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Short communication

Quantification of roxatidine in human plasma by liquid chromatography electrospray ionization tandem mass spectrometry: Application to a bioequivalence study

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ABSTRACT

A sensitive and specific method using a one-step liquid–liquid extraction (LLE) with ethyl acetate followed by high-performance liquid chromatography (HPLC) coupled with positive ion electrospray ionization tandem mass spectrometry (ESI-MS/MS) detection was developed and validated for the determination of roxatidine in human plasma using famotidine as an internal standard (IS). Data acquisition was carried out in multiple reaction monitoring (MRM) mode, by monitoring the transitions m/z 307.3 → 107.1 for roxatidine and m/z 338.4 → 189.1 for famotidine. Chromatographic separation was performed on a reverse phase Hydrosphere C_{18} column at 0.2 mL min^{-1} using a mixture of methanol–ammonium formate buffer as mobile phase (20:80, v/v; adjusted to pH 3.9 with formic acid). The achieved lower limit of quantification (LLOQ) was 1.0 ng mL^{-1} and the standard calibration curve for roxatidine was linear (r^2 = 0.998) over the studied range (1–1000 ng mL^{-1}) with acceptable accuracy and precision. Roxatidine was found to be stable in human plasma samples under short-, long-term storage and processing conditions. The developed method was validated and successfully applied to the bioequivalence study of roxatidine administrated as a single oral dose (75 mg as roxatidine acetate hydrochloride) to healthy female Korean volunteers.

1. Introduction

Roxatidine acetate hydrochloride (2-acetoxy-N-[3-[m-(1-piperidinylmethyl) phenoxy] propyl]acetamide hydrochloride) is a histamine H_2-receptor antagonist that is used to treat gastric and duodenal ulcers [1]. This compound is rapidly converted to its active metabolite, roxatidine, by esterases in the small intestine, plasma, and liver, and thus, cannot be found in plasma samples taken from volunteers after oral administration [2]. Early publications have described methods of analyzing roxatidine in biological samples. The techniques adopted include gas chromatography–mass spectrometry (GC–MS) [3], high-performance liquid chromatography with an UV detector (HPLC-UVD) [4], and capillary gas chromatography with nitrogen selective detection [5]. The latest assay using liquid chromatography–mass spectrometry (LC–MS) was reported [6]. However, these methods suffer from a number of disadvantages, e.g., they are time-consuming and require complex sample preparation [3], they need a large volume of plasma (≥0.1 mL) [4–6], have long run times (>4 min) [3–6], or have poor sensitivities [3–6]. Moreover, to the best of our knowledge, no liquid chromatography–tandem mass spectrometry (LC–MS/MS) based method has been previously described in the literature for the detection of roxatidine.

The method described in this paper provides a simple and accurate LC–MS/MS method to determine plasma roxatidine concentrations using LLE, with the capacity to analyze a wide concentration range (1–1000 ng mL^{-1}). In the present study, the devised method was utilized in a roxatidine acetate hydrochloride (75 mg) bioequivalence study, which was conducted in accordance with Korea Food and Drug Administration (KFDA) guidelines [7].

2. Experimental

2.1. Chemicals and reagents

Roxatidine (98.6% purity) and famotidine were obtained from DAE HWA Pharm. Co., Ltd., (Kyunggi-Do, Korea) and Sigma–Aldrich (St. Louis, MO, USA), respectively. The structures of roxatidine and famotidine (IS) are shown in Fig. 1. Methanol and ethyl acetate of HPLC grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA), and water was obtained using a Milli-Q water purification

