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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PIRPROFEN AND FIVE OF ITS METABOLITES IN HUMAN PLASMA WITHOUT HYDROLYSIS AND IN HUMAN URINE BEFORE AND AFTER CHEMICAL HYDROLYSIS

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SUMMARY

Selective high-performance liquid chromatographic methods for the simultaneous determination of pirprofen and five of its metabolites either in plasma or in urine before and after chemical hydrolysis were developed After addition of an internal standard and a buffer, the compounds were extracted from plasma using reversed-phase C_{18} Bond-Elut columns and from urine using pre-packed silica Extrelut 1 columns, back-extraction into sodium hydroxide and acidification of the alkaline phase before injection Pirprofen, its five metabolites and the internal standard were separated using a linear elution gradient chromatographic system and wavelength programming The analysis of spiked samples demonstrated the good accuracy and precision of the methods with limits of quantitation of 100 or 200 ng/ml for the different compounds in plasma, 200 or 360 ng/ml in urine without hydrolysis and 1 or 1 8 μg/ml in urine after chemical hydrolysis

INTRODUCTION

Pirprofen, 2-[3-chloro-4-(3-pyrrolin-1-yl)phenyl]propionic acid (Rengasil) is a non-steroidal anti-inflammatory drug Its metabolic pathways were described by Egger et al. [1] (Fig. 1)

A high-performance liquid chromatographic (HPLC) method for the determination of pirprofen and one of its metabolites (the pyrrole metabolite) in plasma was described in a previous paper [2] This paper describes methods for the determination of pirprofen and five of its metabolites in human plasma without hydrolysis and in human urine before and after chemical hydrolysis

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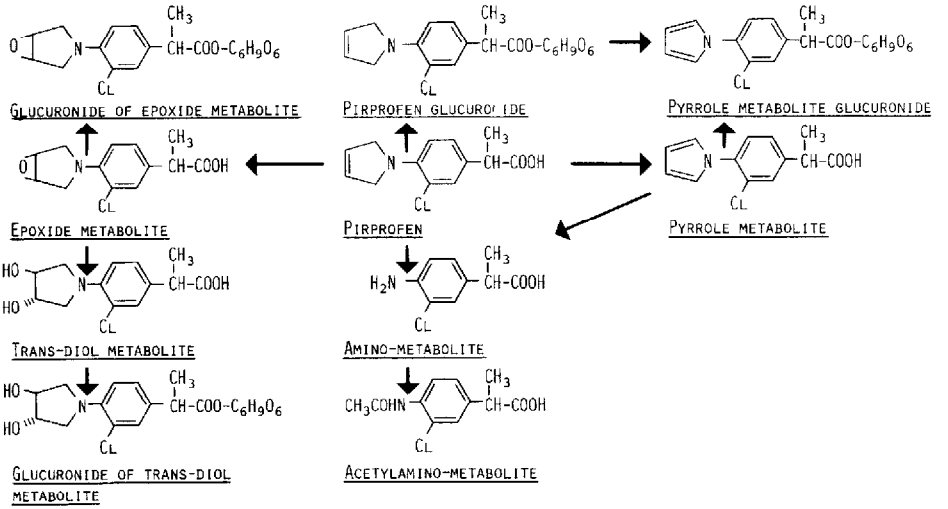

Fig 1 Metabolic pathways of pirprofen

EXPERIMENTAL

Chemicals and reagents

Pirprofen ($C_{13}H_{14}ClNO_2$, mol wt 251 71), its pyrrole metabolite ($C_{13}H_{12}ClNO_2$, mol.wt 249 70), acetylamino metabolite ($C_{11}H_{12}ClNO_3$, mol wt 241 67), *trans*-diol metabolite ($C_{13}H_{15}ClNO_4Na$, mol. wt 307 71), amino metabolite ($C_9H_{10}ClNO_2$, mol wt. 199 64) and epoxide metabolite ($C_{13}H_{14}ClNO_3$, mol wt 267 71) and the internal standard ($C_{14}H_{14}ClNO_2$, mol wt 263 72) were supplied by Ciba-Geigy (Basle, Switzerland)

