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DETERMINATION OF PIRPROFEN IN BIOLOGICAL MATERIAL BY GAS–LIQUID CHROMATOGRAPHY WITH NITROGEN-SPECIFIC AND ELECTRON-CAPTURE DETECTION

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SUMMARY

Pirprofen, 2-[3-chloro-4-(3-pyrrolin-1-yl)phenyl]propionic acid, is an analgesic and anti-inflammatory agent. Most of the pirprofen is present in plasma in the unchanged form (80–90%), the remainder in the form of the pyrrole derivative. However, pyrrole is also formed during sample manipulation by oxidation of pirprofen. Thus, a method based on conversion of pirprofen to pyrrole by oxidation with 2,3-dicyano-5,6-dichlorobenzoquinone and subsequent measurement of the total pyrrole concentration was developed. The method is based on the introduction of an internal standard, extraction and esterification followed by oxidation. The methyl ester of the pyrrole is then determined by gas–liquid chromatography. A nitrogen-specific detector is generally used. However, for small sample sizes (0.1 ml of plasma), an electron-capture detector may be utilized. With this detector measurements of concentrations as low as 0.02 nmol/g (5 ng/g) are still possible.

The kinetics of the degradation of pirprofen to its pyrrole derivative were investigated. Plasma levels of pirprofen after a single oral dose of 400 mg in a healthy volunteer were measured by a method described previously as well as by the new method.

INTRODUCTION

Pirprofen, 2-[3-chloro-4-(3-pyrroline-1-yl)phenyl]propionic acid, is an analgesic and anti-inflammatory agent. An assay procedure for the simultaneous determination of pirprofen and its oxidation product, the pyrrole analogue, using flash methylation, has been described[1].

Most of the pirprofen is present in plasma in the unchanged form (80–90%), the remainder in the form of the pyrrole derivative. However, the pyrrole analogue is also formed during sample manipulation by oxidation of pirprofen. Thus, a method based on conversion of pirprofen to the pyrrole analogue by oxidation with 2,3-dicyano-5,6-dichlorobenzoquinone and subsequent measurement of the total pyrrole

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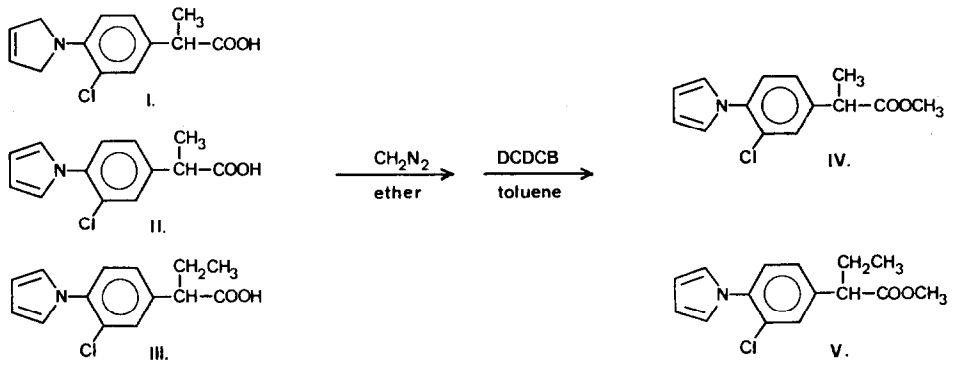


Fig. 1. Structures of pirprofen (I), its pyrrole oxidation product (II), the internal standard (III) and the corresponding derivatives (IV and V). DCDCB = 2,3-dicyano-5,6-dichlorobenzoquinone, used to oxidize pirprofen to its pyrrole analogue.

analogue concentration, was developed (Fig. 1). The butyric acid analogue (GP 48921) of pyrrole is used as an internal standard.

Nitrogen-specific detection (NPD) is generally used. However, for small sample sizes (0.1 ml of plasma) or low concentrations of pirprofen, electron-capture detection (ECD) may be utilized. With ECD measurements of concentrations as low as 0.02 nmol/g are possible.

