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Piroxicam quantitation in human plasma by high-performance liquid chromatography with on- and off-line solid-phase extraction

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Abstract

A comparative study of two analytical methodologies for piroxicam quantitation in plasma by off-line and on-line solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) is described. The SPE cartridges contained C_8 for both extraction methods. The analytes piroxicam and tenoxican (internal standard) were separated on a C_{18} column with a mobile phase consisting of acetonitrile:20 m*M* phosphate buffer pH 3.1 (50:50, v/v) followed by UV detection at 360 nm. The validation of the methods demonstrated good recoveries (over 90%), sensitivity (limits of quantification of 0.05 μg/ml with on-line SPE and 0.1 μg/ml with off-line SPE, based on a 100 μl and 200 μl sample volume, respectively), accuracy and precision (better than 9.5%). Both methodologies have been used for bioequivalence studies.

Keywords: Solid-phase extraction; Piroxicam

1. Introduction

Piroxicam (4-hydroxy-2-methyl-*N*-(2-pyridyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-di-oxide) is a non-steroidal anti-inflammatory and analgesic agent. Its efficacy has been demonstrated in humans for the treatment of various inflammatory diseases and osteoarthritis [1,2].

Several methods have been described for determination and quantification of piroxicam in plasma and most of them involve reversed-phase HPLC with UV detection [3–22]. The majority of these methods required liquid–liquid extraction with evaporation of the extract [3–16], some employ protein precipitation [17–20], another one is based on solid-phase extraction (SPE) [21], and finally there is another method that works on-line SPE. This procedure has been developed with a laboratory-made, column-switching station [22] and implies the drugs are concentrated on an extraction precolumn; after washing the precolumn is automatically back-flushed with the mobile phase to transfer the analytes to the analytical column The problem connected with repeated use of the precolumn for sample concentration and clean-up is that it may deteriorate due to precipitation of protein and the risk of blocking [23]. So, the precolumn must be changed often.

The present paper describes a fully automated method for determination of piroxicam and tenoxicam (internal standard) in human plasma using an on-line solid-phase extraction with exchangeable cartridges for sample concentration and clean-up,

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and this is compared with the same chromatographic method using off-line SPE.

Two methods are suitable to quantify drug levels in pharmacokinetic studies, but the on-line method saves a lot of time and needs less manipulation.

2. Experimental

2.1. Reagents and materials

Piroxicam (PRX) was purchased from Impex Quimica (Pamplona, Spain), Tenoxicam (TNX) (internal standard), was obtained from Solvay laboratory (Barcelona, Spain). Acetonitrile and methanol (HPLC grade), disodium hydrogenphosphate and orthophosphoric acid were purchased from Scharlau (Barcelona, Spain), dihydrogen sodium phosphate from Merck (Darmstadt, Germany), all analytical grade. Water was deionized and purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Chromatography

Analysis by HPLC was performed using an autosampler Triathlon (Spark-Holland, The Netherlands) and pumps 515, variable wavelength UV detector 486 (off-line) and 484 (on-line) operating at 360 nm, Millenium 2010 was the acquisition and processing software used, all of Waters (Milford, MA, USA).

The analytical column was a Kromasil C_{18}, 5 μm, 150×4 mm I.D. and the guard column was of the same phase (Teknokroma, Spain). The mobile phase was acetonitrile–phosphate buffer 20 m*M* pH 3.1 (50:50 v/v), delivered at a flow-rate of 1 ml/min.

2.3. Solutions

Two 1-mg/ml PRX stock solutions (A and B) were prepared in methanol: one for the calibration curve, spiked samples and chromatographic standards (A), another one for quality controls (B). A stock standard solution of TNX was prepared in acetonitrile.

Chromatographic standards, recovering study and spiked samples were made by dilutions of stock solution with mobile phase, until concentrations of 100 and 10 ng/μl PRX; 30 ng/μl of TNX were reached as the internal standard solution.

All solutions were stored at 4°C in a refrigerator where they were stable for at least two months.

