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Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometric Determination of Lornoxicam in Human Plasma

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A rapid, sensitive and selective liquid chromatography-electrospray ionization tandem mass spectrometric (LC-ESI-MS/MS) method for the determination of lornoxicam in human plasma was developed. Lornoxicam and isoxicam (internal standard) were extracted from human plasma with ethyl acetate at acidic pH and analyzed on a Sunfire C_{18} column with the mobile phase of methanol:ammonium formate (10 mM, pH 3.0) (70:30, v/v). The analyte was detected using a mass spectrometer, equipped with electrospray ion source. The instrument was set in the multiple-reaction-monitoring mode. The standard curve was linear (r = 0.9998) over the concentration range of 0.50-500 ng/mL. The coefficient of variation and relative error for intra- and inter-assay at four QC levels were 0.7 to 4.2% and -4.5 to 5.0%, respectively. The recoveries of lornoxicam and isoxicam were 87.8% and 66.5%, respectively. The lower limit of quantification for lornoxicam was 0.50 ng/mL using a 200 μL plasma sample. This method was successfully applied to a pharmacokinetic study of lornoxicam after oral administration of lornoxicam (8 mg) to humans.

Key words: LC-ESI-MS/MS, lornoxicam, Human plasma, Pharmacokinetics

INTRODUCTION

Lornoxicam (6-cholro-4-hydroxy-2-methyl-*N*-2-pyridyl-2*H*-thieno[2,3-e]-1,2-thiazine-3-carboxamide 1,1-dioxide) is a member of the oxicam group of nonsteroidal anti-inflammatory drug (NSAIDs) against various arthritic conditions and post-operative inflammation (Radhofer-Welte and Rabasseda, 2000). Lornoxicam has a short elimination half-life of 3-5 h unlikely other oxicams such as piroxicam, meloxicam and tenoxicam (Skjodt and Davies, 1998) and is metabolized to 5'-hydroxy-lornoxicam by CYP2C9 (Bonnabry *et al.*, 1996). For the determination of lornoxicam in biological fluids, high performance liquid chromatography (HPLC) methods with UV detection (Radhofer-Welte and Dittrich, 1998), electrochemical detection (Suwa *et al.*, 1993) and atmospheric pressure chemical ionization (APCI)-tandem mass spectrometry (MS/MS) (Zeng *et al.*, 2004) were reported. LC-APCI-MS/MS method presented insufficient sensitivity (limit of quantitation, 2.0 ng/mL) and the use of large plasma volumes (0.5 mL plasma). A sensitive and rapid method in plasma is necessary to evaluate pharmacokinetics of lornoxicam which clinically single doses are 4 or 8 mg.

The purpose of this study was to develop a rapid and sensitive LC-electrospray ionization (ESI)-MS/MS method with simple sample preparation for the determination of lornoxicam in human plasma to support a pharmacokinetic study after oral administration of lornoxicam.

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EXPERIMENTAL

Materials and reagents

Lornoxicam and isoxicam were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ethyl acetate and methanol (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, U.S.A.) and the other chemicals were of the highest quality available.

Preparation of calibration standards and quality control samples

Primary stock solutions of lornoxicam and isoxicam (1

mg/mL) were prepared in acetonitrile. Working standard solutions of lornoxicam were prepared by diluting primary stock solution with acetonitrile. The working solution for isoxicam (internal standard, 0.2 μg/mL) was prepared by diluting an aliquot of stock solution with acetonitrile. All standard solutions were stored at ca 4°C in polypropylene tubes in the dark when not in use.

Human plasma calibration standards of lornoxicam (0.50, 1.00, 2.00, 5.00, 10.0, 50.0, 100, 200 and 500 ng/mL) were prepared by spiking the working standard solutions into a pool of drug-free human plasma. Quality control (QC) samples at 0.50, 1.50, 40.0 and 400 ng/mL were prepared in bulk by adding 250 μL of the appropriate working standard solutions (0.01, 0.03, 0.80, 8.0 μg/mL) to drug-free human plasma (4750 μL). The bulk samples were aliquoted (200 μL) into polypropylene tubes and stored at -20°C until analysis.

Sample preparation

200 μL of blank plasma, calibration standards and QC samples were mixed with 10 μL of internal standard working solution and 200 μL of 0.5 M HCl. The samples were extracted with 900 μL of ethyl acetate in 2.0 mL-polypropylene tubes by vortex-mixing for 5 min at high speed and centrifuged at 5000 *g* for 5 min at room temperature. 800 μL of the organic layer was transferred and evaporated to dryness using vacuum concentrator at 30°C. The residues were dissolved in 40 μL of 70% methanol in water by vortex-mixing for 2 min, centrifuged at 5000 *g* for 5 min, transferred to injection vials, and 10 μL were injected into the HPLC column.

