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Simultaneous determination of piroxicam, meloxicam and tenoxicam in human plasma by liquid chromatography with tandem mass spectrometry

Hye Young Ji, Hye Won Lee, Young Hoon Kim, Dong Won Jeong, Hye Suk Lee *

Drug Metabolism and Bioanalysis Laboratory, College of Pharmacy and Phytofermentation Research Center, Wonkwang University, Shinyongdong, Iksan 570-749, Republic of Korea

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Abstract

A rapid, sensitive and selective liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the determination of piroxicam, meloxicam and tenoxicam in human plasma was developed. Piroxicam, meloxicam, tenoxicam and isoxicam (internal standard) were extracted from human plasma with ethyl acetate at acidic pH and analyzed on a Sunfire column with the mobile phase of methanol:ammonium formate (15 mM, pH 3.0) (60:40, v/v). The analytes were detected using a mass spectrometer, equipped with electrospray ion source. The instrument was set in the multiple-reaction-monitoring (MRM) mode. The standard curve was linear ($r = 1.000$) over the concentration range of 0.50–200 ng/ml. The coefficient of variation (CV) and relative error (RE) for intra- and inter-assay statistics at three QC levels were 1.0–5.4% and −5.9 to 2.8%, respectively. The recoveries of piroxicam, meloxicam and tenoxicam ranged from 78.3 to 87.1%, with that of isoxicam being 59.7%. The lower limit of quantification for piroxicam, meloxicam and tenoxicam was 0.50 ng/ml using a 100 μl plasma sample. This method was successfully applied to a pharmacokinetic study of piroxicam after application of transdermal piroxicam patches to humans.

Keywords: LC–MS/MS; Piroxicam; Meloxicam; Tenoxicam; Human plasma

1. Introduction

The oxicam group of non-steroidal anti-inflammatory drugs (NSAIDs) has been used as a highly effective class of drugs against various arthritic conditions and post-operative inflammation. Piroxicam (4-hydroxy-2-methyl-*N*-2-pyridinyl-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide), meloxicam [4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide] and tenoxicam (4-hydroxy-2-methyl-*N*-2-pyridinyl-2H-thieno[2,3-e]-1,2-thiazine-3-carboxamide 1,1-dioxide) are the representative drugs belonging to the oxicam group.

Since the transdermal delivery avoids the gastrointestinal side effect and first-pass effect, many studies have been carried out in order to develop the percutaneous preparations of NSAIDs, including piroxicam and tenoxicam [1–6]. A number of high performance liquid chromatography (HPLC) methods with UV detection [7–16], amperometric detection [17] and tandem mass spectrometry (LC–MS/MS) [16,18–20] were reported for the determination of piroxicam, meloxicam or tenoxicam in biological fluids; however, most of those methods presented insufficient sensitivity (limit of detection; 0.72–50 ng/ml), the use of large biological fluid volumes (0.25–1 ml plasma or urine) or chromatographic interferences. There was no LC–MS/MS method reported for the simultaneous determination of meloxicam, piroxicam and tenoxicam in biological samples. A sensitive and rapid method for the determination of these oxicam drugs in biological fluids is necessary to evaluate pharmacokinetics in transdermal permeation studies.

The purpose of this study was to develop a rapid and sensitive LC–MS/MS method with simple sample preparation for the determination of piroxicam, meloxicam and tenoxicam in human plasma to support a pharmacokinetic study after transdermal application as well as oral administration of piroxicam, meloxicam and tenoxicam.

* Corresponding author. Tel.: +82 63 850 6817; fax: +82 63 851 2013.
E-mail address: hslee@wonkwang.ac.kr (H.S. Lee).

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2. Experimental

2.1. Materials and reagents

Piroxicam (purity; 98.4%), meloxicam (purity; 99.2%), tenoxicam (purity; 99.5%) and isoxicam (purity; 99.1%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethyl acetate and methanol (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of the highest quality available.

2.2. Preparation of calibration standards and quality control samples

Primary stock solutions of piroxicam, meloxicam, tenoxicam and isoxicam (1 mg/ml) were prepared in acetonitrile. Working standard solutions of piroxicam, meloxicam and tenoxicam were prepared by combining aliquots of each primary stock solution and diluting with acetonitrile. The working solution for isoxicam (internal standard, 0.1 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 piroxicam, meloxicam and tenoxicam (0.50, 1.00, 5.00, 10.0, 40.0, 100 and 200 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, 2.00, 20.0 and 80.0 ng/ml were prepared in bulk by adding 250 μl of the appropriate working standard solutions (0.01, 0.04, 0.40, 1.60 μg/ml) to drug-free human plasma (4750 μl). The bulk samples were aliquoted (100 μl) into polypropylene tubes and stored at −20 °C until analysis.

