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High-performance liquid chromatographic determination with amperometric detection of piroxicam in human plasma and tissues

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Abstract

After repeated topical application of a piroxicam gel preparation to the knee, piroxicam was quantified in plasma, subcutaneous tissue, synovial capsule and synovial fluid, using specimens obtained during knee surgery. Electrochemical detection was used and the limit of quantification (LOQ) was 0.72 ng/ml in plasma at a signal-to-noise ratio of 10:1. The chromatographic method was optimised to determine piroxicam in all four matrices, and the analyte was quantified using a calibration line constructed from plasma calibration standards. Levels in subcutaneous tissue, synovial capsule and synovial fluid were compared to plasma steady-state levels and expressed as a ratio, in order to ascertain bioavailability. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Piroxicam

1. Introduction

Piroxicam [4-hydroxy-2-methyl-*N*-(2-pyridyl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide] is a non-steroidal anti-inflammatory drug (NSAID) that has been used to treat various arthropathies and inflammatory diseases in humans [1].

A number of high-performance liquid chromatography (HPLC) methods have been employed to determine piroxicam in biological samples [1–8], and while electrochemical detection (ED) of piroxicam is by no means novel [2], UV detection is most commonly employed [1,3–8], as it is sufficiently sensitive to detect levels associated with common oral dosage regimens. As is the case with some commonly used NSAIDs, the detection limit of piroxicam improves by between five- and 20-fold when ED is used instead of UV detection [2]. Low systemic concentrations, associated with topical dosage regimens, necessitated the development of a more sensitive assay method.

The present paper describes a multiple liquid–liquid extraction using dichloromethane–hexane (1:4). While the extraction efficiency from the four matrices concerned (ca. 60% from all the matrices) was inferior to that of most existing methods, the benefits of a clean extract far outweighed the disadvantages of inferior recovery and a laborious extraction procedure. The purity of the extracts allowed for the determination of piroxicam in all four matrices, without any adjustments to the mobile phase or HPLC column.

Considering the small amounts of biological sam-

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ple ethically attainable during surgery, and the lower piroxicam levels associated with topical applications, electrochemical detection was considered the method of choice, using an oxidation potential of +0.650 V. A much improved limit of quantitation (LOQ) of 0.72 ng/ml in plasma was attained at a signal-to-noise ratio of 10:1.

2. Experimental

2.1. Reagents and materials

Piroxicam was supplied by Francochim (Blagnac, France). HPLC grade tetrahydrofuran, methanol, hexane and dichloromethane (B&J brand) were obtained from Baxter (Muskegon, MI, USA). Potassium chloride (Merck, Darmstadt, Germany) and 85% orthophosphoric acid (Fluka, Buchs, Switzerland), were used without further purification. All water used was purified by RO 20SA reverse osmosis system and Milli-Q polishing system (Millipore, Bedford, MA, USA).

2.2. Apparatus

Mobile phase was delivered by a series 1100 isocratic pump (Hewlett-Packard, Palo Alto, CA, USA) at 0.5 ml/min. The mobile phase consisted of 30 m*M* orthophosphoric acid–methanol–tetrahydrofuran (600:320:80). Potassium chloride (150 mg/l) was added to the mobile phase and the apparent pH adjusted to 2.70 using 4 *M* NaOH. Electrochemical detection was by way of a Hewlett-Packard 1049A programmable electrochemical detector, in amperometric mode, set at an oxidation potential of +0.650 V. A Hewlett-Packard series 1050 autosampler injected 20 μl onto the HPLC column. While on the autosampler, samples were cooled to 4°C using a Lauda RM 6 circulatory cooling system (Lauda, Köningshofen, Germany). A Higgins Haisil 120 BD C_{18}, 120 mm×3.0 mm I.D., 5 μm column was used (Higgins Analytical, CA, USA), and was maintained at 30°C using a CTO 6A column oven (Shimadzu, Kyoto, Japan). Where necessary, sample ultrasonication was done using a Sonorex RK 100 ultrasonicator (Bandelin, Berlin, Germany). Homogenisation of sample was done using a T25 Ultra-Turrax (Janke and Kunkel, IKA Labortechnik, Staufen, Germany), fitted with an IKA UT disperser (O.D. 8 mm). The centrifuging of ampoules was done in a Megafuge 1.0R (Heraeus, Hanau, Germany). High speed centrifuging of microfuge tubes was performed by a centrifuge 5416 (Eppendorf, Hamburg, Germany). Sample freezing was done on a Fryka Polar KP 250 cooling plate (Kältetechnik, Esslingen, Germany). A Speed Vac concentrator (Savant, Holbrook, NY, USA) was used for sample concentration.