LC-MS/MS Analysis

For LC-MS/MS analysis, the chromatographic system consisted of a Nanospace SI-2 pump, a SI-2 autosampler and a S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on a Sunfire C_{18} column (5 μm, 2.1 mm i.d. × 100 mm, Waters, CA, U.S.A.) using a mixture of methanol:ammonium formate (10 mM, pH 3.0) (70:30, v/v) at a flow rate of 0.2 mL/min. The column and autosampler tray were maintained at 50°C and 4°C, respectively. The analytical run time was 3.5 min. The eluent was introduced directly into the electrospray source of a tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd, UK) that was set in the positive mode. The ion source and desolvation temperatures were set at 120°C and 350°C, respectively. The capillary voltage was 3.0 kV and the optimum cone voltages were 35 and 33 V for lornoxicam and isoxicam, respectively. The molecular ions for lornoxicam and isoxicam were fragmented at collision energies of 18 and 17 eV using argon as collision gas. Multiple-reaction-monitoring (MRM) mode was used for the quantification by monitoring the transitions: *m/z* 371.9→95.7 for lornoxicam and *m/z* 336.6→99.2 for isoxicam (internal standard). Peak areas for all components were automatically integrated using MassLynx version 3.5 software (Micromass UK Ltd.).

Method validation

Batches, consisting of triplicate calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 0.50, 1.50, 40.0 and 400 ng/mL were assayed in sets of six replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the coefficient of variation (CV) serve as the measure of accuracy and precision. The selectivity was evaluated by analyzing blank plasma samples obtained from twelve different sources.

The absolute and relative matrix effect and recoveries of lornoxicam and isoxicam were assessed by analyzing three sets of standards at four concentrations (0.50, 1.50, 40.0 and 400 ng/mL) according to the approach of Matuszewski *et al.* (2003). The absolute matrix effect for lornoxicam and isoxicam was assessed by comparing mean peak areas of the analyte at four concentrations spiked after extraction into plasma extracts originating from five different humans (set 2) to mean peak areas for neat solutions of the analytes in 70% methanol (set 1). The variability in the peak areas of the analyte spiked post-extraction into five different plasma extracts (set 2) expressed as CVs (%), was considered as a measure of the relative matrix effect. Recoveries of lornoxicam and isoxicam were determined by comparing mean peak areas of analytes spiked before extraction into the same five different sources as set 2 (set 3) with those of the analyte spiked post-extraction into different blank plasma lots at four concentrations (set 2).

Application

The developed LC-ESI-MS/MS method was used in a pharmacokinetic study after oral administration of lornoxicam (8 mg) to five healthy male volunteers. Following oral administration, blood samples (2 mL) were withdrawn from the arm at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h, transferred to Vacutainer plasma glass tubes (sodium heparin, BD, NJ, U.S.A.) and centrifuged. Following centrifugation (3000 *g*, 15 min, 4°C), plasma samples were transferred to polypropylene tubes and stored at -70°C prior to analysis.

The following pharmacokinetic parameters were determined for each subject: the maximum plasma concentration (C_{max}), the time taken to reach C_{max} (T_{max}), half-life ($t_{1/2}$) and area under the plasma concentration-time curve (AUC). C_{max} and T_{max} were determined by visual inspection,

and $t_{1/2}$ and AUC were calculated by WinNonlin program.

RESULTS AND DISCUSSION

LC-ESI-MS/MS

The positive electrospray ionization of lornoxicam and isoxicam produced abundant protonated molecular ions (MH^+) at *m/z* 371.9 and 336.6, respectively, without any evidence of fragmentation and adduct formation. Protonated MH^+ ions from lornoxicam and isoxicam were selected as the precursor ions and subsequently fragmented in MS/MS mode to obtain the product ion spectra yielding useful structural information (Fig. 1). The fragment ions at *m/z* 95 ([pyridin-2-ylamine+H]$^+$) and 99 ([5-methyloxazol-2-ylamine +H]$^+$) were produced as the prominent product ions for lornoxicam and isoxicam, respectively. The quantification of the analytes was performed using MRM mode due to the high selectivity and sensitivity. Two pairs of MRM transitions were selected: *m/z* 371.9→95.7 for and *m/z* 336.6→99.2 for isoxicam (internal standard).

The Sunfire column with a mobile phase consisting of methanol and ammonium formate (10 mM, pH 3.0) (70:30, v/v) resulted in short chromatographic run time (3.5 min) with satisfactory separation of lornoxicam and isoxicam.

