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The determination of fenspiride in human plasma and urine by liquid chromatography with electrochemical or ultraviolet detection*

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Abstract: A selective and sensitive method for the determination of fenspiride in biological fluids is described. The method involves liquid–liquid extraction followed by separation on a reversed-phase column with electrochemical detection for low levels of the drug in plasma (≤100 ng ml^{-1}) or UV absorption for higher concentrations in plasma or urine. The method is suitable for pharmacokinetic analyses and drug monitoring studies.

Keywords: *Fenspiride; reversed-phase liquid chromatography; dual detection.*

Introduction

Fenspiride is an effective anti-inflammatory and anti-bronchoconstrictor drug (Fig. 1). It is used as the hydrochloride salt in tablet, syrup or injectable solution (Pneumorel®) in the treatment of diseases of the upper or lower respiratory tracts [1–3].

Until now, pharmacokinetic studies were performed using the radiolabelled compound [4, 5] or high-performance liquid chromatographic (HPLC) methods which lack sensitivity or specificity for the determination of the parent drug (F. Lodi, personal communication). The drug is not extensively metabolized and represents the major component in human plasma and urine.

This paper reports a new liquid chromatographic (LC) method for the analysis of fenspiride in human plasma and urine with two detection modes. An electrochemical detector (EC) to enable measurements of low levels in plasma (2–100 ng ml^{-1}), whereas ultraviolet absorbance (UV) was chosen for the determination of high drug levels in plasma and urine (up to, respectively, 1 and 10 $\mu g\ ml^{-1}$).

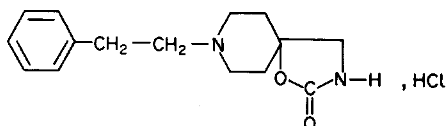

Figure 1
Chemical structure of fenspiride.

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Experimental

Reagents and materials

The following solvents and reagents were used: diethyl ether and dichloromethane Chromasol (SDS, Peypin, France), acetonitrile HPLC grade and sodium hydroxide Normex from Carlo Erba (Milan, Italy), hydrochloric acid 0.1 M Titrisol and potassium dihydrogen phosphate buffer from Merck (Darmstadt, FRG). The water used was prepared by deionization and photoxidation (UHQ Elgastat, Elga, Villeurbanne, France).

All solvents and solutions for LC were filtered through 0.2-μm membrane filters (Millipore, Molsheim, France). Prior to use, the components of the solvent system were mixed and degassed under reduced pressure, and a helium sparge was maintained through the solvent for the duration of the chromatographic analysis.

Fenspiride and its internal standard, {4-[(benzodioxen-6-yl)-methyl]-piperazin-1-yl}-2 pyrimidine, both as hydrochloride salts, were checked before use for their chemical purities in the Department of Analytical Chemistry of Technologie Servier.

Stock solutions of the drug and its internal standard were prepared by accurate weighing at a concentration of 1 mg ml^{-1} in water. Both the stock solutions were stable for at least 4 weeks, provided they were maintained at +4°C and kept away from direct sunlight when not in use. Each working day, standard solutions were prepared by appropriate dilution of the stock solutions over the range of desired concentrations.

Extraction procedure

An aliquot of plasma or urine (1 ml accurately weighed) was placed into a 10-ml centrifuge tube and spiked with 100 μl of an internal standard solution. The concentration of the latter was chosen such that the final concentration of the internal standard was in the middle of the concentration range to be studied. The biological sample was basified with 40 μl of 1 M sodium hydroxide, and liquid–liquid extraction was performed with 5 ml of a mixture of diethyl ether–dichloromethane (60:40, v/v). The tubes were shaken for 15 min on a table-top shaker (Ferrylab Prolabo, France) and then centrifuged at 1500*g* for 5 min. The organic layer was transferred to another tube and washed with 0.5 ml of 0.01 M hydrochloric acid. Following vortexing for 1 min and centrifuging, the upper organic phase was discarded and a 50-μl aliquot of the aqueous phase injected into the LC system.

At this stage of the pretreatment of the biological samples, the extracts were clean enough to analyse fenspiride in plasma by LC–UV. However they were insufficiently clean for the analysis of traces of the drug in plasma by LC–EC or in urine by LC–UV. In these cases, the aqueous phase was subsequently back-extracted at alkaline pH with 5 ml of the mixture diethyl ether-dichloromethane then the organic phase was evaporated at 40°C under nitrogen stream. The residue was reconstituted in 0.5 ml of the LC mobile phase and after vortexing 50 μl was injected into the chromatograph.