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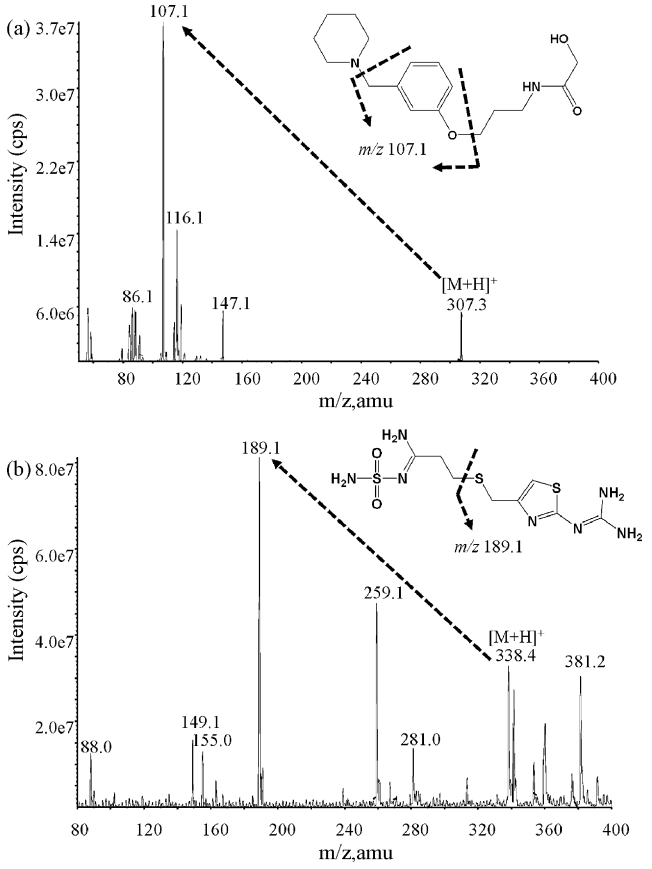

Fig. 1. Product ion spectra of the $[M+H]^+$ ions and the proposed patterns of fragmentation of (a) roxatidine and (b) famotidine.

system (Millipore Corp., Bedford, MA, USA). All other chemicals and solvents were of the highest analytical grade available.

2.2. Stock solutions and working standards

The stock solutions of roxatidine and famotidine (IS) were prepared at 1.0 mg mL^{-1} in methanol. This stock solution of roxatidine was then serially diluted with methanol to obtain working solutions for calibration with concentrations of 10, 20, 50, 100, 200, 500, 1000, 2000, 5000 and 10,000 ng mL^{-1}. Calibration curves were prepared by spiking pooled blank plasma with working solutions to final roxatidine concentrations of 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng mL^{-1}. Quality control (QC) standards (3, 400 and 800 ng mL^{-1}) were prepared in the same manner. The stock solution of IS was further diluted with distilled water to a final concentration of 10 μg mL^{-1}. All solutions were stored at −70 °C until required.

2.3. Sample preparation and extraction procedure

Human plasma samples were stored at −70 °C and thawed at room temperature before analysis. A 20-μL volume of human plasma was introduced into a 1.7-mL Eppendorf tube and 5 μL of the IS working solution (10 μg mL^{-1} of famotidine) was added and vortexed for 30 s. Ethyl acetate was then added (1.3 mL) and extraction was performed by vortex mixing for 10 min. Tubes were then centrifuged for 10 min at 10,000 rpm. The upper organic phase was transferred to clean microtubes and evaporated to dryness using a nitrogen flow in a TurboVap LV (Caliper Life Sciences, Mountain View, CA, USA) evaporation system at 40 °C. The residues obtained were then dissolved in 100 μL of distilled water, reconstituted by vortexing for 10 min and centrifuged at 10,000 rpm for 10 min. Clear supernatants were transferred to a 96-well plate and 10 μL of the supernatant was then injected onto the analytical column.

2.4. LC–MS/MS instrumentation and operating conditions

An Agilent (Palo Alto, USA) 1100 series HPLC system and an API 2000 triple quadrupole mass spectrometer (Foster City, USA) were used. A 10-μL aliquot of each plasma extract was injected into the YMC (Kyoto, Japan) Hydrosphere C_{18} (75 mm × 2 mm i.d., S-5 μm, 12 nm) analytical column operated at 40 °C. The mobile phase was prepared by mixing methanol with 10 mM ammonium formate buffer (adjusted to pH 3.9 with formic acid) at a ratio of 20:80 (v/v). A flow rate of 0.2 mL min^{-1} was found to be adequate for the analysis. The temperature of the autosampler was kept at 4 °C. Working solutions of roxatidine (1 μg mL^{-1}) and IS (1 μg mL^{-1}) were infused separately into the stainless-steel sample capillary of the electrospray source at a constant flow rate of 10 μL min^{-1}, using a 1-mL syringe pump (Hamilton; Bonaduz, Switzerland). Optimum tandem MS parameters, i.e., resolution, ion energy entrance potential, exit potential, and collision energy were set to maximize the intensities of product ions. Two channels were used in positive ion MRM mode to detect roxatidine and famotidine. Analyst software (version 1.4.1) was used for data management.