The kinetics of the degradation of pirprofen to its pyrrole derivative were investigated. Plasma levels of pirprofen after a single oral dose of 400 mg in a healthy volunteer were measured by the method described previously and by the new method. In an additional experiment, urinary elimination of the sum total of pirprofen, the pyrrole analogue and the respective conjugates were measured after chemical hydrolysis.

EXPERIMENTAL AND RESULTS

Reagents and chemicals

For pirprofen ($C_{13}H_{14}ClNO_2$; mol.wt. 251.71) and GP 48 921 ($C_{14}H_{14}ClNO_2$; mol.wt. 263.72), 1 mg was dissolved in 5 ml of 0.1 mol/l sodium hydroxide solution, 4 ml of 0.1 mol/l hydrochloric acid were added and the solution was made up to 100 ml with water. Further dilutions were made with water.

Acetate buffer of pH 5.3 (0.25 *M*) was prepared by dissolving 20.52 g of sodium acetate in 700 ml of water, adjusting the pH to 5.3 with a solution of 4.2 ml of acetic acid in 300 ml water and diluting to 1 l with water.

Toluene, diethyl ether, dichloromethane and *n*-hexane were distilled over a 1-m Vigreux column.

Diazomethane was prepared daily using a diazomethane generator (Pierce, Rockford, IL, U.S.A.) by the method of Fales *et al.*[2].

A 0.1% solution of 2,3-dicyano-5,6-dichlorobenzoquinone in toluene was prepared daily.

Laboratory-grade hydrochloric acid (0.1 and 2 mol/l) and sodium hydroxide solution (0.1 and 2 mol/l) were used.

Procedures

Plasma. A 1-ml volume of plasma, 0.1 ml of internal standard solution (containing GP 48 921, 22.6 nmol if NPD is used or 0.94 nmol if ECD is used), 4 ml of acetate buffer and 4 ml of diethyl ether–dichloromethane (4:1, v/v) are shaken for 15 min at 180 rpm on a mechanical rotary shaker (Infors). After brief centrifugation, the organic phase is transferred into a clean vial and evaporated to dryness under a stream of nitrogen at 40°C.

A 0.4-ml volume of diazomethane in diethyl ether ($1.5 \cdot 10^{-2}$ mmol) is added to the dry residue and left at room temperature for 40 min. The diazomethane and ether are then evaporated under a stream of nitrogen followed by addition of 0.2 ml of dicyanodichlorobenzoquinone (0.1% in toluene) ($1 \cdot 10^{-3}$ mmol), mixed to reconstitute a solution of the residue and left to react at room temperature for 5 min.

A 1.8-ml volume of *n*-hexane and 2 ml of water are added to the reaction mixture and shaken for 10 min at 220 rpm. After brief centrifugation, the organic phase is transferred into a clean vial and again evaporated to dryness. The residue is dissolved in 150 µl of toluene and aliquots of 2 µl are injected into the gas chromatograph.

Urine. Hydrolysis of the pirprofen and pyrrole conjugates are achieved as follows: 0.05–1.00 ml of urine and 1 ml of 2 mol/l sodium hydroxide solution are mixed and left at room temperature for 1 h. After neutralization with 2 ml of 2 mol/l hydrochloric acid the samples are treated as described for plasma.