All solvents were of analytical-reagent grade methanol for spectrophotometry (Carlo Erba, Milan, Italy), isopropyl alcohol for UV spectroscopy (Prolabo, Paris, France), *n*-hexane Uvasol (Merck, Darmstadt, F R G), dichloromethane Uvasol (Merck), acetonitrile for HPLC Ultra Gradient Grade (Baker, Deventer, The Netherlands) and water for use in HPLC (Baker) Acetate buffer (pH 5 3) was prepared from two stock solutions, A and B (20 80, v/v), solution A was obtained by diluting 17 1 ml of acetic acid (Merck) with water (Baker) to 100 ml and solution B by dissolving 24 6 g of anhydrous sodium acetate (Merck) in water to a final volume of 100 ml

Extraction columns

Bond-Elut C_{18} columns (500 mg) with a capacity of 2 8 ml (Analytichem, Harbor City, CA, U S A) were used for plasma and Extrelut 1 (Merck) glass columns pre-packed with silica for urine

Chromatographic equipment and conditions

Chromatography was performed on a Kratos module system composed of two solvent-delivery systems (SF 400 pumps) and a programmable multi-wavelength detector (SF 783G) equipped with a gradient controller An automatic variable-volume injector (Waters 712 WISP) equipped with a cooling system was connected to the mixing chamber of the Kratos module and the analytical column A Hewlett-Packard integrator (HP 3388) recorded the chromatograms and calculated the peak heights The pre-packed analytical column was a stainless-steel tube (15 cm×3 9 mm I D) filled with Novapak C_{18} (4 μm) (Waters) The compounds were eluted at a constant flow-rate of 1 2 ml/min, using a linear elution gradient with acetonitrile–pH 5 5 phosphate buffer (5 10^{-3} mol/l KH_2PO_4) and wavelength programming (Table I) The conditions depended on the performance of the column and could be slightly modified Chromatography was carried out at laboratory temperature (18–21 °C)

Stock solutions and working solutions

Stock solutions were prepared by dissolving pirprofen and its five metabolites in a few drops of methanol and diluting to the final volume with distilled water Working solutions were obtained by diluting the corresponding stock solutions with distilled water The internal standard was dissolved in a few drops of methanol and diluted with distilled water

TABLE I

LINEAR ELUTION GRADIENT AND WAVELENGTH PROGRAMME FOR PLASMA AND URINE

Sample	Time (min)	Mobile phase acetonitrile (%)	Time (min)	Wavelength (nm)
Plasma	0	11	0	234
	3	11	4 5	250
	21	32	6 8	234
	30	32	10 5	244
	31	11	22	250
	50	11	40	234
Urine	0	8	0	234
	3	8	6	250
	20	30	8	234
	32	30	12	244
	33	8	22	250
	50	8	40	234

Calibration graphs

In plasma and urine without hydrolysis Aliquots of the working or stock solutions and a constant amount of internal standard (1 μg) were added to 0 5 ml of plasma or urine to produce reference samples in the concentration range 0 1–10 μg/ml in plasma and 0 2–10 μg/ml in urine for all compounds

In urine after chemical hydrolysis Aliquots of the working or stock solutions and a constant amount of internal standard (1 μg) were added to 0 1 ml of urine to produce reference samples in the concentration range 1–50 μg/ml for all compounds

The calibration graphs remained valid for 1 week. Every day, the validity of the calibration graphs was checked by duplicate analyses of samples spiked with a low and a high concentration of the compounds If these spiked samples gave results that deviated too much (accuracy $>115\%$ or $<85\%$), a new calibration graph had to be prepared

Extraction procedure

In plasma A 50-μl volume of internal standard solution, 0.5 ml of plasma and 0 4 ml of pH 4 citrate buffer were introduced into a glass tube, mixed on a vortex mixer for 5 s and centrifuged at 2000 *g* for 3 min Extraction was performed with a Bond-Elut C_{18} column conditioned with 3 ml of methanol and 2 ml of pH 4 citrate buffer The upper phase of the plasma sample was loaded on the column and aspirated through by applying a vacuum for 5 min, the column was then washed successively with 2 ml of water, 1 ml of 0 1 mol/l sodium acetate and 0 55 ml of methanol. This volume of methanol is necessary to eliminate water through the column; the volume of 0 55 ml should not be exceeded The compounds were eluted with 0 7 ml of methanol into a conical glass tube. After concentration of the methanolic phase to about 100 μl at 30 °C under a stream of nitrogen, 500 μl of water were added The tube was shaken on a vortex mixer for 5 s and 20 μl were injected on to the analytical column.