2.3. Preparation of calibration standards

Spiking solutions of piroxicam were prepared in methanol, and calibration standards were prepared by spiking blank plasma, and then making a series of plasma dilutions that yielded 13 calibration standards, spanning a concentration range of 0.72–600 ng/ml.

A set of seven quality controls, that spanned the same range, were independently made using the same methodology, and used to verify the intra-day and inter-day assay method performance. The calibration standards and quality controls were stored at −20°C until assayed.

As the extraction recovery of piroxicam from all four matrices was similar (ca. 60%), calibration standards and quality controls were prepared in plasma only, and all samples were quantified from the plasma calibration line, applying appropriate multiplication factors where necessary.

Sufficient calibration standards and quality controls were prepared to develop and validate the assay method, and to assay all study samples. A new calibration line, along with quality controls, was included in each assay batch.

2.4. Sample preparation

2.4.1. Plasma extraction

Step 1: To 0.5 ml plasma, in a 5-ml amber ampoule, was added 0.2 ml 0.1 *M* HCl. Step 2: 4 ml dichloromethane–hexane (1:4) was added to the sample, vortexed for 1 min, and centrifuged for 1 min at 650 *g*, 4°C. Step 3: The aqueous layer was frozen and the organic layer decanted into a second 5-ml amber ampoule containing 0.2 ml 0.1 *M* NaOH.

Step 4: The sample was vortexed for 1 min and centrifuged for 1 min (650 *g*, 4°C). Step 5: The aqueous layer was frozen, and the organic supernatant discarded. Step 6: To the remaining aqueous layer was added 0.5 ml 0.1 *M* HCl and the sample placed in a water bath (37°C) for 1 min. Step 7: 3 ml dichloromethane–hexane (1:4) was added and the sample vortexed for 1 min and centrifuged (650 *g*, 4°C) for 1 min. Step 8: The final aqueous layer was frozen, the organic layer decanted into a third 5-ml ampoule, and the sample evaporated to dryness. Step 9: The residue was reconstituted in 0.2 ml mobile phase, vortexed for 10 s, and 0.02 ml injected onto the HPLC column.

2.4.2. Subcutaneous tissue (SCT) and synovial capsule (SC) extraction

Step A: To approximately[1] 0.2 g tissue sample (accurately weighed) in a polypropylene tube (55× 12 mm) was added 0.1 *M* NaOH (volume equivalent to five-times the mass of the tissue sample), and the sample ultrasonicated for 1 h. Parafilm ''M'' (Chicago, IL, USA) was used to seal the polypropylene tube while in the ultrasonicator, to circumvent sample loss by spluttering and evaporation. Step B: The sample was homogenised at 22 000 rpm for 1 min. Step C: The sample was transferred into an Eppendorf microfuge tube and centrifuged at 8500 *g* for 5 min. Step C: 0.5 ml of the aqueous layer[2] was transferred into a 5-ml amber ampoule. The sample was acidified by adding 0.2 ml 1.0 *M* HCl, and the remainder of the extraction performed as per steps 2–9, described in Section 2.4.1.

2.4.3. Synovial fluid (SF) extraction

Step I: Prior to initial pipetting, the sealed sample was ultrasonicated for 20 min, rendering it more manageable when using a conventional air–interface pipette. Step II: 0.2 ml sample was pipetted into a polypropylene tube, and 1 ml 0.1 *M* NaOH added. Step III: The tube was briefly vortexed, sealed with Parafilm ''M'', and ultrasonicated for 1 h. Step IV: The sample was again briefly vortexed, 0.5 ml transfered into a 5-ml amber ampoule, and acidified by adding 0.2 ml 1.0 *M* HCl. The remainder of the extraction was performed as per steps 2–9, described in Section 2.4.1.

