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# Determination of the novel non-steroidal anti-inflammatory drug lornoxicam and its main metabolite in plasma and synovial fluid

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**Abstract**

A rapid and sensitive HPLC method for the determination of the non-steroidal anti-inflammatory drug lornoxicam in plasma samples of humans and laboratory animals is described. After addition of the internal standard (tenoxicam) the plasma sample is acidified and extracted either by dichloromethane via Extrelut columns or by solid-phase extraction using $C_{18}$ columns. After evaporation of the solvent the separation is performed on a $C_{18}$ column in isocratic mode with a mobile phase consisting of 0.1 *M* phosphate buffer (pH 6.0)–methanol and detection at 372 nm. The limit of determination was set to 10 ng/ml using 0.5 ml of sample but can be extended down to 2.0 ng/ml plasma. Using solid-phase extraction with $C_{18}$ columns both lornoxicam and its main metabolite 5′-hydroxylornoxicam can be determined while extraction via Extrelut was used in studies where only lornoxicam was to be determined. This method was used successfully in several thousand samples of pharmacokinetic and bioavailability studies in animals and in humans. © 1998 Elsevier Science B.V.

*Keywords:* Lornoxicam; 5′-Hydroxylornoxicam

## 1. Introduction

Lornoxicam (rINN, 6-chloro-4-hydroxy-2-methyl-*N*-2-pyridyl-2H-thieno[2, 3-*e*]-1, 2-thiazine-3-carboxamide-1,1-dioxide) is a novel non-steroidal anti-inflammatory drug (NSAID) with marked analgesic properties [1]. Lornoxicam is a yellow crystalline substance with a p$K_a$ of 4.7 and a partition coefficient of 1.8 determined in octanol–phosphate buffer pH 7.4. During development of a new chemical entity the performance of preclinical as well as clinical pharmacokinetic studies is required. A major part of these studies is the determination of the drug concentrations in body fluids at specified time points after dosing. Therefore analytical methods for the determination of the parent drug as well as the main metabolite in plasma of different species were developed and validated.

Lornoxicam belongs to the chemical class of the oxicams like piroxicam, tenoxicam and meloxicam. Methods for determination of these compounds with reversed-phase high-performance liquid chromatography (HPLC) have been published. As sample preparation methods protein precipitation [2–7], liquid–liquid extraction [8–21], liquid–liquid extraction using Extrelut columns [22] and off-line as well as on-line solid-phase extraction (SPE) [23–25] have been described. However the sensitivities of these methods are only in the range of 20 ng/ml to 200 ng/ml with the exception of the method described in

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Ref. [15], where a limit of determination of 10 ng/ml was reported.

Lornoxicam is more potent than other oxicams, clinically single doses of 4 or 8 mg are used. As a consequence a sufficiently sensitive method for the determination of lornoxicam had to be developed. In the course of the investigations this method had to be complemented by an extraction for the determination of both lornoxicam and its main metabolite 5′-hydroxylornoxicam which was achieved with the use of SPE on a $C_{18}$ phase.

## 2. Experimental

### *2.1. Reagents and solvents*

Analytical grade chemicals and solvents were used: methanol (analytical-reagent grade), dichloromethane LiChrosolv and 25% ammonia from Merck (Darmstadt, Germany), sodium dihydrogenphosphate monohydrate (analytical-reagent grade) from Merck, sodium hydroxide and orthophosphoric acid (both analytical-reagent grade) from J.T. Baker (Deventer, Netherlands). Deionised water was further purified by the STILL plus device (Purite, Thame, UK).

### *2.2. Standard compounds*

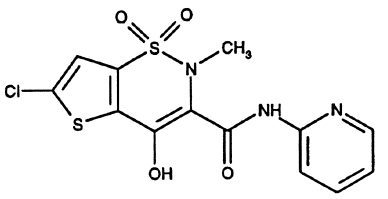
Lornoxicam (Fig. 1, **I**), the metabolite 5′-hydroxylornoxicam (Fig. 1, **II**) and the internal standard tenoxicam (Fig. 1, **III**) were synthesized at Nycomed Austria (Linz, Austria).

