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Simultaneous high-performance liquid chromatographic determination of lornoxicam and its 5′-hydroxy metabolite in human plasma using electrochemical detection

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ABSTRACT

A selective and sensitive high-performance liquid chromatographic method with coulometric detection is described for the simultaneous quantitation of lornoxicam, a new oxicam class drug, and its 5′-hydroxy metabolite in human plasma. The two analytes and the internal standard are extracted from plasma at pH 4.0 by liquid–liquid extraction and separated on a C_{18} column. Absolute detection limits using 100 μl of plasma are found to be 5 and 10 ng/ml for lornoxicam and 5′-hydroxylornoxicam, respectively. The assay described has been successfully applied to samples from clinical studies.

INTRODUCTION

Lornoxicam (rINN, 6-chloro-4-hydroxy-2-methyl-N-2-pyridyl-2*H*-thieno[2,3-*e*]-1,2-thiazine-3-carboxamide-1,1-dioxide, Lx, Fig. 1) is a new, highly potent non-steroidal anti-inflammatory drug (NSAID) [1] of the oxicam class. Oxicams display many of the clinical properties of aspirin-like drugs, including analgesic, antipyretic and anti-inflammatory activities. Two of this class of NSAIDs presently on the market, piroxicam (Px) and tenoxicam (Tx), are known to be low plasma clearance drugs. Many assay systems involving high-performance liquid chromatography with UV detection (HPLC–UV) have been developed to determine plasma levels of these drugs [2–7]. However, the clinical dose of Lx is lower, owing to its strong activity and its relatively faster elimination from plasma than any other oxicam [8,9]. This indicates that the determination of Lx in a limited volume of clinical sample requires a more lower detection limit. First we examined HPLC–UV to detect plasma levels of Lx in a limited plasma sample (0.1 ml), but this was not applicable to pharmacokinetic studies of Lx owing to its low sensitivity. Although electrochemical detection (ED) of Px has been reported as another way to detect oxicams

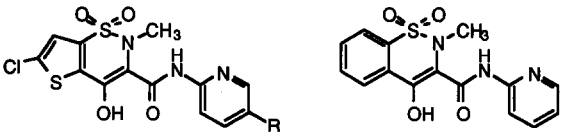

Fig. 1. Structures of lornoxicam, its 5′-hydroxy metabolite and the internal standard piroxicam.

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[10], even in this procedure the lower limit of quantitation was 0.1 μg/ml owing to poor reproducibility at lower levels.

To overcome these problems, we report here a more sensitive HPLC–ED method, which can detect Lx at levels as low as some hundreds of picograms per injection with good precision. This method is also able to determine simultaneously the 5′-hydroxy metabolite, known to be the only metabolite of Lx in human plasma [8]. By using this procedure, Lx and its metabolite were successfully determined in a small amount of plasma in healthy volunteers after oral administration. It is anticipated that this assay will provide a more sensitive alternative to detect this class of NSAIDs.

EXPERIMENTAL

Chemicals and reagents

Lx, its 5′-hydroxy metabolite and internal standard (I.S.), Px, were supplied by Hafslund Nycomed Pharm (Austria). Organic solvents were of HPLC grade and all other chemicals were of analytical-reagent grade (Wako, Japan). Aqueous solutions were prepared using doubly deionized water from a Milli-QSP system (Millipore, Bedford, MA, USA).

Standard solutions

Pure standard stock solutions of Lx and its metabolite (1 mg/10 ml) were prepared in water and 50 μl of 0.1 *M* sodium hydroxide, and stored at 4°C. Working solutions (10, 1, 0.1, 0.01 μg/ml) were made by further dilution in water.

A standard stock solution of the I.S. (1 mg/10 ml) was prepared in the same way and further diluted in water to yield the working solution (0.1 μg/ml). All solutions were tested weekly for degradation of the drug.

Chromatographic apparatus and conditions

The chromatographic system consisted of a constant-flow, single-piston pump LC-6A (Shimadzu, Kyoto, Japan), equipped with an automatic injector (SIL-6B autoinjector, Shimadzu), and a Coulochem 5100A electrochemical detector linked to a 5010 analytical cell (Environmental Science Assoc., Bedford, MA, USA). The potentials of the screen electrode (detector 1) and the sample electrode (detector 2) were varied from 0 to 0.8 V to find the optimal detection conditions. The response time was 10 s. The 1-V output from detector 2 was connected to a Chromatocorder 12 integrator (SIC, Tokyo, Japan). A prepacked Sumipax ODS A-212 (5 μm particle size) column (15 cm × 6 mm I.D., Sumika, Osaka, Japan) was used.