3. Results and discussion

3.1. Extraction

Given the nature of the matrices, a decision was made from the outset to optimise a liquid–liquid extraction procedure for plasma and tissue homogenates. Although piroxicam contains a variety functional groups that predispose the molecule to amphoteric tendencies, the homogenisation of tissue samples was done in a basic, aqueous medium.

A number of extraction solvents were investigated, and although *tert*.-butyl methyl ether and dichloromethane gave good recovery, samples were not sufficiently clean. Various combinations of *tert*.-butyl methyl ether and hexane were investigated, but a combination of hexane–dichloromethane (4:1) proved to be the most successful compromise between recovery and a clean extract.

3.2. Method validation

Based on piroxicam peak area, the calibration line was found to be linear over the plasma concentration range (0.72–600 ng/ml). Due to the fact that little data exist regarding the expected tissue concentrations of piroxicam associated with such a regimen, a calibration range was chosen which was wider than literature would suggest, regarding the expected plasma concentrations. The calibration line produced from nine calibration standards was characterised by the equation $y=(0.727 \text{ to } 1.439)x+(-0.197 \text{ to } 0.574)$, with a mean $r^2=0.992$ and a relative standard deviation (RSD) of 24.7% for $n=8$, over a period of three months, when using a $1/\text{concentration}^2$ weighting.

[1]When adding 0.1 *M* NaOH to the sample, the volume was adjusted so that the ratio of sample (g) to volume 0.1 *M* NaOH added (ml) remained constant at 1:5.

[2]The aqueous layer was found between a supernatant layer of congealed matter, that was cream in colour, and an underlying layer of protein. This congealed, supernatant layer was carefully removed with a wooden applicator, exposing the aqueous layer.

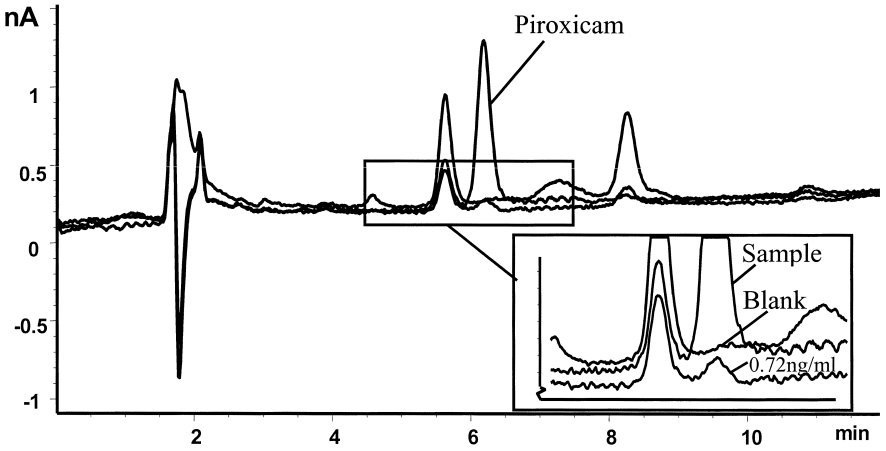

Fig. 1. Overlaid chromatograms of plasma extracts, showing good resolution between endogenous plasma components and piroxicam. A calibration standard of 0.72 ng/ml produces a peak that is well defined from a blank plasma extract. The sample reflects steady-state plasma levels, following repeated topical application of piroxicam.

3.3. Chromatography

Good chromatographic results were obtained from the extracts of all four matrices (Figs. 1 and 2). In none of the four matrices assayed was any endogenous peak found to co-elute with piroxicam. Although it was not possible to obtain blank tissue samples from each volunteer, blank SC, SCT and SF were obtained and assayed prior to commencing with batches. No interference was observed in these three matrices. A blank plasma sample from each volunteer, obtained prior to dosing, was assayed with the post-dosage samples and no interference was observed.