In urine without hydrolysis A 50-μl volume of the internal standard solution, 0 5 ml of urine, ca 15 mg of ascorbic acid and 0 2 ml of pH 5 3 acetate buffer were introduced into a glass tube and mixed on a vortex mixer for 5 s. Extraction was performed with an Extrelut 1 column conditioned with 6 ml of dichloromethane containing 10% (v/v) of isopropyl alcohol and dried at 100 °C overnight The urine sample was loaded on the column; after 5 min of equilibration at room temperature, the compounds were eluted with 6 ml of dichloromethane containing 10% (v/v) of isopropyl alcohol; the eluate dripped slowly into a conical tube and was then concentrated to a final volume of about 100 μl at 30 °C under a stream of nitrogen A 2-ml volume of *n*-hexane and 0.5 ml of 1 mol/l sodium hydroxide were added, the tube was shaken mechanically (Infors shaker) for 30 min at 300 rpm and centrifuged for 3 min at 2000 *g* The organic phase was discarded; 0 4 ml of the aqueous phase was introduced into a glass tube, 0 2 ml of pH 5 3 acetate buffer and 0 2 ml of 2 mol/l hydrochloric

acid were added to obtain a final pH of 5 5 and 20 μl were injected on to the analytical column

In urine after chemical hydrolysis The chemical hydrolysis of pirprofen has been described previously [3] This method was modified by adding ascorbic acid

After addition of the internal standard solution to 0 1 ml of urine, 15 mg of ascorbic acid and 0 25 ml of 2 mol/l sodium hydroxide were introduced into the glass tube The tube was shaken on a vortex mixer for 5 s and left at room temperature for 1 h, then 0.2 ml of pH 5 3 acetate buffer and 0.25 ml of 2 mol/l hydrochloric acid were added and the tube was shaken on a vortex mixer The same extraction procedure as described above for urine without hydrolysis was then applied

Quantitative evaluation

Quantification was based on the peak-height ratio of the different compounds and of the internal standard Calibration graphs were obtained by plotting the peak-height ratio versus the concentration of each compound and calculated by weighted linear regression with a weighting factor of $1/(\text{concentration})^2$

RESULTS AND DISCUSSION

Separation from plasma and urine components

An example of a chromatogram of extracts from blank plasma and plasma spiked with 1 μg of each compound is shown in Fig 2, and an example of extracts from blank urine and urine spiked with 1 6 μg of each compound, after hydrolysis, is shown in Fig 3. No interfering peaks derived from endogenous components were observed in plasma and urine except for urine after hydrolysis because of an interfering peak at the retention time of the acetylamino metabolite, for this reason, the aliquot of urine sample must be limited to 0.1 ml

Calibration graphs

Calibration graphs for plasma were linear in the range 0 2–10 μg/ml for pirprofen and the pyrrole metabolite and 0 1–10 μg/ml for the other metabolites Calibration graphs for urine without hydrolysis were linear in the range 0 2–10 μg/ml for five of the six compounds (0.36–10 μg/ml for the diol metabolite) and for urine after chemical hydrolysis in the range 1–50 μg/ml for the amino, epoxide and pyrrole metabolites and 1.8–50 μg/ml for pirprofen and the acetylamino and diol metabolites

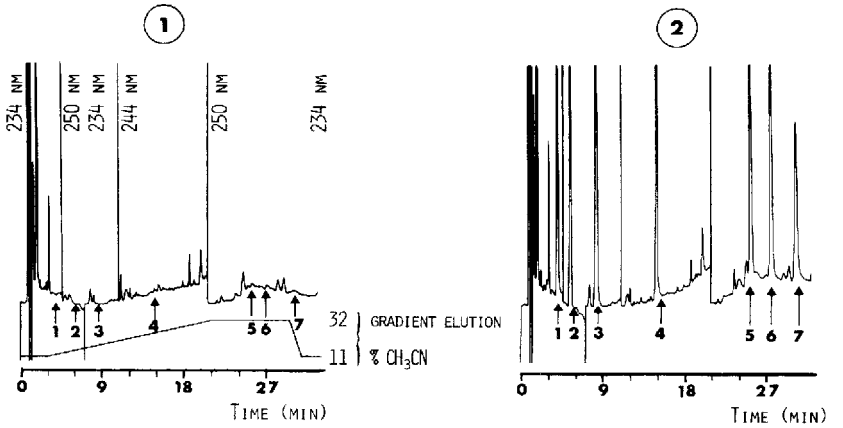

Fig 2 Examples of chromatograms in plasma 1 = Extract of 0 5 ml of human plasma blank (with gradient elution and wavelength programming) 2 = Extract of 0 5 ml of human plasma spiked with 1 μg of the acetylamino (1), diol (2), amino (3), epoxide (4) and pyrrole (5) metabolites, pirprofen (6) and internal standard (7) Detector sensitivity, 10^{-3} a u f s , integrator attenuation, 4 (160 μV/cm)