### *2.3. Standard solutions*

For the lornoxicam stock solution, lornoxicam (5 mg) is weighed into a 100-ml flask and dissolved in 0.25 ml of 0.2 *M* NaOH and 0.625 ml of water. This solution is then diluted with water to a final volume of 100 ml.

For the tenoxicam and 5′-hydroxylornoxicam stock solutions, tenoxicam (4 mg) or 5′-hydroxylornoxicam (5 mg) are dissolved as described above for lornoxicam.

Further dilutions are made with water. The solutions were kept in the dark at 4°C and were found to be stable under these conditions for at least four weeks.

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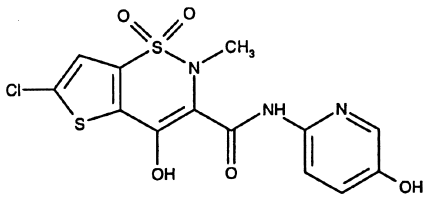


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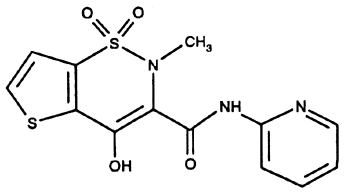


III

Fig. 1. Chemical structures of lornoxicam (**I**), 5′-hydroxylornoxicam (**II**) and tenoxicam (**III**).

### *2.4. Chromatography*

The HPLC system consisted of a gradient pump, column oven, autosampler, variable-wavelength UV detector and a data system.

For analysis of samples after extraction on Extrelut-1 columns the HPLC system LC 5060 (Varian Instruments, San Fernando, CA, USA) equipped with either a Rheodyne valve Model 7125 or the autosampler Model 9090 (Varian Instruments), the UV detector Spectroflow 783 (Kratos Analytical,

Ramsey, NJ, USA), column oven with cooling option (W.O. Industrial Electronic, Langenzersdorf, Austria) and the data system Vista 401 (Varian Instruments) was used. For analysis of samples after SPE the HP-1050 System was used (Hewlett-Packard, Waldbronn, Germany) consisting of a quartenary pump, autosampler and variable-wavelength detector, column oven (W.O. Industrial Electronic) and the data system HP ChemStation (Hewlett-Packard). The samples were separated on a $C_{18}$ column (ODS Hypersil 5 μm, 250×4.6 mm I.D.; Bischoff Analysentechnik und Geräte, Leonberg, Germany) with 0.1 *M* sodium dihydrogenphosphate buffer (pH 6)–methanol (50:50, v/v) at a flow-rate of 1.5 ml/min. The column was thermostatted at 20°C. The detection was by absorption at 372 nm.

### *2.5. Sample preparation*

#### *2.5.1. Extraction on Extrelut column*

To 0.5 ml of plasma 0.100 ml of internal standard solution containing 400 ng tenoxicam was added and filled to a final volume of 1.1 ml with 0.5 *M* phosphate buffer pH 4. The samples were mixed in 12-ml conical glass tubes, homogenised on a vortex mixer and centrifuged (900 *g*). An aliquot of 1 ml of this mixture was put onto Extrelut-1 columns (Merck) equipped with cannules. After 5 min the sample was eluted twice with a 5-ml volume dichloromethane. The solvent was evaporated at 35°C under a stream of nitrogen (99.999 vol%). The residue was dissolved in 0.120 ml of the mobile phase by vortexing. An aliquot of 0.050 or 0.100 ml was injected onto the HPLC column.

For determination of linearity lornoxicam and tenoxicam were added to 0.5 ml of human plasma to give final concentrations of 10, 15, 20, 100, 200, 1000 and 2000 ng lornoxicam/ml plasma ($n=6$) and 800 ng tenoxicam/ml as internal standardisation.

#### *2.5.2. Automatic off-line SPE on $C_{18}$ column*

To up to 0.5 ml of plasma or synovial fluid 0.01 ml of internal standard solution containing 200 ng tenoxicam was added and filled to a final volume of 1.1 ml with 0.5 *M* phosphate buffer pH 2. The samples were mixed in 12-ml conical glass tubes, homogenised on a vortex mixer and centrifuged at 2500 rpm. The samples were placed in the ASPEC device (Gilson Medical Electronics, Villiers-Le-Bel, France) and extracted on $C_{18}$ SPE columns (100 mg solid-phase material; Phenomenex, Torrance, CA, USA) in the batch mode as follows:

The columns were conditioned with 1 ml of methanol, followed by 1 ml of water and 0.5 ml of 0.5 *M* phosphate buffer pH 2. Then an aliquot of 1 ml of the clean supernatant of the diluted sample was transferred to the column, washed with 1 ml of water, dried with 2 ml of air and eluted with 1.25 ml of acetonitrile containing 10% (v/v) 25% ammonia. After evaporation of the solvent under vacuum at 35°C the residue was dissolved in 0.100 ml of 0.1 *M* phosphate buffer (pH 8)–methanol (50:50, v/v) and 0.090 ml were injected for chromatography.