The mobile phase, 0.05 *M* potassium dihydrogenphosphate–acetonitrile–methanol (60:23:17, v/v/v) was prepared daily, degassed, and pumped at a flow-rate of 1.5 ml/min. The column temperature was maintained at 35°C.

Sample preparation

In a glass centrifuge tube, 850 μl of acetate buffer (pH 4.0), 50 μl of internal standard solution and 5 ml of dichloromethane were added to 0.1 ml of plasma. The tube was stoppered and shaken for 10 min, then centrifuged at 1600 *g* for 5 min at 4°C. The organic phase was transferred to another tube, and the residue was again extracted with 5 ml of dichloromethane. The combined organic layers were evaporated to dryness *in vacuo*. The residue was reconstituted in 200 μl of mobile phase, and 80 μl were injected into the analytical column.

Calibration graphs

Lx and its 5′-hydroxy metabolite were added to 0.1 ml of control human plasma to obtain final concentrations ranging from 5 to 1000 ng/ml for Lx and from 10 to 250 ng/ml for its metabolite. These were extracted as described above. Calibration graphs were generated by least-squares regression of the analyte/I.S. peak-height ratio against the concentration of the analyte.

Recovery

The recovery of Lx and its metabolite from human plasma was determined in triplicate at concentrations within the range of the calibration graphs (50, 250 and 1000 ng/ml). Blank plasma samples spiked with known amounts of analytes

were extracted and compared with blank plasma extracts subsequently spiked with the same amounts of analytes. The two sets of extracts were evaporated to dryness, and the residues were reconstituted in mobile phase. The recovery was assessed by comparing the peak heights in the two sets of extracts. The recovery of the I.S. was assessed in the same way as described above, at 50 ng/ml.

Precision and accuracy

The intra-assay precision was assessed using triplicate spiked samples at the same concentrations as for the calibration graphs. The inter-assay precision was studied at three different concentrations ranging from 50 to 1000 ng/ml, which were independently analysed at least twelve times in a fourteen-day period.

Control plasma was spiked with Lx and its metabolite to give at least 24 concentrations ranging from 10 to 1000 ng/ml. The samples were assayed, and concentrations were derived from the calibration graphs. The accuracy was evaluated by comparing the estimated concentration with the known concentration of the analyte.

Clinical study

This method of determination of Lx and its metabolite in plasma was used in a pharmacokinetic study. The results obtained with one subject are presented. Following oral administration of Lx at a dose of 4 mg to the subject, blood samples were collected in heparinized tubes and centrifuged, and the plasma was stored at −20°C until analysis.

RESULTS AND DISCUSSION

The specific and sensitive method reported here for the determination of Lx and its 5′-hydroxy metabolite employs HPLC and a coulometric detector with two working electrodes. The two electrodes were used in the oxidative screen mode, and the optimal oxidation potentials were determined through repeated on-column injections of stock solutions of Lx, the 5′-hydroxy metabolite and the I.S. diluted in water. The resulting hydrodynamic voltammograms at detector 2 are shown in Fig. 2. At potentials higher than +0.55 V, the current attained a plateau phase except for the 5′-hydroxy metabolite: although the latter seemed to have a second phase due to two hydroxyl groups at higher than +0.55 V, an operating oxidation potential of +0.5 V for the second electrode was chosen to avoid the background noise induced by higher potentials. The first electrode was set at 0.4 V to screen for possible oxidizable impurities in the mobile phase and/or the biological sample.

Despite the high resolving power of HPLC and the selectivity of the detector, plasma must be purified prior to injection. For the sample preparation, liquid–liquid extraction under acidic conditions was used. This was found to be highly selective and reproducible for the extraction of both analytes. The recoveries of Lx and the 5′-hydroxy metabolite were excellent (Table I).

On the column, the limit of detection was as low as 100 pg for both compounds. The lower limit of quantitation using 100 μl of plasma was 5 ng/ml for Lx and 10 ng/ml for the metabolite, whereas using UV detection at 371 nm it was less than 30 ng/ml. Although many assay systems have been reported for this class of NSAIDs [2–7,10], the use of ED proved to be more sensitive.