Chromatographic conditions

Two chromatographs were set up. Both were identical except the detection mode. Each chromatograph consisted of a SF 400 pump (Kratos ABI, Roissy, France) with a pulse damper in line, an SP 8780 autosampler (Spectra-Physics, Les Ulis, France) with data acquisition on a SP 4290 computing integrator (Spectra-Physics) and automation by means of Vax Multichrom software (VG Laboratory Systems, Cheshire, UK).

Chromatographic separations were performed on a reversed-phase column Ultrasphere CN (250 × 4.6 mm, i.d.; particle size 5 μm) from Beckman (Gagny, France). The mobile phase of phosphate buffer (20 mM, pH 4.6)–acetonitrile (40:60, v/v) was delivered at a flow-rate of 1.3 ml min^{-1} at 20°C.

The detection system consisted of either a Kratos SF 783 variable wavelength UV detector set at 210 nm or a Coulochem Model 5100 A electrochemical detector equipped with a Model 5020 guard cell and a Model 5011 analytical cell (ESA, Cunow, Cergy Saint-Christophe, France). The analytical cell consisted of two electrodes in series: the first was operated in the screen mode at a potential of +0.40 V and the second, the measuring electrode, was operated at a potential of +0.65 V. A potential of +1.0 V was used for the guard cell, which was placed between the pump and the injector.

Standard curves and quantitation

Calibration curves were prepared daily by spiking blank human plasma (provided by the Centre Départemental de Transfusion Sanguine, Orléans, France) or urine (from drug-free volunteers) with known amounts of the standard solutions of fenspiride and its internal standard. The concentration ranges covered were usually 0–100 ng ml^{-1} for plasma analysis by LC–EC, 0–1000 ng ml^{-1} for plasma analysis by LC–UV, and 0–10000 ng ml^{-1} for urine analysis by LC–UV. The concentration of the internal standard was always set at the middle of the range studied. The samples were assayed by the above procedure. The peak height ratios (y) of fenspiride and the internal standard were determined and plotted against the concentrations of fenspiride (x). Data were fitted by regression analysis to the equation $y = ax^n + b$, and statistical analysis was performed to evaluate the linearity (n) of the curve, the slope (a) and significance of the intercept (b) at the origin. The regression equation was used to calculate the concentration of fenspiride in unknown samples. Three quality control samples, one at low, intermediate and high levels were daily run together with the calibration standards.

Results and Discussion

Typical chromatograms for the analysis of plasma samples with either electrochemical detection or UV absorbance are shown in Fig. 2. In both cases, the compounds were well resolved and the baseline was free from interferences at the positions of analyte and standard.

Other drugs such as theophylline and mucolytics, which commonly are administered with Pneumorel, did not interfere with the assay because they were either not extracted or not detected under the same conditions. Therefore the assay is selective.

The stability of fenspiride and its internal standard in plasma extracts were checked at two concentrations, 100 and 1000 ng ml^{-1}. Samples were injected after storage under different conditions: immediately after extraction, 24 and 48 h at ambient temperature or at +4°C. In all cases, the chromatographic responses were not significantly different. Thus the good stability of the drug and its internal standard in the pretreated samples would enable the analysis of a large series of samples with an automated injector system.

Extraction efficiency

The percentage recovery of the extraction procedure for the unchanged drug was determined at six concentrations ranging from 5 to 10,000 ng ml^{-1}. Peak height ratios obtained from extracted plasma samples were compared with those obtained by injection

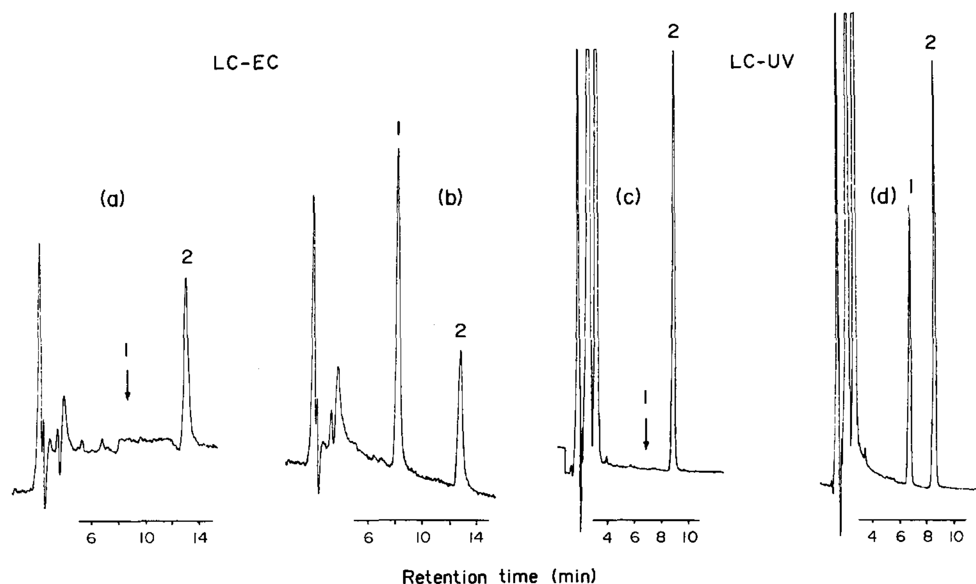

Figure 2
Representative LC–EC and LC–UV chromatograms obtained with blank human plasma (a and c), blank plasma spiked with 50 ng ml^{-1} (b) or 500 ng ml^{-1} (d) of fenspiride (peak 1) and its internal standard (peak 2).