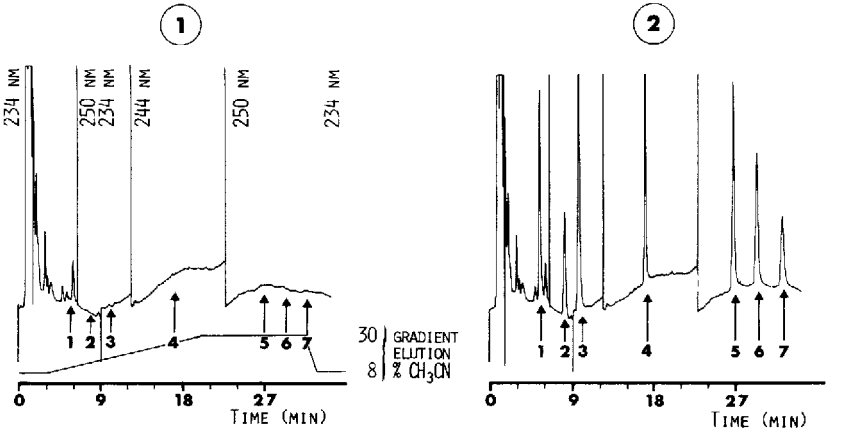

Fig 3 Examples of chromatograms in urine after hydrolysis 1 = Extract of 0 1 ml of human urine blank (with gradient elution and wavelength programming) 2 = Extract of 0 1 ml of human urine spiked with 1 6 μg of the acetylamino (1), diol (2), amino (3), epoxide (4) and pyrrole (5) metabolites, pirprofen (6) and internal standard (7) Detector sensitivity, 10^{-3} a u f s , integrator attenuation, 4 (160 μV/cm)

Within-day accuracy and precision

Plasma or urine samples spiked with different amounts of pirprofen and its five metabolites were analysed six times on the same day For plasma the overall accuracy is characterized by the mean recovery, ranging from 93 6 to 103 9% The precision of the method is shown by the overall relative standard deviation

TABLE II

DAY-TO-DAY ACCURACY (MEAN RECOVERY, %) AND PRECISION (R S D , %, IN PARENTHESES) OF THE DETERMINATION OF PIRPROFEN AND ITS FIVE METABOLITES

Biological fluid	Acetylamino metabolite	Diol metabolite	Amino metabolite	Epoxide metabolite	Pyrrole metabolite	Pirprofen
Plasma	98 3 (8 8) ($n=24$)	94 2 (7 6) ($n=20$)	102 4 (6 8) ($n=20$)	101 3 (4 9) ($n=25$)	99 0 (9 9) ($n=14$)	99 6 (7 6) ($n=19$)
Urine before hydrolysis	99 3 (7 1) ($n=25$)	105 4 (5 9) ($n=20$)	99 0 (5 7) ($n=25$)	101 7 (4 2) ($n=25$)	98 4 (6 2) ($n=25$)	99 7 (6 4) ($n=20$)
Urine after hydrolysis	102 3 (6 5) ($n=16$)	97 1 (8 1) ($n=19$)	95 9 (6 6) ($n=25$)	95 7 (4 2) ($n=25$)	98 6 (4 2) ($n=25$)	101 6 (6 0) ($n=20$)

(R S D.) of 3 5–10.7% For urine without hydrolysis, the mean recovery was 98 3–101 5% and the R S D 3 1–9 3%

Day-to-day accuracy and precision

Plasma or urine samples spiked with different amounts of pirprofen and its five metabolites were analysed on five days For plasma, the overall accuracy was 94 2–102.4% (Table II) and the R.S D 4 9–9 9% For urine with or without hydrolysis, the overall accuracy was 95 7–105 4% and the R.S D 4 2–8 1% (Table II)

These results demonstrate the good accuracy and precision of the method with the concentrations tested