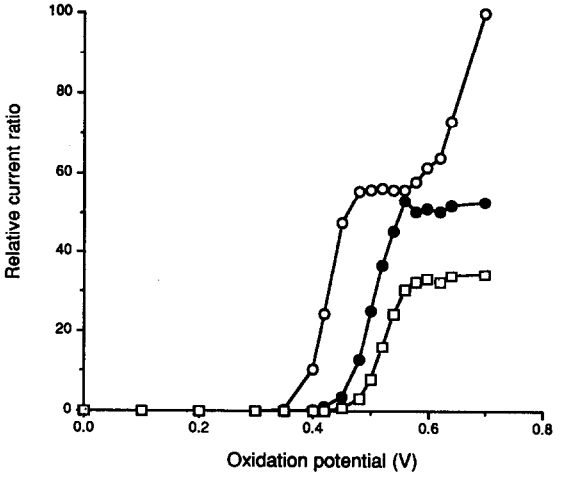

Fig. 2. Hydrodynamic voltammograms of lornoxicam (□), its 5′-hydroxy metabolite (○) and the I.S. (●).

TABLE I

RECOVERY OF LORNOXICAM AND 5'-HYDROXYLORNOXICAM FROM HUMAN PLASMA SAMPLES AND INTRA-ASSAY VARIABILITY

Concentration added (ng/ml)	Lornoxicam		5'-Hydroxylornoxicam	
	Recovery (mean ± S.D., n = 3) (%)	C.V. (%)	Recovery (mean ± S.D., n = 3) (%)	C.V. (%)
50	103.9 ± 0.6	0.6	103.9 ± 0.9	0.9
250	103.1 ± 1.4	1.4	101.1 ± 0.8	0.8
1000	99.5 ± 8.3	8.4	–	–

Calibration graphs were prepared from triplicate plasma samples spiked with Lx ranging from 5 to 1000 ng/ml or with the 5'-hydroxy metabolite from 10 to 250 ng/ml. In all cases, the graphs were linear: least-squares analysis yielded the following characteristics: for Lx, $y = 0.21356 + 98.873x$, $r^2 = 1.000$; and for 5'-hydroxy metabolite, $y = -2.9393 + 47.794x$, $r^2 = 0.999$ (y = concentration of Lx and its metabolite in ng/ml; x = ratio of peak height; r = correlation coefficient). To evaluate the reproducibility of the system and the method, intra-assay precision studies were conducted. The coefficient of variation (C.V.) ranged from 0.9 to 4.3% for Lx and from 1.2 to 5.5% for the 5'-hydroxy metabolite (Table II). The inter-assay reproducibility had a mean C.V. of 8.7% for Lx and 7.8% for the 5'-hydroxy metabolite.

The mean accuracies for Lx and its metabolite were 100.2 ± 6.4% (mean ± S.D., n = 36) and 99.4 ± 5.7% (mean ± S.D., n = 24), respectively (Table III).

Fig. 3 shows representative chromatograms of extracts of drug-free plasma spiked or unspiked

TABLE II

CALIBRATION GRAPHS FOR LORNOXICAM AND 5'-HYDROXYLORNOXICAM IN PLASMA AND INTRA-ASSAY VARIABILITY

Concentration (ng/ml)	Lornoxicam		5'-Hydroxylornoxicam	
	Peak-height ratio (mean ± S.D., n = 3)	C.V. (%)	Peak-height ratio (mean ± S.D., n = 3)	C.V. (%)
5	0.06 ± 0.00	2.8	–	–
10	0.11 ± 0.00	2.9	0.29 ± 0.02	5.5
50	0.53 ± 0.00	0.9	1.10 ± 0.02	2.0
100	1.06 ± 0.05	4.3	2.30 ± 0.06	2.8
250	2.45 ± 0.03	1.1	5.33 ± 0.06	1.2
500	5.26 ± 0.06	1.2	–	–
1000	10.33 ± 0.29	2.8	–	–

TABLE III

INTER-ASSAY PRECISION AND ACCURACY OF LORNOXICAM AND 5'-HYDROXYLORNOXICAM

Compound	Added (ng/ml)	Found (ng/ml)	C.V. (%)	Accuracy[a] (%)	Replicates (*n*)	Period of time (weeks)
Lornoxicam	51.16	53.52	4.7	4.61	12	2
	255.80	250.60	5.2	−2.03	12	2
	1023.20	1004.26	7.5	−1.85	12	2
5'-Hydroxylornoxicam	50.75	49.74	7.2	−1.99	12	2
	253.72	255.81	3.6	0.82	12	2

[a] Deviation between added and found.

with Lx and its metabolite. The retention times of the 5'-hydroxy metabolite, the I.S. and Lx were 8.0, 9.0 and 10.4 min, respectively. The total time for a chromatographic run was less than 12 min.