2.4. Sample preparation

2.4.1. Off-line extraction

Solid-phase extraction (SPE) cartridges, Bond-Elut C_8 100 mg (Varian, Harbor City, CA, USA) were attached to a SPE vacuum station Vac-Elut SPE24 (Varian) and activated with 2 ml methanol and conditioned with 2 ml milli-Q water and 2 ml phosphate buffer (0.15 m*M*) at pH 3.5.

Then, 200 μl of plasma with 1 μg/ml of internal standard (blank, spiked samples and volunteer samples) were transferred to the cartridges. After the entire plasma had been aspired through the cartridges, 1 min of interaction was allowed.

The cartridges were washed with 2 ml of the same buffer and dried under full vacuum, then they were eluted with 400 μl of mobile phase and 50 μl were injected into HPLC system. The temperature of the injector carrousel was 6°C.

2.4.2. On-line extraction

The apparatus consisted of a solvent delivery unit (SDU) with a purge pump and six-port solvent selection valve, an autosampler and a programmable on-line solid-phase extraction unit (Prospekt), all three units purchased from Spark-Holland.

The exchangeable cartridges were of 10×2 mm I.D. and were filled with C_8 bonded phase (Varian) of 40-μm particles size.

The microvials containing 300 μl of plasma with internal standard were placed in the autosampler, programmed to apply 100 μl of plasma to every cartridge. The temperature of the carrousel was the same used for the off-line extraction. One millilitre of acetonitrile–water (5:95, v/v) was used for the needle wash.

Before application of plasma, the cartridge was activated with methanol, flow-rate of 1.5 ml/min for 2.50 min; and with water with the same conditions.

Then the flow-rate descended to 1 ml/min and the cartridge was flushed for 4 min with phosphate buffer (0.15 *M*, pH 3.5); at that time, the flow was directed through the loop of the autosampler and

plasma transported to the cartridge, the flow was stopped for 30 s for plasma interaction.

The cartridge was rinsed with phosphate buffer (0.075 *M*, pH 3.5) at 1 ml/min. The analytes were eluted with mobile phase for 3 min.

The Prospekt cannot be programmed to use the cartridge more than once in a cycle but in order to be able to reuse it (for six times) in a new series, it was purged with 2 ml of water and 3 ml of methanol. The cartridge was automatically changed for a new one, and a new cycle started.

3. Results and discussion

3.1. Chromatographic separation

Fig. 1A shows typical chromatograms of chromatographic standards dissolved in mobile phase, Fig. 1B blank plasma, free of interfering peaks and Fig. 1C, spiked samples with 0.5 μl/ml PRX and 1 μg/ml TNX (S.I.).

The retention times for chromatographic standards were 1.8±0.92 and 1.94±0.08 for TNX on- and off-line respectively and 3.6±0.04 and 3.8±0.19 for PRX on- and off-line respectively.

3.2. Recovery and linearity

The piroxicam and internal standard peak areas were measured after injection of PRX and TNX solution in mobile phase. These were compared with those obtained with blank plasma spiked with known amounts of two compounds.

Table 1 shows the recovery results in both methods, for TNX, 78.52%±9.06 and 91.34%±3.93 in off- and on-line, respectively, and 101.54%±8.21 and 91.64%±1.06 for 0.5 μg/ml of PRX, and 97.06%±5.75 and 92.94%±1.18 for 1.5 μg/ml of PRX, off- and on-line, respectively.

The linearity for PRX was checked with a calibration curve made with blank plasma spiked with 0, 0.05., 0.1, 0.15, 0.2, 0.5, 1, 1.5 and 2.5 μg/ml of piroxicam, six determinations for each concentration were made using the two methods. In the four following days, each level of calibration curve was analysed by triplicate. The response ratio of the peak area PRX and the internal standard versus effective concentration was fitted by a weighted least-squares linear regression to the equation:

$$Y = bx \pm a$$

The weighting factor used was $1/C^2$.

Table 2 shows the results obtained in the assays for on- and off-line extraction, with a good linearity in both cases ($r^2 = 0.9930$ and 0.9878, respectively) over the entire range tested. On average, the slope of the calibration graph obtained on five different days did not vary by more than 7.13% (off-line) and 4.12% (on-line), demonstrating the good stability of the measuring systems, the on-line method being slightly better.