2.3. Sample preparation

Hundred microlitres 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 1 ml 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. The organic layer was transferred and evaporated to dryness under nitrogen at 35 °C. The residues were dissolved in 40 μl of methanol:water (1:1, v/v) 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.

2.4. 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 column (5 μm, 2.1 mm i.d. × 100 mm, Waters, CA, USA) using a mixture of methanol:ammonium formate (15 mM, pH 3.0) (60:40, v/v) at a flow rate of 0.2 ml/min. The column and autosampler tray temperatures were 45 and 4 °C, respectively. The analytical run time was 6.0 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 and 350 °C, respectively. The capillary voltage was 3.0 kV and the optimum cone voltages were 29, 30, 30 and 33 V for piroxicam, meloxicam, tenoxicam and isoxicam, respectively. The molecular ions of piroxicam, meloxicam, tenoxicam and isoxicam were fragmented at collision energies of 18, 17, 18 and 17 eV using argon as collision gas. Specific precursor/product ion transitions were employed. Multiple-reaction-monitoring (MRM) mode was used for the quantification by monitoring the transitions: m/z 332 → 95 for piroxicam, m/z 352 → 115 for meloxicam, m/z 338 → 121 for tenoxicam and m/z 336 → 99 for isoxicam (internal standard). Peak areas for all components were automatically integrated using MassLynx version 3.5 software. (Micromass UK Ltd.)

2.5. 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 2.00, 20.0 and 80.0 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 absolute recoveries of piroxicam, meloxicam and tenoxicam were determined by individually comparing the peak area of six extracted samples at the concentrations of 2.00, 20.0 and 80.0 ng/ml with the mean peak area of recovery standards. Three replicates of each of the recovery standards were prepared by adding piroxicam, meloxicam, tenoxicam and internal standard to blank human plasma extracts.

The relative matrix effects for piroxicam, meloxicam and tenoxicam were assessed by analyzing standards spiked at six concentrations into different plasma extracts originating from five different lots of blank plasma and comparing the peak areas of the analytes according to the approach of Matuszewski et al. [21]. The variability in the peak areas of the analytes, expressed as CVs (%), was considered as a measure of the relative matrix effect for these analytes.

2.6. Application

The developed LC–MS/MS method was used in a pharmacokinetic study after the application of transdermal piroxicam patches (48 mg) to clean and dry sites on the upper arms of four healthy male volunteers for 24 h. Following patch application, blood samples (2 ml) were withdrawn from the arm contralateral to the patch application site at 0, 2, 4, 6, 8, 10, 12, 24, 33 and 48 h, transferred to Vacutainer™ plasma glass tubes (sodium heparin, BD, NJ, USA) and centrifuged. Following centrifugation (3000 × *g*, 15 min, 4 °C), plasma samples were transferred to polypropylene tubes and stored at −20 °C prior to analysis.

The following pharmacokinetic parameters were determined for each subject: the maximum plasma concentration (C_{max}), minimum plasma concentration (C_{min}), the time taken to reach C_{max} (T_{max}) and area under the plasma concentration–time curve (AUC). C_{max}, C_{min} and T_{max} were determined by visual inspection, and AUC was calculated by the linear trapezoidal method from 0 to 48 h.

3. Results and discussion

3.1. LC–MS/MS

The positive electrospray ionization of piroxicam, meloxicam, tenoxicam and isoxicam produced abundant protonated molecular ions (MH^+) at *m/z* 332, 352, 338 and 336, respectively, without any evidence of fragmentation and adduct formation. Protonated MH^+ ions from piroxicam, meloxicam, tenoxicam 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]$^+$), 115 ([5-methylthiazol-2-ylamine+H]$^+$), 121 ([pyridin-2-ylisothiocyanate+H]$^+$) and 99 ([5-methyloxazol-2-ylamine+H]$^+$) were produced as the prominent product ions for piroxicam, meloxicam, tenoxicam and isoxicam, respectively. The quantification of the analytes was performed using MRM mode due to the high selectivity and sensitivity. Four pairs of MRM transitions were selected: *m/z* 332 → 95 for piroxicam, *m/z* 352 → 115 for meloxicam, *m/z* 338 → 121 for tenoxicam and *m/z* 336 → 99 for isoxicam.