2.5. Method validation

Method validation was conducted in accordance with the currently accepted U.S. Food and Drug Administration (USFDA) guideline for Industry (Bioanalytical Method Validation) [8].

2.5.1. Specificity, linearity, precision and accuracy

The specificity of the method was measured by analysis of six blank plasma samples of different origin for interference at the retention times of the analyte and IS. Specificity was assessed by comparing the chromatograms obtained from the sample spiked with a concentration of roxatidine and IS at LLOQ with those obtained from blank samples. LLOQ was determined as the lowest concentration which gave a signal to noise ratio. (S/N) ≥ 10.

The linearity method was determined by analysis of standard plots associated with a ten-point standard calibration curve, ranging from 1 to 1000 ng mL^{-1}. A linear least-squares regression with a weighting index of $1/x^2$ was applied to the peak area ratios of roxatidine and IS versus the concentrations of roxatidine in plasma to generate a calibration curve. Calibration curves had to have correlation coefficients (r^2) of 0.99 or better.

Intra- and inter-day accuracies and precisions were determined by performing five separate analyses per day for 5 days using roxatidine concentrations of 1, 2, 10, 100 and 1000 ng mL^{-1}. Variance of precision was calculated from estimated concentrations, and precision is expressed as coefficient of variance (CV), at each level. The accuracy of the assay was defined as a percentage of the measured concentration over the theoretical concentration. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value, except for LLOQ, which was set at 20%.

2.5.2. Recovery, matrix effect and process efficiency

Recovery, matrix effect and process efficiency were estimated in a post-extraction addition approach, as proposed in Ref. [9]. Three sets of samples were prepared at QC concentrations. Samples in set A consisted of neat standards containing the analytes in distilled water. For preparation of the samples in the set B, blank plasma samples were first extracted and spiked after extraction.

For preparation of the samples in the set C, blank plasma samples were spiked before extraction. The percentage recovery of roxatidine and IS was determined by comparing the mean area of extracted set B with mean area of set C samples (ratio of C/B). These spike-after-extraction samples represented 100% recovery. The matrix effect was investigated in the following two ways: (1) The 'absolute' matrix effect of analyte was calculated by comparing response ratio (roxatidine peak area/IS peak area) of set B from the post-extraction samples with set A at the same concentrations (ratio of (B − A)/A). (2) To assess 'relative' matrix effect, eight different lots of blank plasma were used to prepare QC samples at three concentrations ($n = 3$). The relative standard deviations (R.S.D.s) of the response ratio of each analyte among the eight lots were calculated to determine inter-lot matrix variability. Value below 0% indicates ion suppression, while values above 0% indicate ion enhancement. Finally, process efficiency (3), which represents a combination of matrix effect and recovery, was estimated by comparing the response ratio of set C with those of corresponding samples in set A (ratio of C/A).

2.5.3. Stability

Stabilities of roxatidine in plasma were assessed by analyzing QC samples after storage for different times and temperatures, as described below. QC samples were prepared in sufficient volume to allow multiple replicates ($n = 5$) at each test condition. Results were compared with those of freshly prepared QC samples, and percentage concentration deviations were calculated. Stability was calculated as the difference from the freshly prepared samples.

Short-term stability. Studies included examinations of the stability of roxatidine in plasma at room temperature (25 °C), 4 and −20 °C for 48 h.

Long-term stability. Stability was assessed after storage at −70 °C for 30 days.

Post-preparative stability. The studies were conducted by reanalyzing extracted QC samples kept under autosampler conditions (4 °C) for 24 h.

Freeze–thaw stability. Roxatidine QC samples were analyzed prior to (control samples, $n = 3$) and after three freeze–thaw cycles. Samples were stored at −70 °C for 24 h and then thawed unassisted at room temperature. When completed thawed, samples were refrozen for 24 h. Samples were analyzed after three freeze–thaw cycles.