3.3. Precision and accuracy

The within-day ($n = 6$) and day-to-day ($n = 5$) precision and accuracy were evaluated from the calibration curve results. The precision and accuracy of both methods were given in terms of coefficients of variation (% C.V.) and percentage bias respectively.

The precision and accuracy results are give in Tables 3 and 4. In the interval of concentrations investigated, all variation coefficients and bias, intro- and inter-day, were better than 9.5%.

3.4. Sensitivity

Based on a 200-μl (off-line) and 100-μl (on-line) sample volume, the lower PRX limit of quantification (LOQ) (% C.V. and percent bias ⩽20% on six replicate extractions) studied were 0.05 and 0.1 μg/ml on- and off-line respectively. It was not necessary to decrease the sensitivity more because of the high levels of PRX in volunteer plasma. With the on-line SPE we could detect 7.5 ng/ml of piroxicam and 25 ng/ml with off-line extraction.

3.5. Stability

3.5.1. Autosampler stability

The stability of PRX and TNX in the autosampler was checked 12 and 24 h at carrousel temperature, for 1 μg/ml piroxicam and tenoxicam concentration, no significant differences appeared between $t = 0$, $t = 12$ and $t = 24$ h.

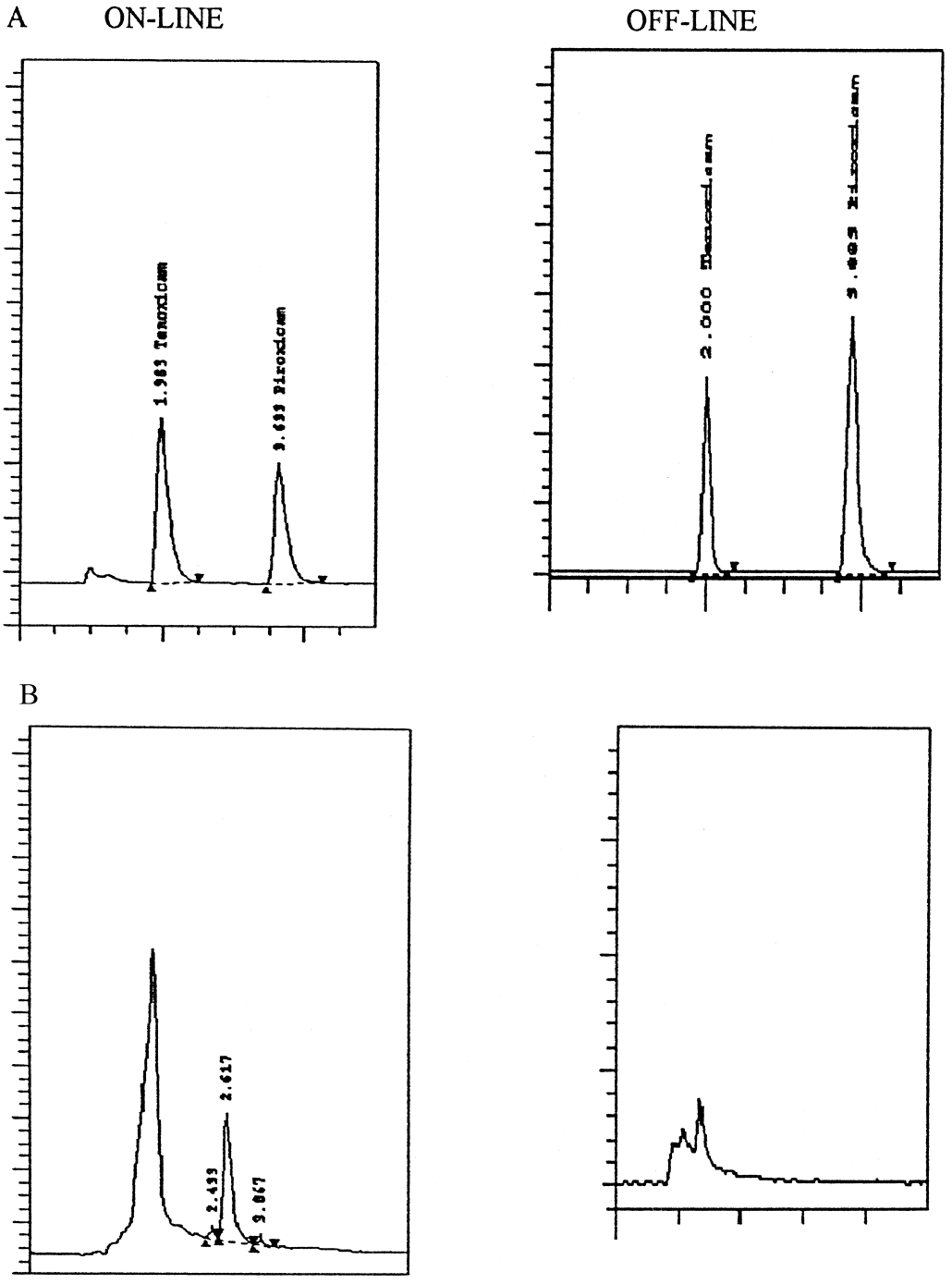

Fig. 1. Chromatographic separation. (A) Shows typical chromatograms of chromatographic standards dissolved in mobile phase; (B) blank plasma, free of interfering peaks; (C) spiked samples with 0.5 μl/ml PRX and 1 μg/ml TNX (S.I.).

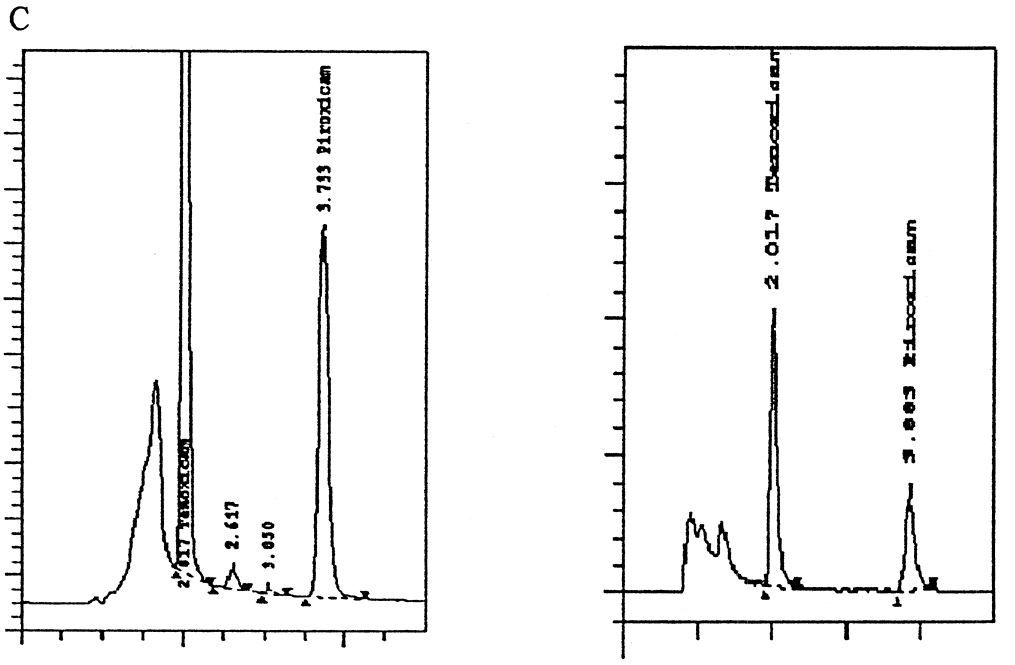

Fig. 1. (*continued*)

Table 1
Recovery results by on- and off-line extraction

Concentration (μg/ml)	1 μg/ml Tenoxicam	0.5 μg/ml Piroxicam	1.5 μg/ml Piroxicam
On-line (R%)[a]	91.34±3.93	91.64±1.06	92.94±1.18
Off-line (R%)[a]	78.52±9.06	101.54±8.21	97.06±5.75

[a] Mean of six determinations ±SD.

3.5.2. Freeze and thaw stability

A plasmatic standard with 1.5 μg/ml of PRX was stored at −80°C, the sample was subjected to three freeze–thaw cycles.