Fig. 2 shows the representative LC-MS/MS MRM chro-

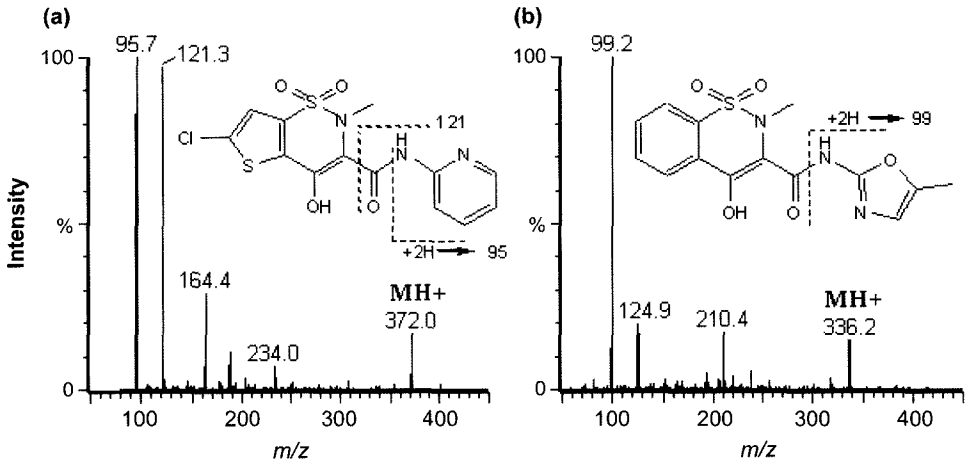

Fig. 1. Product ion mass spectra of (a) lornoxicam and (b) isoxicam

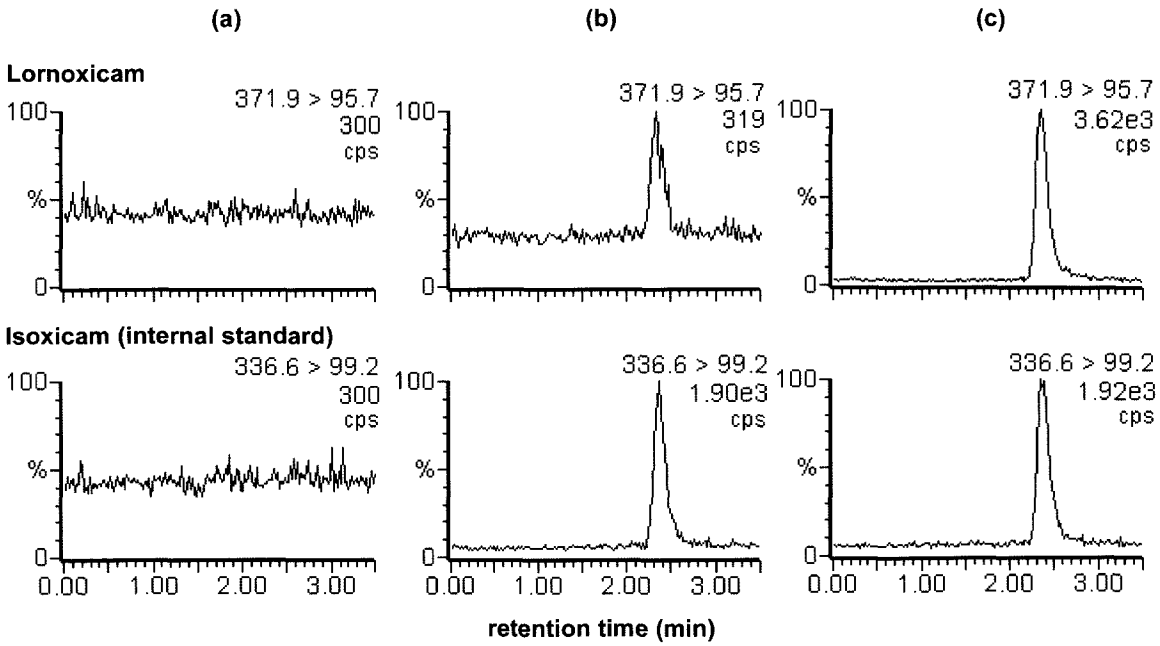

Fig. 2. MRM chromatograms of (a) a human blank plasma, (b) human plasma sample spiked with 0.5 ng/mL of lornoxicam and (c) a plasma sample obtained 30 min after oral administration of lornoxicam (8 mg) to a male volunteer

matograms obtained from the analysis of blank human plasma and human plasma samples spiked with lornoxicam at 0.50 ng/mL. The analysis of blank plasma samples from sixteen different sources did not show any interference at the retention times of lornoxicam (2.3 min) and isoxicam (2.3 min), confirming the specificity of the present method. Sample carryover effect was not observed.