2.6. Application to bioequivalence study

The bioequivalence protocol used was approved by the Korea Food and Drug Administration. The described method was used to analyze the plasma samples of 24 healthy female Korean volunteers with an average age of 21.9 years and an average weight of 54.4 kg. Subjects were enrolled in this study after performing a medical history assessment, a physical examination, and standard laboratory (hematology, biochemistry, electrolytes, and urinalysis) testing. Each volunteer was administered 75 mg of roxatidine acetate hydrochloride orally (a gastric cap. [DAE HWA Pharm. Co., Ltd., Korea] and a Roxane [Handok Pharmaceuticals Co., Ltd., Korea]) using a standard 2 × 2 cross-over model in randomized order. A 1-week washout period was allowed between administrations. Approximately 7 mL blood samples were collected from each volunteer using a cannula inserted into the median cubital vein at the following times; predose and then at 0.5, 1, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12 and 24 h after drug administration. The blood samples were collected in separate vacutainers containing heparin as anticoagulant and centrifuged immediately. Then plasma samples were frozen at −70 °C until required for LC–MS/MS analysis.

Finally, C_{max} (maximum plasma concentrations) and T_{max} (times to C_{max}) were determined using individual subject plasma concentration–time profiles. A non-compartmental model for extravascular input, provided by WinNonlin Professional 3.1 software, was used to calculate pharmacokinetics parameters, i.e., AUC_{0-24} (area under the plasma drug concentration–time curve between 0 and 24 h), extrapolated AUC_{inf} (AUC from 0 to infinity), and $t_{1/2}$ (drug half-life). For the purpose of the bioequivalence analysis, ANOVA (Analysis of Variance) was performed using the K-BE test 2002 program at a significant level of 0.05 [10].

3. Results and discussion

3.1. Optimization of MS detection and chromatographic conditions

Mass spectrometric parameters were optimized to achieve the maximum abundances of product and fragment ions. The optimized instrument parameters for monitoring roxatidine were as follows: curtain gas, 10; nebulizing gas (GS1), 40; TurboIonSpray gas (GS2), 50; declustering potential (DP), 47 V; collision energy (CE), 53 V; collision cell exit potential (CXP), 0 V. Most optimized instrument parameters for IS except for some parameter including DP, 55 V; CE, 25 V; CXP, 2 V. Full scan mass spectra and product ion scan spectra of roxatidine and IS were obtained by direct infusion into the mass spectrometer of 1 μg mL^{-1} diluted in mobile phase at a flow rate of 0.2 mL min^{-1}. Roxatidine and IS mass spectra exhibit protonated species $[M+H]^+$ at m/z 307.3 and m/z 338.4, respectively, and these were chosen as precursor ions. The two compounds fragmented to produce intense product ion signals at m/z 107.1 and m/z 189.1, respectively. Ionization and fragmentation were found to be highly efficient, and as a result, a substantial detection response was obtained at the lower limit of quantitation (1 ng mL^{-1}). Product ion mass spectra of roxatidine and IS are shown in Fig. 1.

3.2. Specificity, linearity, precision and accuracy

Fig. 2 shows the typical chromatograms of a drug-free plasma sample, a blank plasma spiked with (2 ng mL^{-1}) and IS, and a plasma sample collected at 1.5 h after drug administration. There is no significant interference from the plasma found at the retention time.

Table 1
Intra- and inter-day assay summary ($n = 5$).

Theoretical concentration (ng mL^{-1})	Intra-day			*Inter − day*		
	Mean concentration found (ng mL^{-1})	CV (%)	Accuracy (%)	Mean concentration found (ng mL^{-1})	CV (%)	Accuracy (%)
1	0.94 ± 0.09	9.87	106.66	0.91 ± 0.06	6.79	110.17
2	2.02 ± 0.15	7.28	99.11	1.97 ± 0.13	6.44	101.54
10	10.44 ± 0.51	4.91	95.79	11.18 ± 1.12	9.97	89.43
100	106.40 ± 4.34	4.08	93.98	108.48 ± 4.76	4.39	92.18
1000	997.80 ± 42.29	4.24	100.22	100.27 ± 68.92	6.71	97.32