The Sunfire column with a mobile phase consisting of methanol and ammonium formate (15 mM, pH 3.0) (60:40, v/v) resulted in short chromatographic run time (6.0 min) with sat-

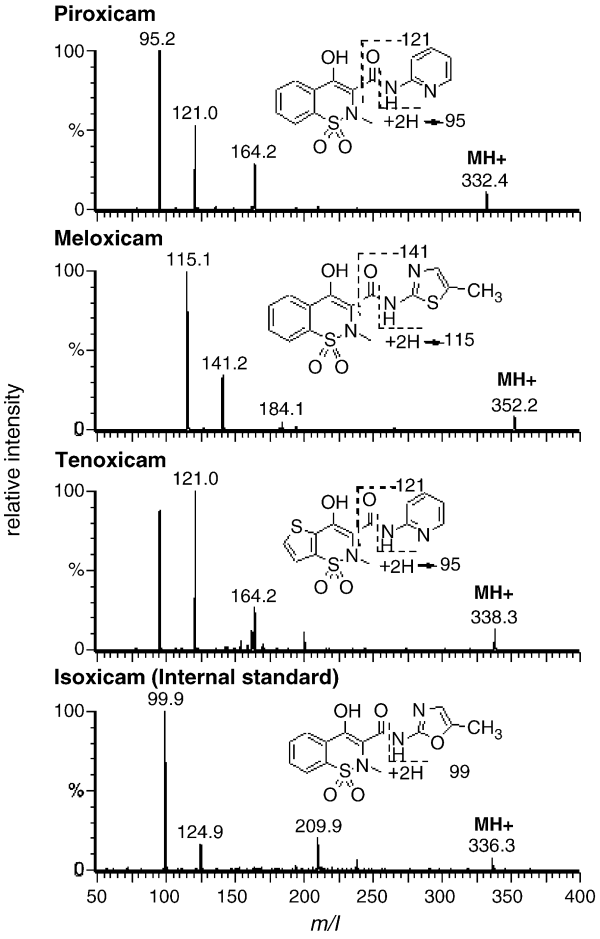

Fig. 1. Product ion mass spectra of piroxicam, meloxicam, tenoxicam and isoxicam.