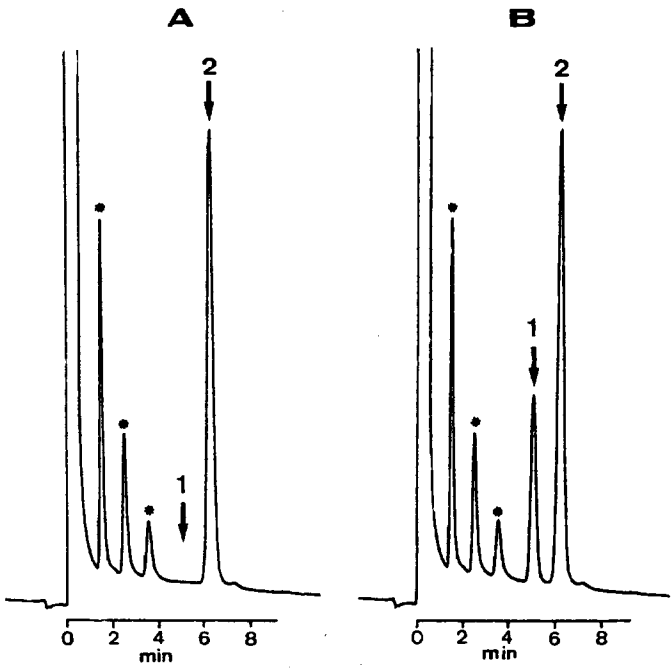


Fig. 2. Chromatograms of extracts of plasma samples containing (A) 22.6 nmol of internal standard (2) only and of (B) 22.6 nmol of internal standard (2) and 10 nmol of pirprofen (1); 1/175th of the final extract injected (NPD system). The peaks marked with an asterisk are unknown constituents of plasma.

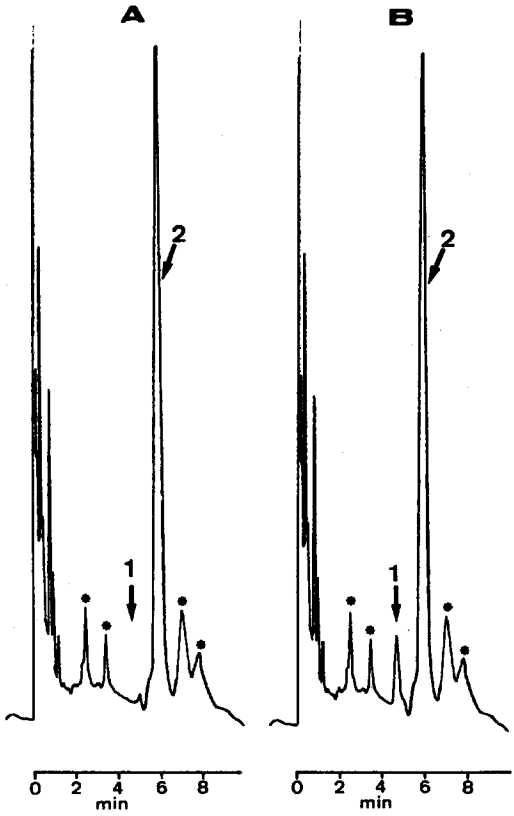


Fig. 3. Chromatograms of extracts of plasma samples containing (A) 0.94 nmol of internal standards (2) only and (B) 0.94 nmol of internal standard (2) and 0.08 nmol of pirprofen (1); 1/40th of the final extract injected (ECD system). The peaks marked with an asterisk are unknown constituents of plasma.

Gas chromatography

Generally, after therapeutic doses of pirprofen in adults, chromatography of plasma samples may be performed using the nitrogen-specific detection. However, if less than 1 ml of plasma (*i.e.* 200 μl) are available or if concentrations of pirprofen are very low, it may be necessary to use ECD. Typical ranges for the two assay procedures are 2–50 nmol/g with NPD and 0.02–0.80 nmol/g with ECD.

NPD. A Perkin-Elmer Sigma 3 instrument equipped with an NPD detector was used. The column was a 2.5 m × 4 mm I.D. Pyrex glass column packed with 3% OV-101 on Supelcoport (100–120 mesh). The temperatures were column 215°C, detector 250°C and injector 225°C. The flow-rates were carrier gas (nitrogen) 15 ml/min, hydrogen 4.4 ml/min and air 120 ml/min. The retention times using these conditions were 5 min for the pyrrole derivative and 6.3 min for the internal standard derivative. Chromatograms are shown in Fig. 2.

ECD. A Pye GCD instrument equipped with a ^{63}Ni electron-capture detector was used. The column was a 2.5 m × 4 mm I.D. Pyrex glass column packed with 3% OV-101 on Supelcoport (100–120 mesh). The temperatures were column 190°C and injector 200°C. The carrier gas (nitrogen) flow-rate was 60 ml/min. The retention