Method validation

Calibration curves were obtained over the concentration range of 0.50 to 500 ng/mL for lornoxicam in human plasma. Linear regression analysis with a weighting of 1/concentration^2 gave the optimum accuracy of the corresponding calculated concentrations at each level (Table I). The low CV value for the slope indicated the repeatability of the method (Table I).

Table II shows a summary of intra- and inter-batch precision and accuracy data for QC samples containing lornoxicam. Both intra-and inter-assay CV values ranged from 0.7 to 4.2% at four QC levels. The intra-and inter-assay RE values for lornoxicam were -4.5 to 5.0% at four QC levels. These results indicated that the present method has the acceptable accuracy and precision. The lower limit of quantitation (LLOQ) was set at 0.50 ng/mL for lornoxicam using 200 μL of human plasma. Representative chromatogram at the LLOQ is shown in Fig. 2b and the signal-to-noise ratio for lornoxicam are higher than 5. This LLOQ value was smaller than that (2 ng/mL) of APCI-MS/MS method reported by Zeng *et al.* (2004) in 500 mL of human plasma. Therefore, the present method enables the pharmacokinetic studies of lornoxicam after oral dosing of lornoxicam (4 mg).

The overall extraction recovery of lornoxicam from rat plasma was 87.8%, which was consistent at four concentration levels, and the recovery of isoxicam (internal standard) was 66.5% (Table III). The one-step liquid-liquid

Table I. Calculated concentrations of lornoxicam in calibration standards prepared in human plasma (n = 9)

Statistical variable	Theoretical concentration (ng/mL)									slope	r
	0.50	1.00	2.00	5.00	10.0	40.0	100	200	500		
Mean (ng/mL)	0.50	0.99	1.96	5.01	9.87	49.4	101	200.	497.	0.2054	0.9998
CV (%)	6.0	5.1	5.1	2.9	4.0	1.5	1.6	0.4	2.1	7.9	
RE (%)	0.0	-1.0	-2.0	0.2	-1.3	-1.2	1.0	0.0	-0.6		

Table II. Precision and accuracy of lornoxicam in human plasma quality control samples

Statistical variable	Intra-batch (ng/mL, n=6)				Inter-batch (ng/mL, n=3)			
	0.50	1.50	40.0	400	0.50	1.50	40.0	400
Mean	0.48	1.53	42.0	387	0.51	1.53	39.1	382
CV (%)	4.2	2.5	2.9	4.0	2.0	0.7	2.4	2.2
RE (%)	-4.0	2.0	5.0	-3.3	2.0	2.0	-2.3	-4.5

Table III. Absolute matrix effect, recovery and precision (CV, %) data for lornoxicam and isoxicam (internal standard) in five different lots of human plasma

Nominal concentration (ng/mL)	Absolute matrix effect[a] (%)		Recovery[b] (%)		Precision[c] (CV, %)					
					Peak area of lornoxicam		Peak area of isoxicam		Peak area ratio	
	lornoxicam	I.S.	lornoxicam	I.S.	Set1	Set2	Set1	Set2	Set1	Set2
0.5	103	101	88.7	65.9	5.3	9.9	5.2	5.9	5.1	5.8
1.5	104	104	87.4	69.9	6.6	8.6	3.5	7.8	4.0	7.5
40	102	101	86.9	65.8	6.5	9.4	6.0	5.2	4.6	4.1
400	103	102	88.0	64.2	4.6	9.4	4.2	3.4	3.4	3.1
Mean	103	102	87.8	66.5	5.8	9.3	4.7	5.6	4.3	5.1

[a]Absolute matrix effect expressed as the ratio of the mean peak area of an analyte spiked post-extraction (set 2) to the mean peak area of same analyte standards (set 1) multiplied by 100.

[b]Recovery calculated as the ratio of the mean peak area of an analyte spiked before extraction (set 3) to the mean peak area of an analyte spiked post-extraction (set 2) multiplied by 100.

[c]Precision of determination of peak areas of lornoxicam and isoxicam, and peak area ratios (lornoxicam/isoxicam) in sets 1 and 2 was a measure of relative matrix effect.

extraction with ethyl acetate at neutral pH has been successfully applied to the extraction of lornoxicam from human plasma.

