47	3.89	97.0
5	2.51	6.53	100.4

3.4. Stability studies

The stability experiments aimed at testing all possible conditions that the samples might be exposed to during sample shipping and handling. Results are summarized in Tables 3 and 4.

Three freeze–thaw cycles and 6 h room temperature storage had no substantial effect on the results. The samples were stable at room temperature for at least 2 weeks (Table 3). Standard solutions of loperamide (1000 ng/ml in acetonitrile) and IS (90 ng/ml in acetonitrile) were analyzed using LC–MS and then stored at room temperature for 6 h. The samples were analyzed to compare with assayed sample before 6 h, and results are presented in Table 4. The difference (%) values in stock solution stability were 1.06 and −0.49% for loperamide and ketoconazole (IS), respectively. The post-preparative samples were stable at room temperature for at least 5 days including the residence time in the autosampler. The stability of loperamide hydrochloride in aqueous solution was previously reported to be maximal at ca. pH 4.5 and its degradation half-life to be 402.6 days at 90 °C, indicating that loperamide is very stable in aqueous solution [15].

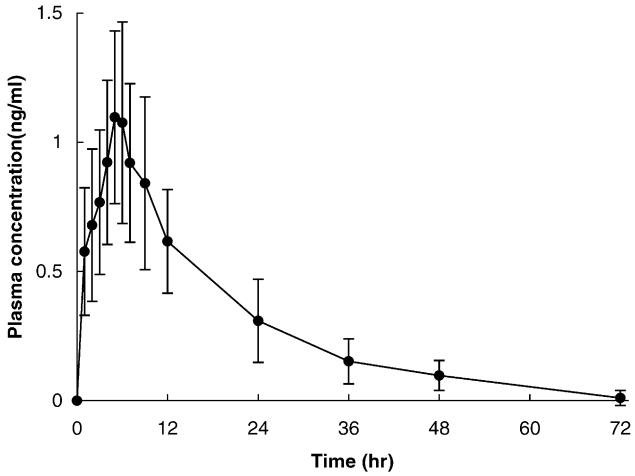

Fig. 3. Plasma concentration–time profile of loperamide hydrochloride after an oral administration of four 2 mg Loperin® capsule (8 mg loperamide) to eight healthy male volunteers. Each point represents a mean ± S.D. (n = 8).

3.5. Pharmacokinetic study

The developed method was successfully used for a pharmacokinetic study in which plasma concentrations of loperamide in eight healthy male volunteers were determined up to 72 h after the oral administration of 8 mg of Loperin® capsules. Plasma drug concentration/time curves are shown in Fig. 3.

Pharmacokinetic studies of loperamide have been performed [1,5,11]. Killinger et al. [5] assayed serum loperamide concentrations using a radioimmunoassay method, and reported the following pharmacokinetic parameters: AUC, 25.2 ± 3.5 ng h/ml; C_{max}, 2.24 ± 0.42 ng/ml; T_{max}, 5.2 ± 0.3 h; and $t_{1/2}$, 11.2 ± 0.8 h, after a single oral dose of four 2 mg capsules (8 mg loperamide). Following the oral administration of eight 2 mg capsules (16 mg loperamide), Doser et al.

Table 3
Data of freeze/thaw, short-term, and long-term stability

Stability	Loperamide in plasma (ng/ml)					
	0.5			5.0		
	Mean (ng/ml)	R.S.D. (%)	Accuracy (%)	Mean (ng/ml)	R.S.D. (%)	Accuracy (%)
Intra-day (n = 5, day 1)	0.524	3.30	104.9	4.996	2.51	99.9
Freeze/thaw stability (n = 3)	0.504	1.70	100.7 (−4.2)	4.63	2.67	92.6 (−7.3)
Short-term stability (n = 3)	0.526	0.54	105.1 (+0.2)	4.62	0.89	92.4 (−7.5)
Long-term stability (n = 3)	0.505	3.40	101.1	4.68	2.83	93.6 (−6.3)

Table 4
Stock solution stability

Sample	0 h		6 h		Difference (%)
	Mean peak area	R.S.D. (%)	Mean peak area	R.S.D. (%)	
Standard, 1000 ng/ml (n = 3)	1909290.9	0.31	1929501.0	0.63	1.06
IS, 90 ng/ml (n = 3)	342662.2	0.75	340979.6	0.07	−0.49

Table 5
Pharmacokinetic parameters of loperamide in eight human volunteers

	Parameter				
	$AUC_{72\,h}$	C_{max}	T_{max}	K_e	$t_{1/2}$
Mean	19.26	1.18	5.38	0.06	11.35
S.D.	7.79	0.37	0.74	0.01	2.06

C_{max}, peak plasma concentration; T_{max}, time to C_{max}; AUCt, the area under the concentration time versus curve; $t_{1/2}$, elimination half-life; and K_e, elimination rate constant.

[11] assayed plasma drug concentrations using reverse-phase HPLC fitted with an electrochemical detection (ECD) device, and reported; $AUC_{60\,h}$, 59.27 ± 23.62 ng h/ml; C_{max}, 3.98 ± 1.72 ng/ml; T_{max}, 4.38 ± 1.13 h; and $t_{1/2}$, 18.43 ± 1.72 h. The pharmacokinetic parameters, $AUC_{72\,h}$, C_{max}, T_{max}, and $t_{1/2}$ were derived from the calculated plasma concentrations. In the present study, $AUC_{72\,h}$, C_{max}, T_{max}, and $t_{1/2}$ for loperamide were 19.26 ± 7.79 ng h/ml, 1.18 ± 0.37 ng/ml, 5.38 ± 0.74 h, and 11.35 ± 2.06 h, respectively (Table 5), which were similar to those reported previously.

4. Conclusion

The purpose of the present study was to develop a standard protocol for the bioequivalence testing of loperamide hydrochloride. We devised and validated, a simple and rapid LC–MS method to determine loperamide hydrochloride levels in human plasma, and used this test to conduct a bioavailability study by administering 8 mg of loperamide hydrochloride to human subjects. The developed assay showed acceptable precision, accuracy, linearity, stability, and specificity.

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