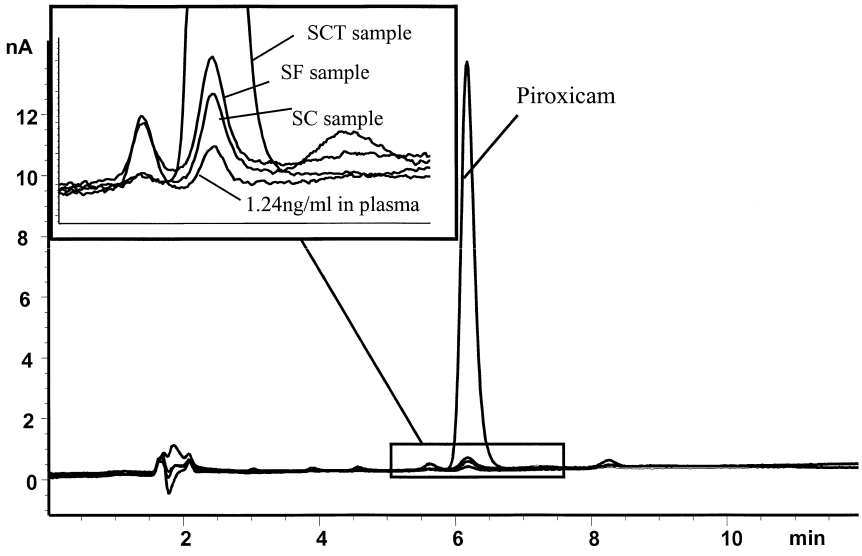

Fig. 2. Overlaid chromatograms of mixed plasma and tissue extracts. Peak areas obtained from SCT, SC and SF, which are all larger than a 1.24 ng/ml calibration standard peak, are yet to be multiplied by approximately the same multiplication factor, in order to obtain absolute concentrations. The large peak area obtained from the SCT sample, and roughly equivalent areas for SC and SF, suggest significant accumulation of piroxicam beneath the skin, and a decrease in tissue concentrations with an increase in tissue depth.

3.4. Recovery

Plasma was spiked with a known amount of piroxicam and then extracted. Recovery experiments with SCT and SC were done by spiking the basic tissue homogenate, obtained in step B of the subcutaneous tissue and synovial capsule extraction section with known amounts of piroxicam, and extracting as described. Recovery from SF was determined by spiking the basic homogenate obtained in step II of the synovial fluid extraction section, and extracting as described. Response factors of extracts were compared to those of appropriate response standard solutions and expressed as a percentage, as set out in Table 1.

Table 1
Extraction recoveries of piroxicam for plasma, SCT, SC and SF

Matrix	Concentration (ng/ml)	Mean recovery, $n=3$ (%)
Plasma	454, 3.47, 0.86	64.3
SCT homogenate	454	64.9
SC homogenate	454	57.8
SF homogenate	127	67.8

3.5. Sensitivity

Compared to previously published methods [1–8], sensitivity was greatly improved. This was due to the fact that ED was employed and optimised (Fig. 3). The LOQ for the method was 0.72 ng/ml in plasma and homogenate, with a signal-to-noise ratio of 10:1. Due to the dilutions necessitated for the assay of the tissue homogenates, this translates into 4.32 ng/g tissue, if 0.2 g tissue is available, and 4.32 ng/ml SF if 0.2 ml SF is obtained.

3.6. Inter- and intra-day accuracy and precision

Within a single batch, the method was found to be both accurate and precise (Table 2). In addition, comparison of calibration standard and quality control data from the various batches (Tables 3 and 4), which were completed over three months, shows a high degree of day-to-day reproducibility.

3.7. Matrix stability

Prior to method development, plasma stability standards were prepared at high, medium and low concentration, and stored at −20°C. After an interval of 16 weeks, fresh standards were prepared in plasma, and assayed together with the pre-prepared stability standards. Response factors of the freshly made standards were compared to those of the 16-week-old standards and no significant difference was observed. It was concluded that when stored at −20°C, piroxicam is stable in human plasma for at least 16 weeks.

3.8. Specificity

Chromatograms from samples obtained from 30 subjects assayed were scrutinised, and no interference was detected. In addition, plasma from six different sources was extracted and no peak was found to elute near the retention time of piroxicam. Similarly, blank SC, SCT and SF from a single source were extracted and no interference was observed.

3.9. On-instrument stability

Piroxicam was found to be stable in the reconstituted extract for the period during which samples were on the instrument (approximately 16 h).

3.10. Application

This assay method, which could be employed in comparative bioavailability studies, was used to determine piroxicam levels in plasma, SCT, SC and SF, following repeated topical application of piroxicam gel to the knee joint (40 mg, applied twice daily, in the seven days preceding knee surgery). Bioavailability was ascertained from the three ratios produced between steady-state plasma concentration, and concentration in the remaining three matrices. Results regarding these ratios are presented in Table 5.