To 0.25 ml of human plasma lornoxicam and 5′-hydroxy lornoxicam were added to give final concentrations of 40, 100, 200, 400 and 2000 ng/ml plasma and 20, 40, 100, 200 and 400 ng/ml, respectively ($n=6$) for determination of the linearity of the method. Tenoxicam as internal standard was added to give 800 ng/ml.

## 3. Results

### *3.1. Selectivity*

Plasma of various species – mouse, rat, rabbit, dog, monkey and man – was used. Under the conditions applied no interference with endogenous peaks was observed (Figs. 2 and 3). The main metabolite 5′-hydroxylornoxicam was clearly separated from lornoxicam (Fig. 3). Retention times were for lornoxicam ca. 5.8–6.1 min (Extrelut method) or ca. 6.7 min (SPE), for tenoxicam ca. 2.6–2.8 min and for 5′-hydroxylornoxicam ca. 5.1 min.

### *3.2. Linearity*

Calibration curves were prepared by analysing spiked plasma samples using internal standardisation with tenoxicam based on peak areas. Linear regression was calculated for the ratio of the peak areas ($\text{area}_{\text{lornoxicam}}/\text{area}_{\text{internal standard}}$) on the ratio of the spiked amounts. After extraction on Extrelut columns the calibration can be described by a slope of