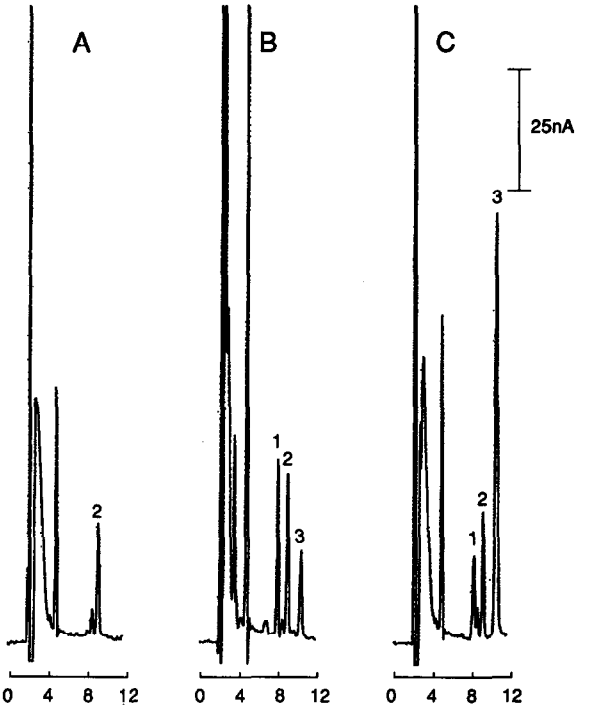

Fig. 3. Chromatograms of (A) a plasma sample collected from a subject before the dose of lornoxicam, (B) drug-free plasma spiked with 50 ng/ml each of lornoxicam, the 5'-hydroxy metabolite and the I.S. and (C) a plasma sample collected from a subject 1 h after a single 4-mg oral dose of lornoxicam. Peaks: 1 = 5'-hydroxy metabolite; 2 = I.S.; 3 = lornoxicam.

This method was applied to a study of the pharmacokinetics of Lx after oral administration of 4 mg to a healthy subject. A plasma sample collected before the dose was free from interferences (Fig. 3A); a chromatogram obtained from a plasma sample taken 1 h after administration is shown in Fig. 3C. As shown in Fig. 4, plasma concentrations of Lx and its metabolite were well within the limit of quantitation of our assay.

In addition, as shown in Fig. 2, the sensitivity to Px, used as the I.S., is even greater than that to Lx. This indicates that, to determine plasma levels of Px, a smaller sample volume could be used without reducing the sensitivity.

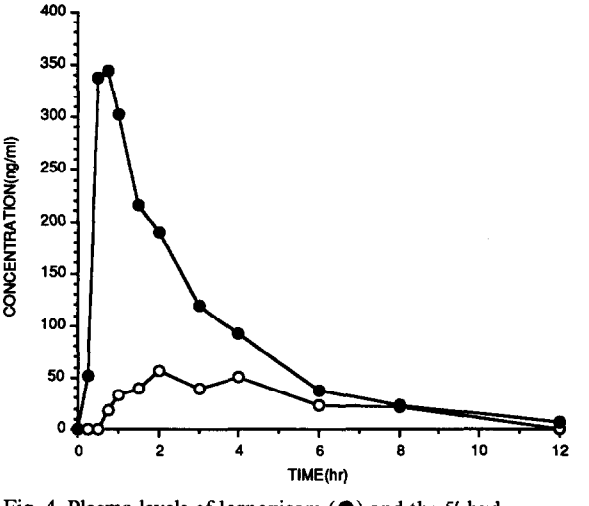

Fig. 4. Plasma levels of lornoxicam (●) and the 5'-hydroxy metabolite (○) following a single 4-mg oral dose of lornoxicam to a healthy subject.

CONCLUSION

A simple and highly selective HPLC assay with electrochemical detection has been developed for the simultaneous determination of Lx and its 5'-hydroxy metabolite in a limited volume (0.1 ml) of plasma sample. The method is reproducible and also more sensitive than the commonly used HPLC–UV technique, and was successfully used in a pharmacokinetic study in a human. This method will also be applied to the determination of therapeutic concentrations of Px in human plasma samples.

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