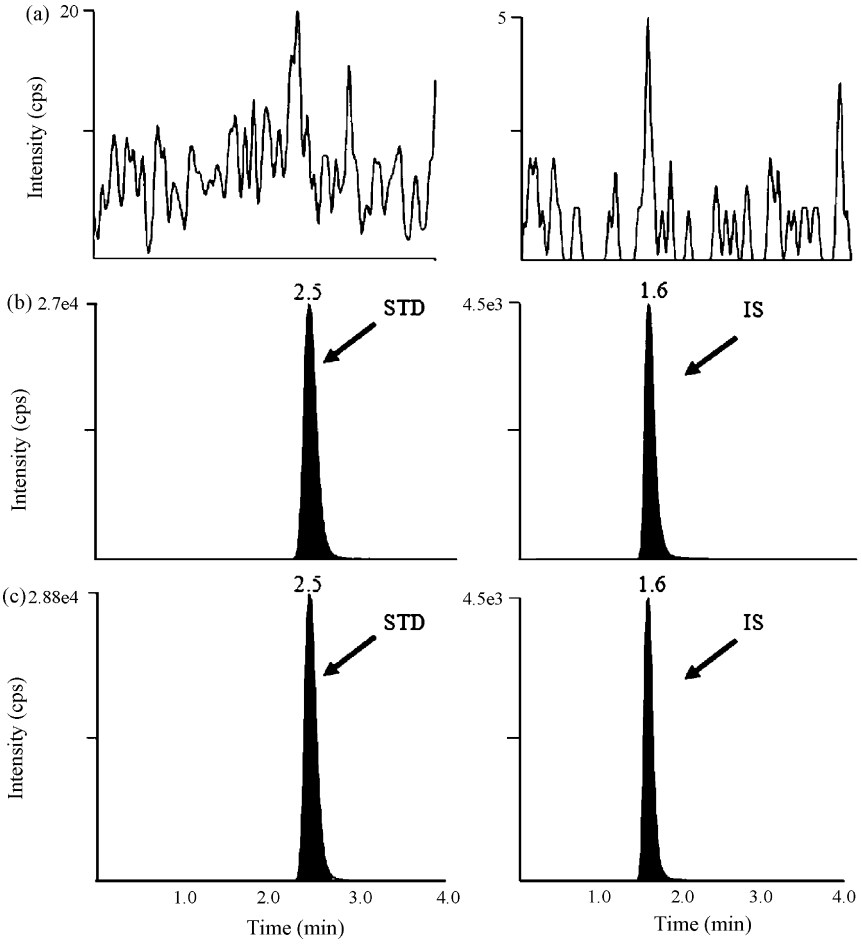

Fig. 2. Chromatograms of (a) blank human plasma, (b) plasma spiked with roxatidine (2 ng mL^{-1}) and famotidine (IS, 10 ng mL^{-1}), and (c) plasma from a volunteer (1.5 h after the oral administration of a 75 mg roxatidine acetate HCl capsule; this corresponds to a roxatidine plasma concentration of 2.64 ng mL^{-1}).

Calibration curves were prepared over the roxatidine concentration range of 1–1000 ng mL^{-1} by linear regression using a $1/x^2$ weighting factor. Correlation coefficients (r^2) were greater than 0.998 ($n = 5$) for all curves and the within- and between-run CVs of the response factors for the concentrations assayed were <15%.

Intra- and inter-day precision and accuracy of the method used for roxatidine analysis are presented in Table 1. Intra-day precisions were between 4.08 and 9.87%, and inter-day precisions between 4.39 and 9.97%. Intra- and inter-day accuracies ranged from 93.98 to 106.66% and from 89.43 to 110.17%, respectively. All results were within the ranges of precision (%) and accuracy (%) specified by the USFDA for bio-analytical applications.

3.3. Recovery, matrix effect and process efficiency

To decrease LLOQ, roxatidine recovery was examined using five different extraction solvents (ethyl acetate, ethyl ether, butyl acetate, dichloromethane, and *tert*-butyl methyl ether). Finally, ethyl acetate was chosen because of its good analyte recoveries and low variation, and using this solvent the LLOQ was reduced to 1 ng mL^{-1}. Standard deviations (S.D.s) observed between recoveries were <20% in the specified concentration range. These results show that the developed approach can be successfully applied to determine roxatidine recoveries by ethyl acetate extraction. The recoveries of roxatidine at 3, 400 and 800 ng mL^{-1} were 93.42 ± 4.57, 102.99 ± 3.94 and 96.26 ± 1.99%, respectively. The mean recovery of IS at these concentrations using ethyl acetate was only 67.91 ± 1.28%. However, the amount of analyte recovered was consistent and reproducible.