Limits of quantitation

The limit of quantitation (R S D $\leqslant 10\%$) was calculated from the results for the within-day accuracy For plasma, the limit of quantitation was 0 2 μg/ml for pirprofen and the pyrrole metabolite and 0 1 μg/ml for the other compounds For urine without hydrolysis, the limit of quantitation was 0 2 μg/ml for all the compounds except the diol metabolite (0.36 μg/ml) For urine after chemical hydrolysis, the limit of quantitation was 1 μg/ml for the amino, epoxide and pyrrole metabolites and 1 8 μg/ml for pirprofen and the acetylamino and diol metabolites

Stability of the extracts of plasma and urine

The extracts of plasma or urine containing pirprofen and its five metabolites must be kept at $-20\,^{\circ}C$ until injection and could be kept for only 6 h on the Wisp injector refrigerated at $2\,^{\circ}C$ After 8 h, the degradation of pirprofen to the pyrrole derivative reached 5–7% in urine

Stability of pirprofen during extraction or hydrolysis

No detectable transformation of pirprofen to one of its metabolites was observed during extraction from plasma The transformation of pirprofen to the pyrrole metabolite could reach 14% during extraction from urine at room tem-

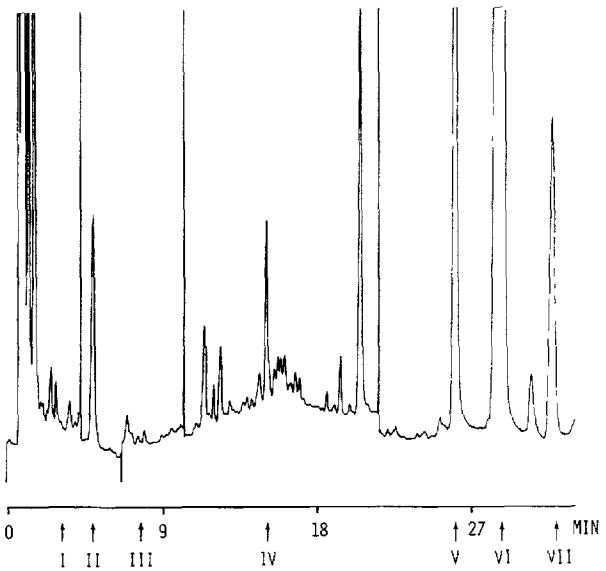

Fig 4 Example of chromatogram obtained from a subject who had received 400 mg of pirprofen orally Sampling time, 3 h after administration I = Acetylamino metabolite (not detected), II = diol metabolite (0 8 μg/ml), III = amino metabolite (not detected), IV = epoxide metabolite (0 38 μg/ml), V = pyrrole metabolite (4 1 μg/ml), VI = pirprofen (36 3 μg/ml), VII = internal standard Detector sensitivity, 10^{-3} a u f s , integrator attenuation, 4 (160 μV/cm)

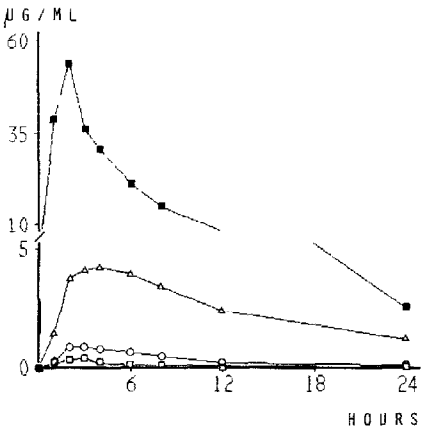

Fig 5 Example of plasma concentration curves for pirprofen and three of its metabolites obtained after oral administration of 400 mg of pirprofen to one subject ○ = diol metabolite, □ = epoxide metabolite, △ = pyrrole metabolite, ■ = pirprofen

perature The addition of ascorbic acid reduced this transformation to 4 5% After chemical hydrolysis, the transformation of pirprofen to the pyrrole metabolite could reach 30–50% The addition of ascorbic acid during hydrolysis and extraction reduced this transformation to 6%

Application

The method described was applied to the determination of pirprofen and its metabolites in plasma after oral administration of 400 mg of pirprofen to one subject No measurable amount of the acetylamino and amino metabolites was found The diol, epoxide and pyrrole metabolites and pirprofen were found over the sampling time Fig 4 shows a chromatogram obtained from the subject 3 h after administration. The plasma concentration curves are shown in Fig 5

CONCLUSION

The described method permits the determination of pirprofen and five of its metabolites in human plasma and urine with acceptable precision and accuracy

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