The mean absolute matrix effect, the ratio of mean peak areas of set 2 to those of set 1 multiplied by 100, was 103% and 102% for lornoxicam and isoxicam, respectively (Table III). A value of 100% indicates that the response in the solvent and in the plasma extracts were the same and no absolute matrix effect was observed. A value of < 100% indicates an ionization suppression and a value of > 100% indicates an ionization enhancement. There was little absolute matrix effect for lornoxicam and isoxicam. The assessment of a relative matrix effect was made based on direct comparison of the peak areas of lornoxicam and isoxicam spiked post-extraction into extracts originating from five different sources of human plasma (set 2). The CVs of determination of set 2 at different concentrations varied from 8.6 to 9.9% for lornoxicam and 3.4 to 7.8% for isoxicam (Table III). This variability seemed to be comparable to the precision of determination of standards injected directly in 70% methanol (set 1) (4.6 to 6.6% for lornoxicam and 3.5 to 6.0% for isoxicam, Table III). These data confirm that the relative matrix effect for lornoxicam and isoxicam was practically absent. The CV of the ratio of lornoxicam to isoxicam for samples spiked post-extraction into extracts from five different lots of plasma varied from 3.1 to 7.5% at different concentrations and was similar to the CV of the ratio of lornoxicam/isoxicam injected directly in 70% methanol (3.4 to 5.1%, set 1 in Table III), confirming that the absolute and relative matrix effects for ratio of lornoxicam and isoxicam have practically no effect on the determination of lornoxicam spiked into five different lots of human plasma.

Stability of lornoxicam during sample handling (three freeze-thaw cycles and short-term temperature storage) and the stability of processed samples were evaluated (Table IV). Three freeze-thaw cycles and short-term (4 h) storage at room temperature of the QC samples at the low and high concentrations before analysis, had little effect on the quantification. Extracted QCs and calibration standards were allowed to stand at 4°C for 24 h prior to injection without affecting the quantification.

Clinical application

This method has been successfully applied to the pharmacokinetic study of lornoxicam in five healthy male volunteers. Representative chromatograms of the extract of a plasma sample obtained 30 min after oral dosing of lornoxicam (8 mg) to human are shown in Fig. 2c. Fig. 3 shows the mean plasma concentration profile of lornoxicam obtained after a single oral dosing of lornoxicam (8 mg) to five healthy male volunteers. C_{max}, T_{max}, $t_{1/2}$ and AUC of lornoxicam were 554 ± 100 ng/mL, 2.4 ± 0.9 h, 4.3 ± 0.9 h and 3089 ± 996 ng·h/mL, respectively.

Table IV. Stability of samples (n = 5)

Statistical variable	Three freeze/thaw stability	Short-term stability (4 h at room temperature)	Post-preparative stability (24 h at 4°C)
1.50 ng/mL			
Mean	1.43	1.42	1.55
CV (%)	4.9	1.9	2.2
RE (%)	-4.7	-5.3	3.3
400 ng/mL			
Mean	381	387	373
CV (%)	2.2	2.8	3.9
RE (%)	-4.8	3.3	-6.8

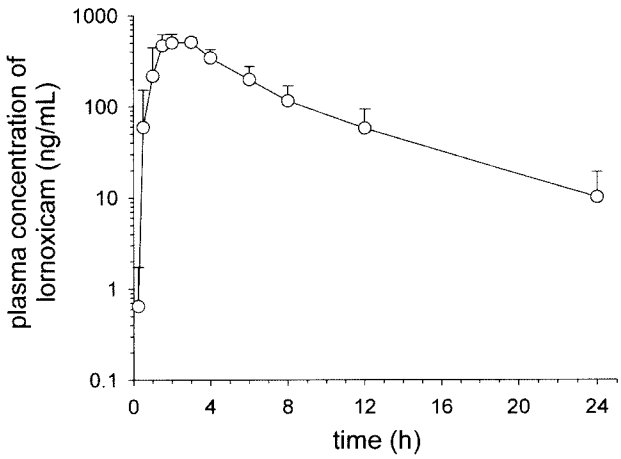

Fig. 3. Mean plasma concentration-time profile of lornoxicam after oral administration of lornoxicam (8 mg) to five male volunteers. Each point represents the mean ± S.D.

In conclusion, a rapid, sensitive and reliable LC-ESI-MS/MS method for the determination of lornoxicam in human plasma has been successfully developed and validated using liquid-liquid extraction with ethyl acetate as sample clean-up procedure. This assay method demonstrated acceptable sensitivity (LLOQ: 0.50 ng/mL), precision, accuracy, selectivity, recovery and stability and a relatively short analysis time. This method was successfully applied to assay human plasma samples from the pharmacokinetic study of lornoxicam.

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