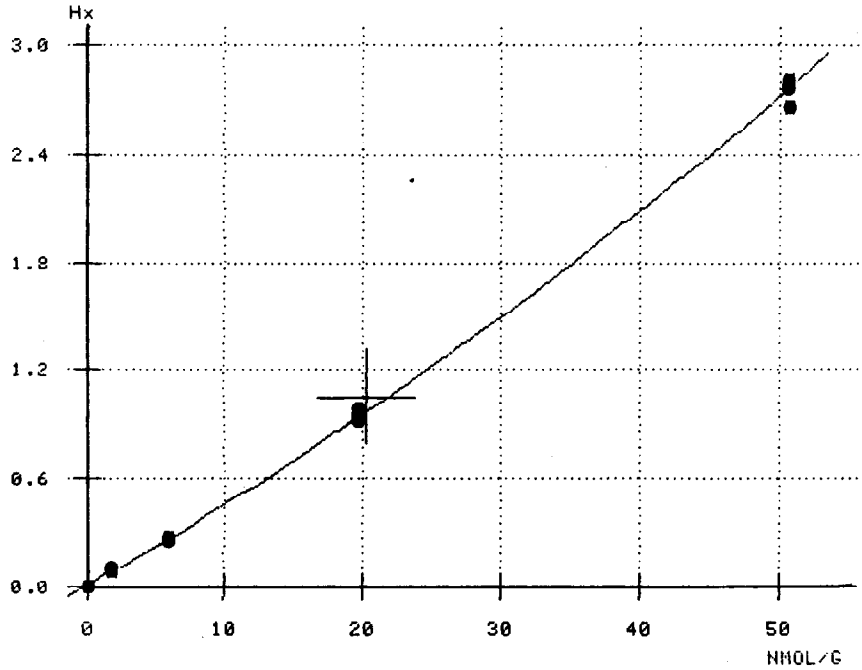


Fig. 4. Calibration graph for the entire analytical procedure (NPD system) for pirprofen. H_x = peak-height ratio (pyrrole derivative/int. standard derivative).

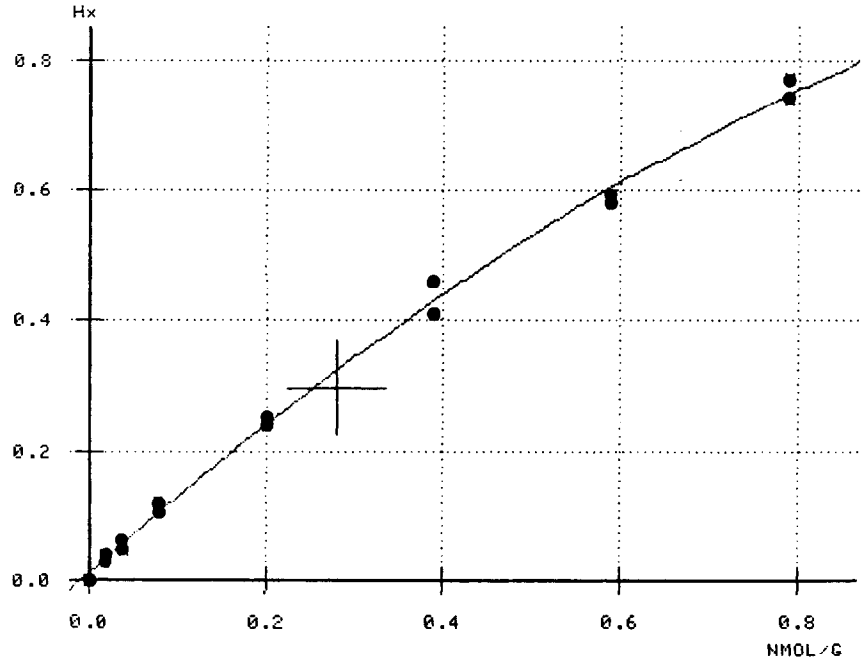


Fig. 5. Calibration graph for the entire analytical procedure (ECD system) for pirprofen. H_x = as in Fig. 4.

times using these conditions were 4.7 min for the pyrrole derivative and 6 min for the internal standard derivative. Chromatograms are shown in Fig. 3.

Calibration graphs

Calibration graphs for plasma and urine determination were prepared as follows. Blank plasma or urine samples were spiked with solutions of pirprofen (0–0.8 nmol/g for ECD and 0–50 nmol/g for NPD calibrations). The samples were processed as described. The peak height of the pyrrole derivative was divided by that of the internal standard derivative and the ratio (H_x) plotted against initial pirprofen concentrations. Figs. 4 and 5 show calibration graphs recorded using NPD and ECD, respectively.