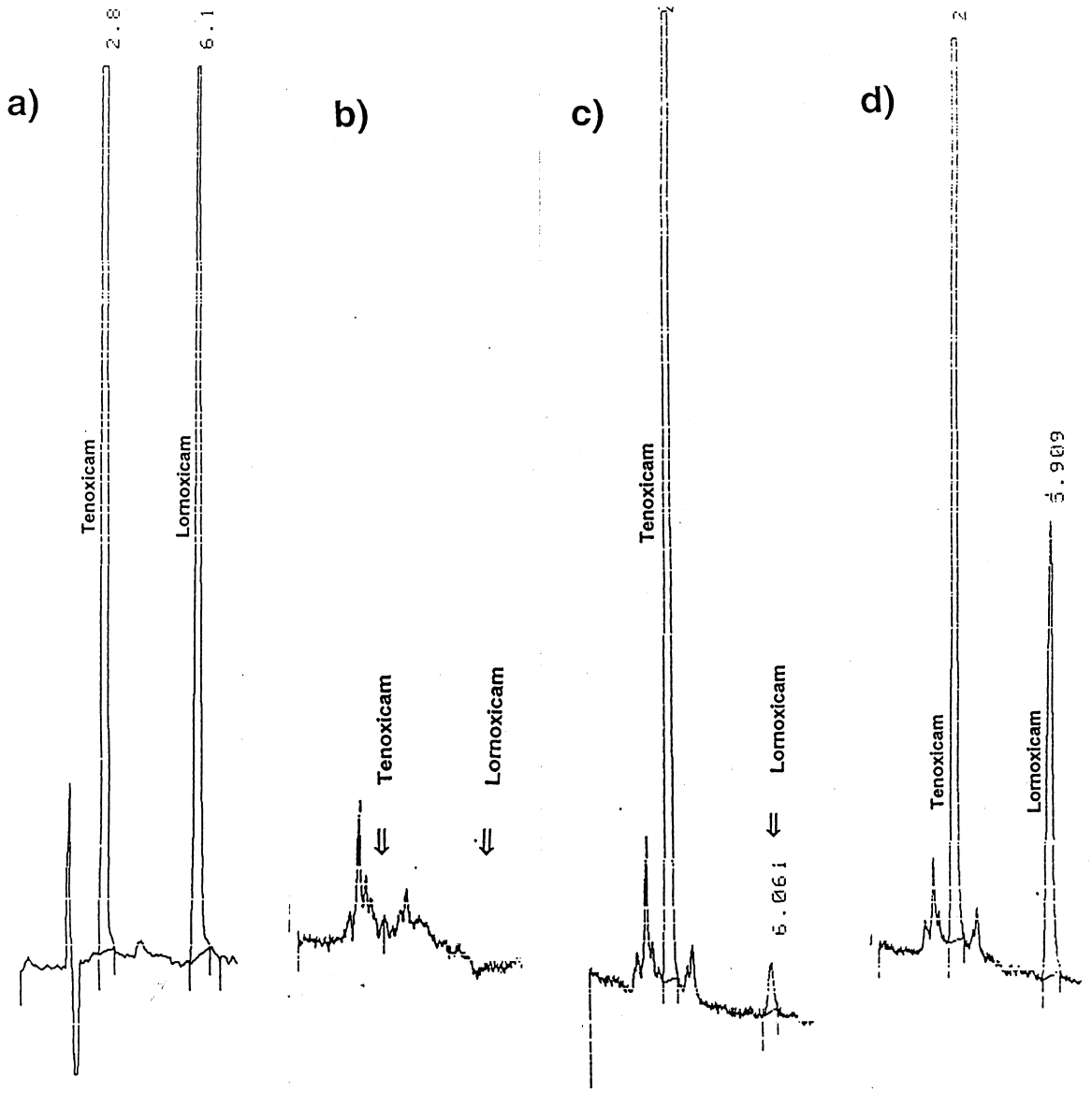


Fig. 2. Chromatograms of plasma samples after extraction on Extrelut-1 columns. *x*-axis: retention time (min); *y*-axis: absorbance. (a) Aqueous solution of tenoxicam and lornoxicam; (b) extract of 0.5 ml blank human plasma; (c) extract of 0.5 ml human plasma spiked with 400 ng tenoxicam and 5 ng lornoxicam; (d) extract of a plasma sample of a treated volunteer (containing 145 ng lornoxicam/ml).

1.159 and an intercept of −0.0034 with a correlation coefficient ($r^2$) of 1.000 ($n$=42). The calibration for lornoxicam after SPE can be described by a slope of 0.927 and an intercept of −0.0021 with $r^2$=0.999 ($n$=30). The respective data for the metabolite gave a slope of 0.870, an intercept of 0.0010 and an $r^2$ of 0.992 ($n$=30).

### *3.3. Precision*

Precision was determined on an intra-day as well as inter-day basis and is expressed as the coefficient of variation (C.V.) of the concentrations found in the spiked samples.