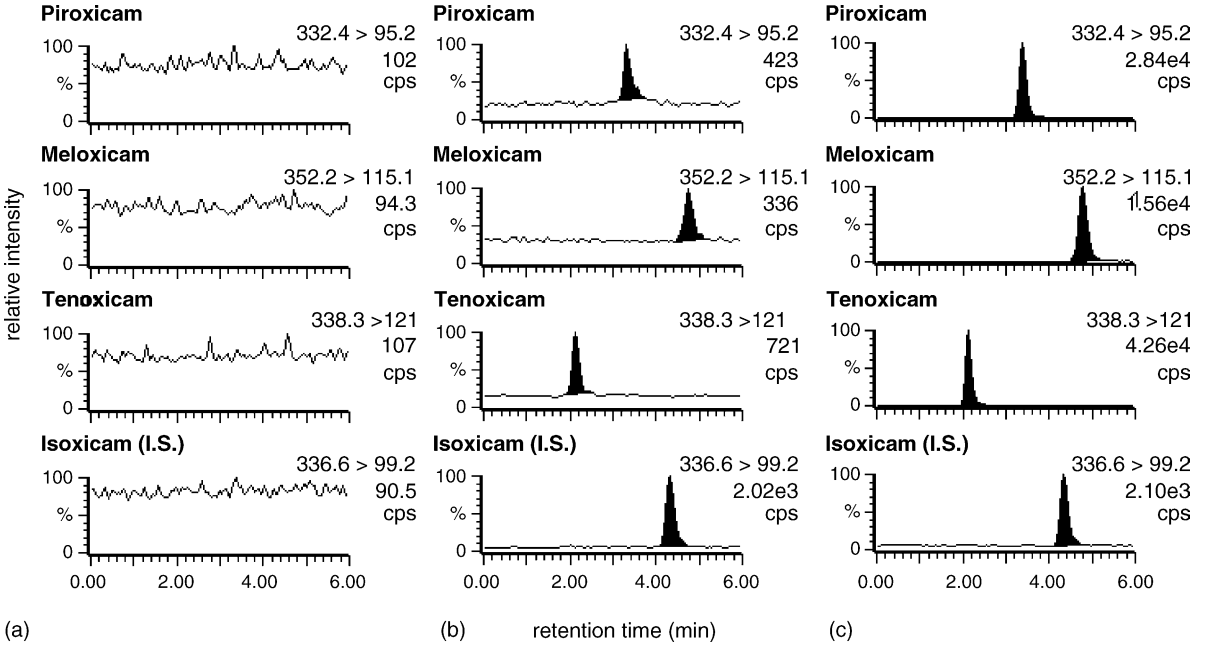

Fig. 2. MRM chromatograms of (a) a human blank plasma and human plasma samples spiked with (b) 0.50 ng/ml and (c) 40 ng/ml of piroxicam, meloxicam and tenoxicam.

Table 1
Calculated concentrations of piroxicam, meloxicam and tenoxicam in calibration standards prepared in human plasma

Analytes	Statistical variable	Theoretical concentration (ng/ml)							Slope	r
		0.50	1.00	5.00	10.0	40.0	100	200		
Piroxicam	Mean (ng/ml)	0.48	1.03	5.04	9.92	40.6	99.1	200	0.3307	1.000
	CV (%)	11.6	5.5	3.1	3.3	1.4	1.4	0.3	0.4	
	RE (%)	−3.1	3.0	0.9	−0.8	1.5	−0.9	0.2		
Meloxicam	Mean (ng/ml)	0.51	0.97	4.96	9.66	39.9	101	200	0.2307	1.000
	CV (%)	4.2	4.3	1.7	3.2	0.7	0.9	0.5	0.3	
	RE (%)	1.2	−2.7	−0.7	−3.4	−0.2	0.9	−0.2		
Tenoxicam	Mean (ng/ml)	0.46	1.00	5.07	10.0	40.0	99.8	200	0.4009	1.000
	CV (%)	3.2	4.5	1.8	2.4	0.3	0.6	0.9	0.4	
	RE (%)	−8.1	0.4	1.5	0.5	0.1	−0.2	0.1		

Values are mean ± S.D. (n = 9).

isfactory separation of piroxicam, meloxicam, tenoxicam and isoxicam without using gradient elution.

Fig. 2 shows the representative LC–MS/MS MRM chromatograms obtained from the analysis of blank human plasma and human plasma samples spiked with piroxicam, meloxicam and tenoxicam at 0.50 and 40 ng/ml. The analysis of blank plasma samples from sixteen different sources did not show any interference at the retention times of piroxicam (3.3 min), meloxicam (4.7 min), tenoxicam (2.1 min) and isoxicam (4.3 min), confirming the specificity of the present method. Sample carryover effect was not observed.

Table 3
Absolute recoveries of piroxicam, meloxicam, tenoxicam and isoxicam (internal standard) from spiked human plasma

Concentration (ng/ml)	Recovery (%, mean ± S.D., n = 6)			
	Piroxicam	Meloxicam	Tenoxicam	Isoxicam
2.00	78.3 ± 5.8	81.8 ± 7.0	80.0 ± 3.8	–
20.0	84.7 ± 3.2	85.0 ± 4.5	87.1 ± 3.6	–
80.0	85.6 ± 5.4	85.5 ± 5.4	86.1 ± 4.3	–
10.0	–	–	–	59.7 ± 4.1

–: not assayed.

3.2. Method validation

Calibration curves were obtained over the concentration range of 0.50–200 ng/ml for piroxicam, meloxicam and tenoxicam 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 1). The low CV value for the slope indicated the repeatability of the method (Table 1).

Table 2 shows a summary of intra- and inter-batch precision and accuracy data for QC samples containing piroxicam, meloxicam and tenoxicam. Both intra- and inter-assay CV values ranged from 1.0 to 5.0% at three QC levels. The intra- and inter-assay RE values for piroxicam, meloxicam and tenoxicam were −5.9 to 2.8% at three 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 piroxicam, meloxicam and tenoxicam using 100 μl of human plasma. Representative chromatograms at the LLOQ are shown in Fig. 2b and the signal-to-noise ratios for piroxicam, meloxicam and tenoxicam are higher than 5. At LLOQ level, CV values were ≤5.4% and RE values were −2.9 to 2.4% for piroxicam, meloxicam and tenoxicam (Table 2). This LLOQ value was smaller than that reported by de Jager et al. [17] in 500 μl of human plasma (0.72 ng/ml for piroxicam), by Wiesner et al. [18] in 100 μl of human plasma (8.96 ng/ml for meloxicam), and by McKinney et al. [20] in 1 ml of equine urine (10 ng/ml for pirox-