Hydrolysis

Hydrolysis of conjugates was optimized by incubation of urine samples, obtained from a volunteer who had been treated with an oral dose of pirprofen, for various time periods and with various amounts of enzyme and sodium hydroxide. Both hydrolysis methods may be used. However, the chromatograms obtained after chemical hydrolysis show less interfering peaks and therefore the following standard conditions were chosen: up to 1 ml urine + 1 ml of 2 mol/l sodium hydroxide solution left for 1 h at room temperature. These conditions were found to be sufficient to hydrolyse all conjugates. During the hydrolysis no losses of pyrrole analogue (and pirprofen) could be detected.

Derivatization

Pirprofen, the pyrrole derivative and the internal standard are esterified almost instantly with diazomethane. The mixture of esters is then oxidized using 2,3-dicy-

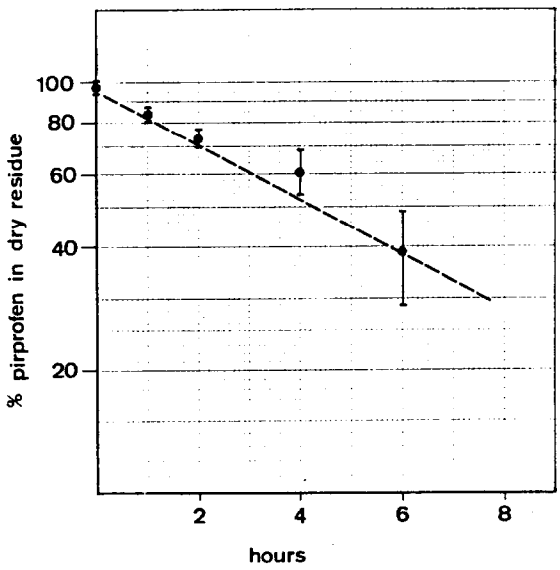


Fig. 6. Semilogarithmic plot of the pirprofen content of a dry residue at room temperature following evaporation of the extraction solvent.

ano-5,6-dichlorobenzoquinone to dehydrogenate the pyrroline to the pyrrole ring (Fig. 1). The oxidation is achieved in less than 5 min. The structure of the pyrrole methyl ester was confirmed by NMR and mass spectrometry.

Extraction

Partition coefficients of pirprofen between organic phases and acetate buffer showed an optimum extraction yield at pH 5.3. Therefore, acetate buffer of pH 5.3 (0.25 mol/l) and diethyl ether–dichloromethane (4:1, v/v) were chosen to extract pirprofen from biological material. The overall yield for the entire analytical procedure is better than 80% as evaluated by gas chromatography.

Stability

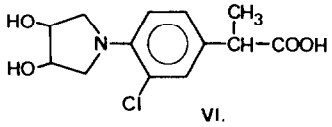
Solutions of pirprofen in 0.1 mol/l sodium hydroxide solution, 0.1 mol/l hydrochloric acid and methanol–water (1:1, v/v) were kept at room temperature for 4 days and the pyrrole formation was measured daily. It was found that no more than 2.2–5.7% of pyrrole was formed in any of the samples.

In plasma pirprofen was found to be stable for over 24 h at 37°C. Following extraction of pirprofen and pyrrole from plasma with the solvent mixture, the organic layer is evaporated to dryness. At this point, as soon as all the solvent has evaporated, pirprofen is extremely vulnerable to oxidation, its half-life then being only about 4.5 h, *i.e.*, after 1 h 15% and after 24 h 74% of the pyrrole degradation product are formed (Fig. 6).

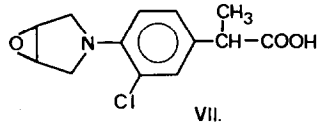
Plasma samples containing pirprofen are stable if stored at −20°C. Twelve samples with concentrations between 4 and 187 nmol/g were analysed, then stored for 3 months at −20°C and re-analysed. Recoveries were 102 ± 20% of the first determinations.