Intra-day C.V.s for lornoxicam were for the whole

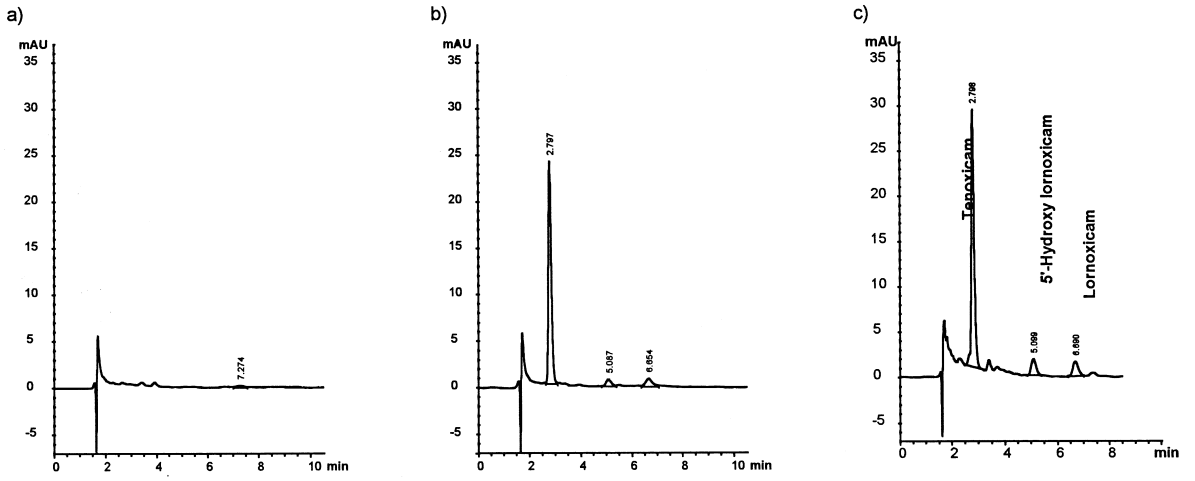


Fig. 3. Chromatograms of plasma samples after SPE. (a) Extract of 0.25 ml blank human plasma; (b) extract of 0.25 ml blank human plasma spiked with tenoxicam, 5′-hydroxylornoxicam and lornoxicam; (c) extract of plasma sample of a treated volunteer (containing 44 ng 5′-hydroxylornoxicam/ml and 57 ng lornoxicam/ml).

calibration range below 4.5% ($n=6$) with both methods, for 5′-hydroxylornoxicam ($n=6$) the respective values were between 4.2 and 11.0. Inter-day C.V.s were in the range of 3.2–7.4% for both compounds (Tables 1 and 2).

### 3.4. Accuracy

Accuracy was determined by comparison of found concentrations to the spiked ones.

On an intra-day basis an average 95 to 107% of the spiked concentrations were found for both methods and both analytes, except for the method with SPE for the lowest concentrations with deviations of +21 and +22%. In the samples measured average inter-day accuracy was 93–101% (Tables 1 and 2).

### 3.5. Extraction yield

Blank plasma was spiked with 400 ng tenoxicam and 40, 400 and 1500 ng lornoxicam/ml ($n=4$; Extrelut method) or with 500 ng/ml of tenoxicam,

Table 1
Intra-day and inter-day precision and accuracy (human plasma, Extrelut method)

| Concentration spiked (ng/ml) | *n* | Concentration found | | C.V. (%) |
|---|---|---|---|---|
| | | Mean value (ng/ml) | % of spiked concentration | |
| *Intra-day* | | | | |
| 10 | 6 | 10.7 | 107 | 2.0 |
| 15 | 6 | 15.5 | 103 | 4.2 |
| 20 | 6 | 20.5 | 103 | 4.5 |
| 100 | 6 | 95.6 | 96 | 1.7 |
| 200 | 6 | 198 | 99 | 1.6 |
| 1000 | 6 | 975 | 98 | 0.6 |
| 2000 | 6 | 1930 | 96 | 1.7 |
| *Inter-day* | | | | |
| 75 | 33 | 74.4 | 99 | 3.2 |
| 450 | 38 | 447 | 99 | 3.5 |

Table 2
Intra-day and inter-day precision and accuracy (human plasma, SPE)

| Analyte | Concentration spiked (ng/ml) | *n* | Concentration found | | C.V. (%) |
|---|---|---|---|---|---|
| | | | Mean value (ng/ml) | % of spiked concentration | |
| *Intra-day* | | | | | |
| Lornoxicam | 40 | 6 | 48.8 | 122 | 4.0 |
| Lornoxicam | 100 | 6 | 101 | 101 | 2.2 |
| Lornoxicam | 200 | 6 | 206 | 103 | 3.5 |
| Lornoxicam | 400 | 6 | 381 | 95 | 2.8 |
| Lornoxicam | 2000 | 6 | 2000 | 100 | 3.0 |
| 5′-Hydroxylornoxciam | 20 | 6 | 24.2 | 121 | 8.5 |
| 5′-Hydroxylornoxciam | 40 | 6 | 39.3 | 98 | 4.2 |
| 5′-Hydroxylornoxciam | 100 | 6 | 105 | 105 | 7.2 |
| 5′-Hydroxylornoxciam | 200 | 6 | 189 | 95 | 11.0 |
| 5′-Hydroxylornoxciam | 400 | 6 | 404 | 101 | 3.4 |
| *Inter-day* | | | | | |
| Lornoxicam | 100 | 27 | 99.6 | 100 | 5.5 |
| Lornoxicam | 400 | 26 | 402 | 101 | 3.9 |
| Lornoxicam | 2000 | 19 | 1960 | 98 | 3.8 |
| 5′-Hydroxylornoxciam | 20 | 9 | 18.6 | 93 | 7.4 |
| 5′-Hydroxylornoxciam | 100 | 27 | 97.7 | 98 | 5.9 |
| 5′-Hydroxylornoxciam | 400 | 26 | 396 | 99 | 5.1 |

lornoxicam and 5′-hydroxylornoxicam ($n=12$; SPE). The extraction yield, calculated in comparison to directly injected standard solutions of the theoretical concentrations, was 70–75% of the theoretical for the Extrelut method and above 90% for the method with SPE.