Table 2
Precision and accuracy of piroxicam, meloxicam and tenoxicam in human plasma quality control samples

Analytes	Statistical variable	Intra-batch (ng/ml, n = 6)				Inter-batch (ng/ml, n = 18)		
		0.50	2.00	20.0	80.0	2.00	20.0	80.0
Piroxicam	Mean	0.49	1.89	19.3	75.6	2.02	19.9	79.8
	CV (%)	3.9	3.2	4.7	2.3	5.0	3.5	4.4
	RE (%)	−2.9	−5.5	−3.6	−5.5	0.8	−0.5	−0.3
Meloxicam	Mean	0.51	2.00	18.8	75.4	2.00	19.2	77.6
	CV (%)	1.1	2.1	2.0	1.0	1.5	2.4	4.1
	RE (%)	2.4	0.1	−5.9	−5.8	0.1	−3.9	−3.0
Tenoxicam	Mean	0.49	2.06	20.2	77.1	2.01	20.1	76.5
	CV (%)	5.4	1.7	2.7	3.6	2.9	3.2	3.4
	RE (%)	−1.8	2.8	1.0	−3.6	0.4	0.5	−4.4

Table 4
Precision (CV, %) of determination of peak areas of piroxicam, meloxicam, tenoxicam and isoxicam, and peak area ratios (analyte/internal standard) in analytes spiked after extraction into extracts from five different human blank plasma lots

Nominal concentration (ng/ml)	Precision (CV, %)						
	Peak area				Peak area ratio		
	Piroxicam	Meloxicam	Tenoxicam	Isoxicam	Piroxicam	Meloxicam	Tenoxicam
1.0	8.0	10.8	8.1	12.3	11.5	12.7	12.8
5.0	11.9	6.6	8.2	8.8	13.8	10.6	12.4
10	11.0	11.8	11.4	10.2	8.5	6.7	12.8
40	12.0	9.8	12.5	12.0	12.5	7.5	12.5
100	9.3	8.8	6.3	5.7	8.3	4.2	8.6
200	9.9	11.8	10.7	7.6	6.8	7.5	10.7

icam and tenoxicam). Therefore, the present method enables the pharmacokinetic studies of piroxicam, meloxicam or tenoxicam in after percutaneous application of piroxicam, meloxicam or tenoxicam.

Although methyl-*tert*-butyl ether and dichloromethane gave good recovery, the use of ethyl acetate resulted in the most successful compromise between extraction recovery and a clean extract. The extraction recoveries of piroxicam, meloxicam and tenoxicam from spiked human plasma were determined using the one-step liquid–liquid extraction with ethyl acetate at acidic pH at the concentrations of 2.00, 20.0 and 80.0 ng/ml in six replicates. The recoveries of piroxicam, meloxicam and tenoxicam ranged from 78.3 to 87.1%, with that of isoxicam being 59.7 ± 4.1% (Table 3).

The assessment of the presence of a relative matrix effect was made based on direct comparison of the peak areas of piroxicam, meloxicam, tenoxicam and isoxicam spiked post-extraction into extracts originating from five different sources of human plasma [21]. The CVs of the determination of peak areas of piroxicam, meloxicam, tenoxicam and isoxicam at six different concentrations varied from 8.0 to 12.0% for piroxicam, 6.6 to11.8% for meloxicam, 6.3 to 12.5% for tenoxicam and 5.7 to 12.0% for isoxicam (Table 4). The CVs of the ratio of analyte/internal standard for standards spiked post-extraction into extracts from five different lots of plasma were 6.8–13.8% for piroxicam, 4.2–12.7% for meloxicam and 8.6–12.8% for tenoxicam (Table 4).

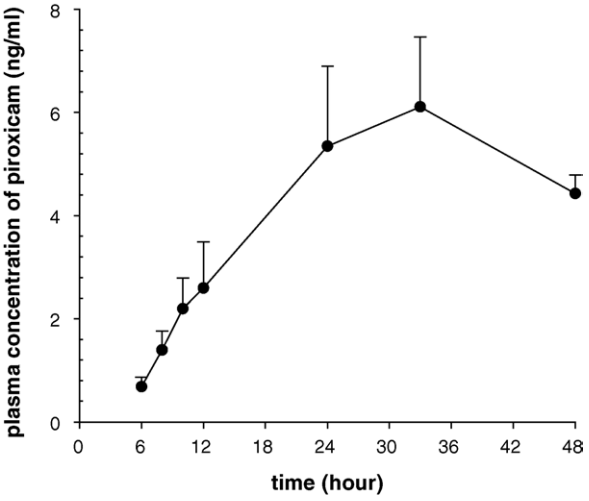

Fig. 3. Mean plasma concentration–time profile of piroxicam after application of transdermal piroxicam patches (48 mg) for 24 h to the upper arms of four male volunteers.