Following the dosage regimen used in this study, a determinable amount of piroxicam is found at the suspected sites of action and it is evident that there is significant accumulation of piroxicam in subcutaneous tissue, with an even distribution of piroxicam between the synovial capsule and synovial fluid.

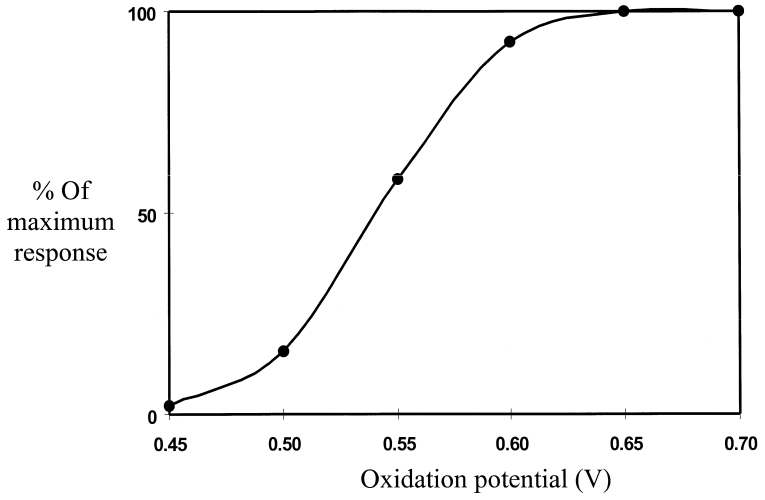

Fig. 3. Piroxicam voltammogram.

Table 2
Intra-day accuracy and precision (n=5) of plasma quality controls

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	RSD (%)
538	543	4.4
300	302	7.4
150	145	3.8
3.47	3.44	10.7
2.61	2.56	6.4
1.74	1.88	4.1
0.86	0.88	7.3

We could find no directly comparable data reported in the literature. Marks and Dykes [9] reported on cutaneous concentrations following topical application of piroxicam gel. They found the highest levels of piroxicam in superficial skin surface biopsies (80–320 μg/g of tissue), with the lowest tissue levels recorded in skin surface biopsies nearest the viable epidermis. Low and often undetectable plasma levels were observed in this study.

Hundal et al. [10] reported on synovial fluid concentrations after oral administration of piroxicam. The levels were in the order of 0.3–4.6 μg/ml of

Table 3
Inter-day accuracy and precision of plasma calibration standards

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	RSD (%)	n
600	584	3.2	8
299	303	3.5	8
199	197	6.9	8
151	148	6.4	8
49.5	49.5	4.4	8
19.8	20.8	6.2	8
4.94	4.82	11.8	7
2.47	2.56	10.1	9
1.24	1.23	8.1	14

Table 4
Inter-day accuracy and precision of plasma quality controls

Nominal concentration (ng/ml)	Mean found concentration (ng/ml)	RSD (%)	*n*
300	291	7.8	16
150	137	13.5	15
3.47	3.46	7.7	15
2.61	2.44	10.8	8
1.74	1.71	9.3	8

fluid, while steady-state total plasma concentrations ranged between 0.5–8.3 μg/ml. The ratio between total synovial fluid and total plasma concentration ranged between 0.39–0.9 with an average synovial fluid/plasma quotient of 0.51 (synovial fluid/plasma 1:1.96). The ratio determined by Hundal et al. differs appreciably from the synovial/plasma ratio of 3.04:1 found in this study (Table 5). This is understandable as Hundal et al. quantified piroxicam in synovial fluid following oral ingestion of piroxicam, while the present study involved the topical application.

4. Conclusion

This assay method is sufficiently sensitive to quantify piroxicam in plasma, SC, SCT and SF, following the repeated topical application of a piroxicam gel formulation. In addition to being sensitive, it is accurate and precise between 0.72 and 600 ng/ml.

Table 5
Ratios of SC, SF and SCT, relative to steady-state plasma concentrations

Ratio expressed	Mean ratio	RSD (%)	*n*
SC/plasma	3.02	136	22
SF/plasma	3.04	77.4	21
SCT/plasma	20.9	150	28

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