### 3.6. Sensitivity

The limit of detection (LOD) was determined as 0.8 ng/injection ($S/N$~2).

Limit of determination was set to 10 ng lornoxicam/ml plasma using 0.5 ml plasma per assay with the sample preparation on Extrelut columns and to 40 ng lornoxicam and 20 ng 5′-hydroxylornoxicam/ml plasma using 0.25 ml plasma per assay with the alternative method.

### 3.7. Stability

Blank plasma was spiked with lornoxicam (20–1000 ng/ml) and 5′-hydroxylornoxicam (20 and 100 ng/ml), analysed immediately after preparation and after storage at −20°C for 2.5 or five months.

Both analytes were found to be stable under these storage conditions (Table 3).

### 3.8. Identity of detected substances

Samples of treated animals and human volunteers were extracted on $C_{18}$ columns and analyzed by HPLC. The effluent of the HPLC column was collected at the above mentioned retention times and investigated by mass spectroscopy. Lornoxicam and 5′-hydroxylornoxicam could be confirmed unequivocally [26].

### 3.9. Comparison of manual extraction on Extrelut-1 columns and automatic SPE on $C_{18}$ columns

The plasma samples from a volunteer study with lornoxicam were analysed after extraction with both of the described procedures. The results obtained with both methods are reasonably comparable (Fig. 4).

Table 3
Stability in human plasma kept at −20°C

| Analyte | Concentration spiked (ng/ml) | Concentration found (ng/ml) | |
|---|---|---|---|
| | | Initial analysis | Final analysis |
| Lornoxicam[a] | 20 | 19.2 | 20.3 |
| Lornoxicam[a] | 50 | 51.7 | 46.6 |
| Lornoxicam[a] | 100 | 96.0 | 92.6 |
| Lornoxicam[a] | 300 | 295 | 290 |
| Lornoxicam[b] | 100 | 93.2 | 103 |
| Lornoxicam[b] | 1000 | 1010 | 1040 |
| 5′-Hydroxylornoxciam[b] | 20 | 20.6 | 20.4 |
| 5′-Hydroxylornoxciam[b] | 100 | 92.0 | 94.9 |

[a] Extrelut method, storage for five months.
[b] SPE, storage for 2.5 months.

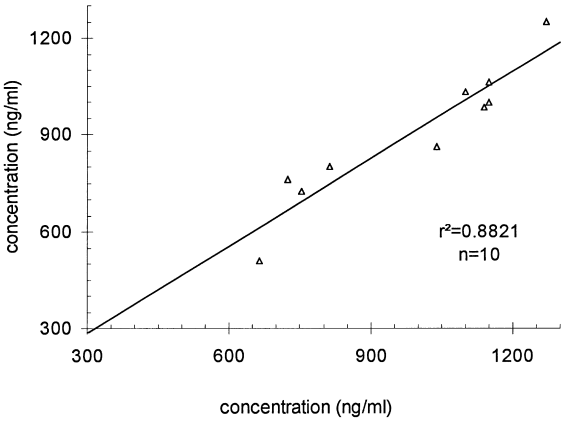


Fig. 4. Plasma concentration of lornoxicam determined after SPE on Bond Elut $C_{18}$ (*y*-axis) and after liquid–liquid extraction via Extrelut-1 columns (*x*-axis).

### *3.10. Application to synovial fluid samples*

A 0.5-ml volume of synovial fluid was extracted on $C_{18}$ cartridges as described for plasma samples. Accuracy and reproducibility were checked by spiked synovial fluid samples and were found to be comparable to those of plasma samples (Table 4).

## 4. Discussion

In the course of preclinical and clinical drug development analytical methods for determination of lornoxicam and its main, pharmacodynamically inactive metabolite 5′-hydroxylornoxicam in plasma were established and validated.