3.3. Clinical application

This method has been successfully applied to the pharmacokinetic study of piroxicam after transdermal application of piroxicam patches (48 mg) for 24 h to the upper arms of four healthy male volunteers. Fig. 3 shows the mean plasma concentration profile of piroxicam in four healthy male volunteers. C_{max}, C_{min}, T_{max} and AUC of piroxicam were 6.1 ± 1.4 ng/ml, 0.67 ± 0.18 ng/ml, 33 h and 191 ± 42.1 ng h/ml, respectively.

4. Conclusion

A rapid, sensitive and reliable LC–MS/MS method for the determination of piroxicam, meloxicam and tenoxicam 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 a pharmacokinetic study of piroxicam after application of transdermal piroxicam patches to humans. This method may also be suitable for pharmacokinetic studies required to develop percutaneous preparations of tenoxicam and meloxicam.

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References

[1] B.P. Wenkers, B.C. Lippold, Arzneimittelforschung 50 (2000) 275.
[2] S.C. Shin, C.W. Cho, I.J. Oh, Int. J. Pharm. 193 (2000) 213.
[3] E. Larrucea, A. Arellano, S. Santoyo, P. Ygartua, Eur. J. Pharm. Biopharm. 52 (2001) 113.
[4] E. Larrucea, A. Arellano, S. Santoyo, P. Ygartua, Drug Dev. Ind. Pharm. 28 (2002) 245.

[5] H.S. Gwak, I.K. Chun, Int. J. Pharm. 236 (2002) 57.
[6] H.A. Cheong, H.K. Choi, Eur. J. Pharm. Sci. 18 (2003) 149.
[7] J.L. Mason, G.J. Hobbs, J. Chromatogr. B 665 (1995) 410.
[8] A. Avgerinos, S. Axarlis, J. Dragatsis, Th. Karidas, S. Malamataris, J. Chromatogr. B 673 (1995) 142.
[9] M. Amanlou, A.R. Dehpour, J. Chromatogr. B 696 (1997) 317.
[10] M. Yritia, P. Parra, J.M. Fernadez, J.M. Barbanoj, J. Chromatogr. A 846 (1999) 199.
[11] T. Velpandian, J. Jaiswal, R.K. Bhardwaj, S.K. Gupta, J. Chromatogr. B 738 (2000) 431.
[12] A. Doliwa, S. Santoyo, M.A. Campanero, P. Ygartua, J. Pharm. Biomed. Anal. 26 (2001) 531.
[13] S. Dadashzadeh, A.M. Vali, N. Rezagholi, J. Pharm. Biomed. Anal. 28 (2002) 1201.
[14] B. Dasandi, Shivaprakash, H. Saroj, K.M. Bhat, J. Pharm. Biomed. Anal. 28 (2002) 999.
[15] W.R.G. Baeyens, G. Van der Weken, E. D'haeninck, A.M. Garcia-Campana, T. Vankeirsbilck, A. Vercauteren, P. Deprez, J. Pharm. Biomed. Anal. 32 (2003) 839.
[16] M. Sultan, G. Stecher, W.M. Stoggl, R. Bakry, P. Zaborski, C.W. Huck, N.M. El Kousy, G.K. Bonn, Curr. Med. Chem. 12 (2005) 573.
[17] A.D. de Jager, H. Ellis, H.K.L. Hundt, K.J. Swart, A.F. Hundt, J. Chromatogr. B 729 (1999) 183.
[18] J.L. Wiesner, A.D. de Jager, F.C.W. Sutherland, H.K.L. Hundt, K.J. Swart, A.F. Hundt, J. Els, J. Chromatogr. B 785 (2003) 115.
[19] N. Van Hoof, K. De Wasch, S. Poelmans, H. Noppe, H. de Brabander, Rapid Commun. Mass Spectrom. 18 (2004) 2823.
[20] A.R. McKinney, C.J. Suann, A.M. Stenhouse, Rapid Commun. Mass Spectrom. 18 (2004) 2338.
[21] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.