Interaction of metabolites

The metabolism of pirprofen has been described in detail[3]. The two metabolites most likely to interfere with the assay are the diol and the epoxide (Fig. 7). The diol is not co-extracted under the given conditions. The epoxide is co-extracted and also chromatographed, but the retention time is long (8.5 min) and the response only about one sixth of that of the pyrrole ester.



VI.



VII.

Fig. 7. Structures of the two metabolites, the 3,4-diol (VI) and the corresponding epoxide (VII).

Other interactions

One major source of interaction is the plasticizer tris(butoxyethyl) phosphate, which is present in some vacutainer stoppers and is easily leached into biological samples. This compound produces a very large interfering peak when using NPD. The interference has been described previously[4] in connection with the determination of theophylline with NPD.

Recovery, precision and limit of quantitation

NPD procedure. The recovery of pirprofen and the precision were evaluated by analysing spiked plasma and urine samples; 44 plasma and 7 urine samples were prepared and analysed. The plasma samples had concentrations between 3.5 and 55 nmol/g and the urine samples between 4.8 and 143 nmol/g.

The differences between the found and the initial concentrations ranged from −4.7 to +3.2% for plasma and from −3.4 to +1.7% for urine.

The correlation between concentrations taken and found is illustrated in Fig. 8 for plasma and in Fig. 9 for urine. Calculation of the linear regression[5] between the taken and found concentrations resulted in correlation coefficients (r) of 0.996 for plasma and 0.998 for urine. Estimated values for the standard deviation (called

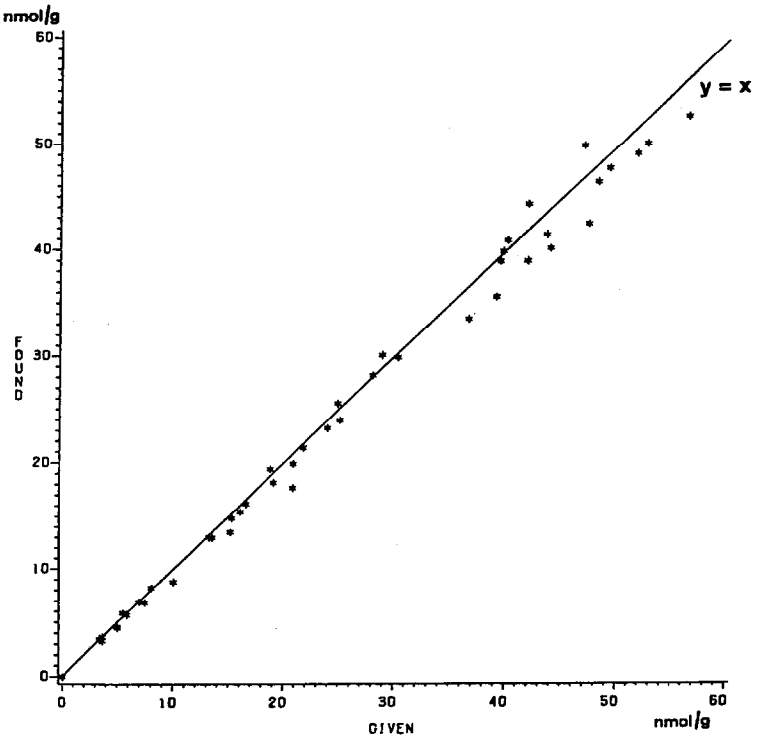


Fig. 8. Method validation for the NPD procedure for plasma. The correlation coefficient between taken and found concentrations (r) is 0.996 and the estimated standard deviation (S_e) is 1.480 nmol/g (10 nmol/g corresponds to 2.52 μg/g).