One method was developed using extraction on Extrelut-1 columns which is an improvement of the classical liquid–liquid extraction in terms of sample preparation time. It was widely used for analysis of plasma samples of animal and human pharmacokinetic studies. As an example the concentrations of lornoxicam in plasma after oral and intravenous administration to rats are given in Fig. 5.

In the course of the investigations 5′-hydroxylornoxicam was identified as the main metabolite in plasma [27] and as this substance could not be

Table 4
Intra-day precision and accuracy (human synovial fluid, $n=3$; SPE)

| Analyte | Concentration spiked (ng/ml) | Concentration found | | C.V. (%) |
|---|---|---|---|---|
| | | Mean value (ng/ml) | % of spiked concentration | |
| Lornoxicam | 100 | 107 | 107 | 4.6 |
| 5′-Hydroxylornoxicam | 100 | 100 | 100 | 4.1 |

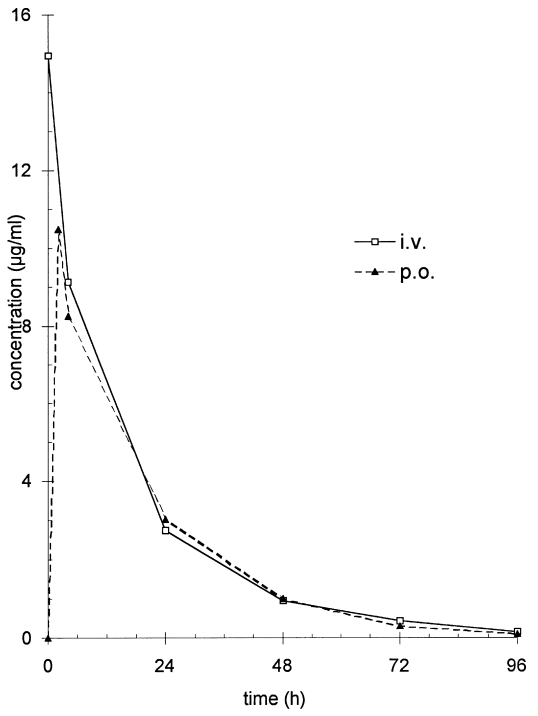


Fig. 5. Plasma concentration of lornoxicam after 2 mg lornoxicam/kg bodyweight given orally or intravenously to one rat each (sample preparation with Extrelut-1 columns).

determined by the above mentioned method, an analytical method for lornoxicam using SPE [25] was modified and adapted for automated sample preparation. As the main metabolite is not pharmacodynamically active, it was not necessary to determine this compound routinely, but only in studies directed to metabolism. For example, it was investigated whether different pharmacokinetic properties of lornoxicam were observed in poor metabolisers of sparteine or mephenytoin compared to extensive metabolisers [28].

Both methods were applied extensively during the development of lornoxicam and validated according to current practice for bioanalytical methods [29,30].

In a clinical study the distribution of lornoxicam to synovial fluid of patients suffering from rheumatoid arthritis or osteoarthritis was investigated and the method with SPE was applied to synovial fluid [31]. The validation however had to be restricted to a minimum because of inaccessibility of larger volumes of the relevant blank biological fluid.

Concentration ranges and determination limits were dependent on the available sample volume and the ranges required for the respective studies. For both methods the limit of determination could be lowered easily if required, e.g., by increasing the sample volume to 0.75 ml down to 2 ng/ml plasma could be determined after Extrelut extraction [32].

Selectivity of the methods obtained by sample preparation and detection by absorption at a relatively selective wavelength was checked by mass spectrometry.

During our investigations Suwa et al. [10] published a method for the determination of lornoxicam and 5′-hydroxylornoxicam in plasma utilizing liquid–liquid extraction and electrochemical detection. Their detection limits were 5 ng/ml for lornoxicam and 10 ng/ml for 5′-hydroxylornoxicam using 100 µl of sample. As in human pharmacokinetic studies the sample volume usually is not a critical factor, these sensitivities can be reached by our methods by using 500 µl of sample or more. In addition the possibility of automated sample preparation is highly desirable for routine analysis with respect to sample throughput and limitation of costly manual work.

Therefore our method represents an easy and routinely applicable method for the determination of lornoxicam and 5′-hydroxylornoxicam in plasma.

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