Matrix effects of roxatidine were investigated by comparing response ratio of roxatidine working solutions with processed blank samples reconstituted with roxatidine working solutions. The mean absolute and relative matrix effects of roxatidine at three different concentrations (3, 400 and 800 ng mL^{-1}) were less than ±10% (Table 2). The result of process efficiency was more than 95.12% (Table 2). These results indicate that no co-eluting endogenous substances significantly influenced the ion suppression in this analytical method. Accordingly, it is considered that the proposed analytical method is reliable and is subject to minimal matrix effect.

3.4. Stability

To evaluate roxatidine stability in human plasma, drug-free plasma samples were spiked with roxatidine at 3, 400 and

Table 2
Matrix effect and process efficiency of roxatidine ($n = 3$).

Analyte concentration (ng mL^{-1})	Absolute matrix effect (%)	Relative matrix effect (%)	Process efficiency (%)
Roxatidine			
3	1.46 ± 4.07	8.87 ± 5.99	95.12 ± 8.28
400	−3.30 ± 1.80	4.09 ± 1.78	99.57 ± 2.64
800	0.59 ± 1.32	6.55 ± 2.64	96.85 ± 3.22

Table 3
Pharmacokinetic parameters of roxatidine after oral administration of roxatidine acetate hydrochloride (75 mg) to female volunteers ($n = 24$).

Pharmacokinetic parameter	Reference drug (mean ± S.D.)	Test drug (mean ± S.D.)	Confidence limit 90%
AUC_{0-24} (μg h mL^{-1})	2.00 ± 0.63	2.21 ± 0.63	105.67–117.46
AUC_{inf} (μg h mL^{-1})	2.06 ± 0.67	2.29 ± 0.68	106.19–118.43
C_{max} (μg mL^{-1})	0.26 ± 0.07	0.27 ± 0.06	99.26–112.47
T_{max} (h)	3.25 ± 0.88	3.64 ± 0.96	–
$t_{1/2}$ (h)	4.23 ± 1.06	4.71 ± 1.80	–

800 ng mL^{-1}. After extraction, samples were arranged in an autosampler and analyzed. After 48 h at 4 °C, roxatidine stabilities at these concentrations were 100.52 ± 3.62, 105.32 ± 3.16 and 105.36 ± 1.32%, respectively. After 48 h at room temperature, stabilities were 101.14 ± 6.35, 103.22 ± 4.46 and 103.96 ± 4.66%, respectively, and after 48 h at −20 °C they were 109.33 ± 4.56, 109.71 ± 0.12 and 110.49 ± 0.66%, respectively. Thus, in terms of short-term stability in human plasma, roxatidine was found to be stable for 48 h at 4 °C, room temperature, and at −20 °C. After 30 days at −70 °C in human plasma, its stabilities at these concentrations were 101.31 ± 4.40, 98.80 ± 4.17 and 103.96 ± 2.33%, respectively. In this long-term stability study, plasma samples spiked with roxatidine showed no loss of peak area. After 24 h at autosampler condition (4 °C) in the extract, its stabilities at these concentrations were 100.62 ± 2.41, 104.30 ± 5.83 and 105.83 ± 3.30, respectively. Moreover, after three freeze–thaw cycles at these concentrations roxatidine stabilities were 105.67 ± 7.13, 107.33 ± 0.65 and 106.92 ± 1.42%, respectively. Summarizing, no significant deterioration was observed under any of the conditions examined.

3.5. Bioequivalence study

The proposed method was applied to the bioequivalence study of roxatidine in human plasma. The developed procedure was found to be sensitive enough for the quantitative analysis of roxatidine in human plasma with acceptable accuracy over a period of 24 h after a single oral administration. Moreover, high-throughput sample analysis is of particular importance for studies that require the analysis of large numbers of samples. The devised method of sample preparation using LLE could resolve this problem. Table 3 presents the pharmacokinetic parameters of roxatidine for reference and test formulations. The 90.0% confidence intervals for the ratios of test drug to reference drug in terms of AUC_{0-24} and C_{max} were within the range 80.0–125.0%, which is acceptable according to USFDA and KFDA [7,8].

4. Conclusions

A simple and sensitive LC–MS/MS method was developed to determine roxatidine levels in human plasma. Moreover, the devised method was found to fully meet USFDA guidelines. Furthermore, the method was found to be highly sensitive and specific, and is capable of analyzing large sample numbers.

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