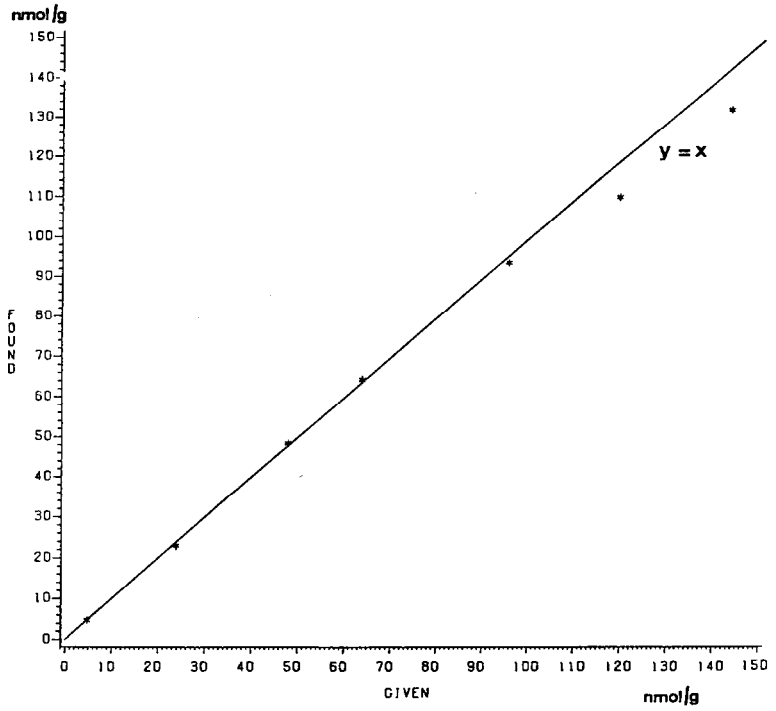


Fig. 9. Method validation for the NPD procedure for urine. The correlation coefficient between taken and found concentrations (r) is 0.998 and the estimated standard deviation (S_e) is 2.811 nmol/g (10 nmol corresponds to 2.52 μg/g).

S_e in ref. 5) were 1.480 nmol/g for plasma and 2.811 nmol/g for urine. The limit of quantitation is about 2 nmol/g.

ECD procedure. Eleven plasma samples spiked with pirprofen at concentrations between 0.03 and 0.75 nmol/g were prepared and analysed. The differences between the found and the initial concentrations ranged from −4.9 to +5.2%. The correlation between the taken and found concentrations is illustrated in Fig. 10. Calculation of the linear regression[5] between the taken and found concentrations resulted in a correlation coefficient (r) of 0.998 and an estimated value of the standard deviation of 0.016 nmol/g. The limit of quantitation is about 0.02 nmol/g.

Application

A healthy volunteer (72 kg, 41 years old) received one single oral dose of 400 mg of pirprofen as a solution (in 100 ml of pH 7.4 phosphate buffer). Plasma concentrations of the total of pirprofen and pyrrole were determined by the NPD procedure described here and also by the flame-ionization detection procedure reported previously[1] (Fig. 11). In an additional experiment, one healthy volunteer (60 kg, 48 years old) received a single oral dose of 400 mg of pirprofen (capsule). The cumulative urinary elimination, measured after hydrolysis, was determined over a 24-h period. It could be shown that 50% of the dose was excreted in the urine in the form of either pirprofen or the pyrrole analogue and the corresponding conjugates.