of standard solutions. The extraction efficiency was not significantly different over the whole concentration range nor with the detection mode used. The mean recoveries (±standard deviation) were 75.9 ± 6.1% (n = 23) and 52.9 ± 3.2% (n = 16), respectively for fenspiride and the internal standard.

Accuracy and precision

Accuracy and precision of the method were determined by processing spiked plasma samples at three concentrations with respect to a calibration curve run each day with both chromatographic detections. The intra- and inter-assay precision expressed as the relative standard deviation (RSD) of the mean value found, and accuracy in terms of relative error of measurement (REM) are reported in Table 1. Within-day RSDs ranged

Table 1
Precision and accuracy data for the determination of fenspiride in plasma using EC and UV detection

Plasma levels (ng ml^{-1})	Intra-assay (n = 12) RSD (%)	Intra-assay (n = 12) REM (%)	Inter-assay (n = 6) RSD (%)	Inter-assay (n = 6) REM (%)
LC–EC				
5	4.9	10.0	7.7	10.0
10	5.3	0.8	6.6	0.4
100	6.8	0.1	7.4	3.3
LC–UV				
25	4.3	0.1	5.9	5.6
500	3.3	0.3	4.1	2.4
1000	3.3	2.9	1.6	0.1

from 3.3 to 6.8%. The between-day variation of samples analysed on 6 different days was 1.6–7.7%.

Linearity and limit of quantitation

Calibration curves were linear in the concentration ranges studied according to the detector used. Electrochemical detector was useful for the analysis of low drug concentrations (<100 ng ml^{-1}). At high levels, the response of this detector became less reliable, whereas UV detector gave linear response in a wider range of concentrations (up to 10,000 ng ml^{-1}). For that reason, the two detectors were not put in line but installed separately on each chromatographic system. Based on a signal-to-noise ratio of 3, the detection limit of the method from 1 ml of plasma was 2 and 10 ng ml^{-1}, using EC and UV detection, respectively. At these levels, the precision was 15 and 2.9% respectively, ($n = 4$).

Comparison of LC–EC and LC–UV

The plasma concentrations of fenspiride were determined by both methods using EC and UV detection. For this comparison, 30 control plasma samples were treated with known amounts of fenspiride in order to cover the common range of concentrations measured by the two detection modes, i.e. from 10 to 100 ng ml^{-1}. Thus the samples were split in two series, each of them being analysed by one method. The comparative results are shown in Fig. 3. A linear relationship was found with a slope of 1.071, an intercept of 0.073, and a correlation coefficient of 0.989. The data determined by the two detection modes are in good agreement in the common level range of detection.

Both the LC–EC and LC–UV methods are rapid, precise and reliable for the quantitative determination of fenspiride in human plasma and urine. Although the quantitation limit was lower with EC detection, the method with UV detection could be usefully employed to determine the drug in a wide range of concentrations. A further determination could be performed using the LC–EC method in plasma samples containing low drug levels (<100 ng ml^{-1}). Hence the use of this procedure would avoid the saturation of the electrochemical detector.

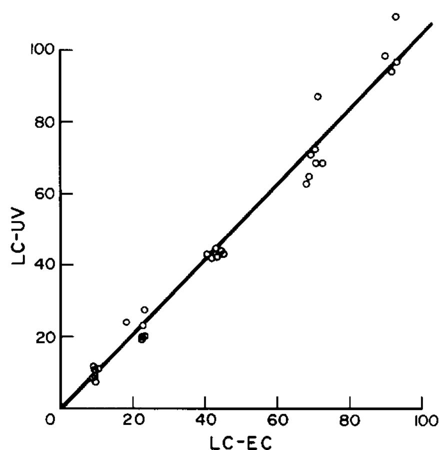

Figure 3
Comparison of fenspiride concentrations determined in the same plasma samples ($n = 30$) by the two detection modes.

Conclusion

The method described in this paper allows the determination of fenspiride in human biological fluids and the choice of the detection mode EC or UV permits its application to pharmacokinetic studies as well as in therapeutic monitoring.

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