The recovery decreased 24% in the third cycle: so, to reduce the number of thaw, volunteer plasma samples were separated and frozen in two aliquots.

3.5.3. Long time stability

The quality controls, plasmatic standards and volunteer samples were stored at −80°C. The samples were stable at −80°C for at least one year.

4. Conclusions

Due to the fact that liquid–liquid extraction takes a lot of time, the latest tendency when we want to separate a drug from its plasmatic matrix, is to

Table 2
Results for on- and off-line extraction

	On-line				Off-line			
	X	SD	CV (%)	*n*	*X*	SD	CV (%)	*n*
Intercept (a)	−0.00158	0.00156	−98.734	5	0.261	0.0367	14.061	5
Slope (b)	0.9466	0.0390	4.120	5	2.7574	0.1967	7.134	5
Coef. correlation (*r*)	0.9965	0.0015	0.151	5	0.9940	0.0027	0.272	5
Coef. determination (r^2)	0.9930	0.0008	0.081	5	0.9878	0.0051	0.516	5

Table 3
Inter-day precision (% CV) and accuracy (% Bias) ($n=5$)

Amount added (μg/ml)	On-line			Off-line		
	Amount found (μg/ml±SD)	CV (%)	Bias (%)	Amount found (μg/ml±SD)	CV (%)	Bias (%)
0.05	0.051±0.003	6.65	5.13	–	–	–
0.10	0.101±0.006	5.81	3.93	0.097±0.009	5.80	7.35
0.15	0.138±0.007	4.97	7.80	0.153±0.013	7.06	6.85
0.20	0.200±0.012	5.94	4.93	0.207±0.013	3.42	5.92
0.50	0.485±0.023	4.85	4.35	0.500±0.030	5.87	5.06
1.00	1.011±0.045	4.44	3.57	0.983±0.049	7.29	4.04
1.50	1.529±0.077	5.02	4.44	1.450±0.061	5.45	3.96
2.50	2.610±0.086	3.31	4.89	2.554±0.174	9.01	6.00

Table 4
Intra-day precision (% CV) and accuracy (% Bias) ($n=6$)

Amount added (μg/ml)	On-line			Off-line		
	Amount found (μg/ml±SD)	CV (%)	Bias (%)	Amount found (μg/ml±SD)	CV (%)	Bias (%)
0.05	0.051±0.005	9.05	4.88	–	–	–
0.10	0.103±0.003	3.15	3.25	0.099±0.007	3.61	7.35
0.15	0.136±0.006	4.43	9.23	0.153±0.006	2.48	6.85
0.20	0.201±0.012	6.14	4.99	0.202±0.009	3.15	5.92
0.50	0.477±0.020	4.18	5.50	0.497±0.043	7.38	5.06
1.00	1.024±0.029	2.81	2.65	0.968±0.062	5.87	4.04
1.50	1.536±0.056	3.61	3.40	1.414±0.052	3.50	3.96
2.50	2.572±0.086	3.35	4.14	2.691±0.112	4.19	6.00

perform SPE. It was investigated in our laboratory what kind of advantages could a totally automatic SPE (on-line) present to quantify piroxicam in plasma, over traditional SPE. Both worked out in our laboratory.

In the described off-line method, due to the fact that PRX plasmatic levels were very high, it was possible to avoid the eluate drying phase. It also could work with a small plasma volume, 200 μl. The technique is simple, easy and robust, as can be proved by the results shown in Tables 1 to 4.

As for on-line SPE, it can be observed in the results that a bigger sensitivity and better internal standard recovery is achieved. It was also possible with a shorter manipulation process and the possibility of working during the night without supervision care for up to 70 samples a day, including the cleaning cartridge; and later utilisation of every cartridge for five more times.

Both methods demonstrated to be highly correlated like it is shown in the equation for the linear regression between the concentrations of piroxicam in plasma determined by the two methods described:

$$Y = -0.00984 + 1.032X$$

$$R = 0.9997$$

On- and off-line SPE have been successfully applied to the investigation of the pharmacokinetics after a single oral administration of a 20-mg piroxicam tablet.

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