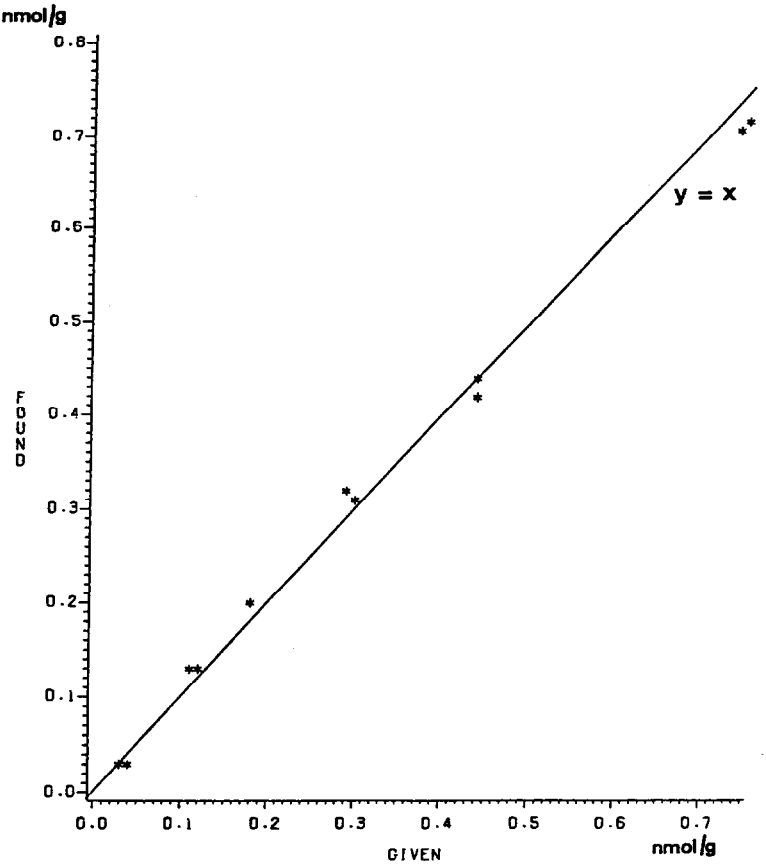


Fig. 10. Method validation for the ECD procedure for plasma. The correlation coefficient between taken and found concentrations (r) is 0.998 and the estimated standard deviation (S_e) is 0.016 nmol/g (0.1 nmol/g corresponds to 25 μg/g).

DISCUSSION

Maximum concentrations of pirprofen in plasma are around 300–400 nmol/g after a single therapeutic dose of 400 mg. It is therefore generally not necessary to utilize highly sensitive ECD. However, for determinations in small samples (clinical) or in other biological fluids such as breast milk, utilization of ECD becomes essential. Although the molecule possesses only one chlorine atom, the ECD sensitivity is excellent, probably owing to the additional electrophore properties of the pyrrole ring.

The oxidized derivative of pirprofen, the pyrrole, can be measured in freshly obtained plasma samples using the specific method reported previously[1]. However, it could be shown that the pyrrole analogue is also easily formed by oxidation during ordinary sample manipulation. Thus, it seems obvious to convert all pirprofen into its degradation product before analysis. Comparison between the two methods has shown good agreement. The method described here is simple, rapid and suitable for the use in combination with an automatic sampling device.

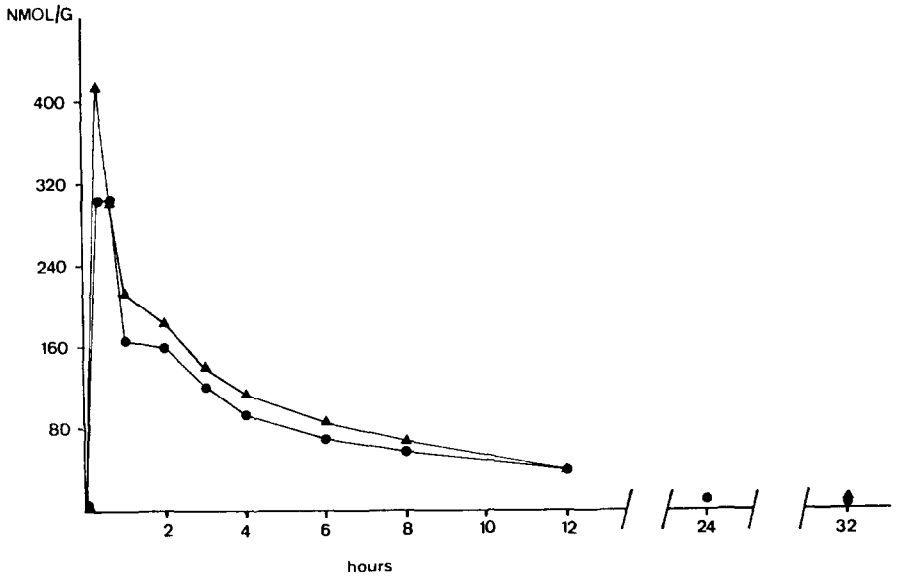


Fig. 11. Comparison of the plasma levels, measured after a single oral dose of 400 mg of pirprofen in solution, by the NPD procedure and by a previously reported method[1] (sum of pirprofen and the pyrrole analogue). ▲, Flash-methylation procedure; ●, NPD procedure.

ACKNOWLEDGEMENTS

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