Journal of Chromatography, 530 (1990) 377–385
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5370

Determination of ximoprofen and its metabolites in human urine by high-performance liquid chromatography with ultraviolet absorbance detection

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(First received February 12th, 1990; revised manuscript received April 28th, 1990)

ABSTRACT

A simple, sensitive and selective method for the determination of ximoprofen and its keto and hydroxy metabolites in human urine has been developed using high-performance liquid chromatography in the reversed-phase mode. The limit of reliable determination of ximoprofen and each of its metabolites in urine is about 1 μg/ml (4 nmol/ml). The method has been applied to urine samples obtained from human volunteers after administration of single intravenous doses of 30 mg of ximoprofen and about 70% dose was accounted for in terms of these compounds and their glucuronic acid conjungates.

INTRODUCTION

Ximoprofen, 2-[4-(3-oximinocyclohexyl)phenyl] propionic acid (Fig. 1) [1], is a new non-steroidal anti-inflammatory agent which belongs to the arylpropionic acid class of anti-inflammatory drugs [2]. Ximoprofen is a potent member of this class of drugs as dosages of 15 mg twice daily have been shown to be clinically effective [3,4]. Ximoprofen is biotransformed mainly by hydrolysis of the oxime group to the keto derivative (keto analogue) which is then reduced to the hydroxy derivative (hydroxy analogue). All three compounds are conjugated with glucuronic acid via the carboxyl function. The keto analogue could also be derived by chemical degradation since ximoprofen is unstable under acid conditions.

A method for the determination of ximoprofen in plasma by gas chromatography has previously been published [5]. This paper describes a rapid, sensitive and selective assay for ximoprofen and its keto and hydroxy analogues in urine using high-performance liquid chromatography (HPLC) in the reversed-phase mode. The assay may be used for quantitation (by difference) of the glucuronic acid conjugates of these compounds after hydrolysis by β-glucuronidase.

0378-4347/90/$03.50

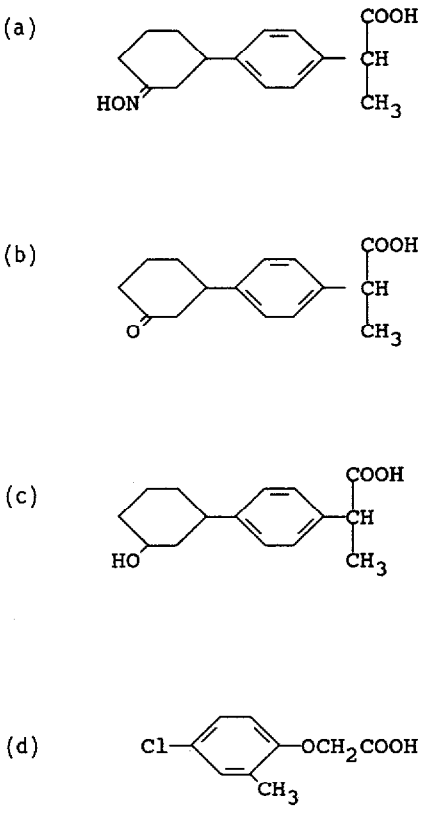

Fig. 1. Structures of (a) ximoprofen, (b) the keto analogue, (c) the hydroxy analogue and (d) 4-chloro-2-methylphenoxyacetic acid (internal standard).

EXPERIMENTAL

Materials

Methyl *tert.*-butyl ether (MTBE) and acetonitrile (far UV grade) were HPLC grade. All other reagents were of analytical grade and all inorganic reagents were prepared in freshly glass-distilled water. Citrate buffers (0.05 *M*, pH 5 and 1 *M*, pH 6.8) were prepared from appropriate amounts of citric acid and sodium hydroxide dissolved in distilled water; the final pH of each solution was adjusted with hydrochloric acid (1 *M*) or sodium hydroxide (1 *M*), as appropriate. Dissolution of appropriate amounts of disodium hydrogen orthophosphate in distilled water yielded a solution (0.05 *M*, pH *ca.* 9.1) from which phosphate buffer (0.05 *M*, pH 7.4) was prepared by pH adjustment with phosphoric acid. β-Glucuronidase, derived from *Escherichia coli*, was suspended (2 mg/ml) in citrate buffer (1 *M*, pH 6.8). Standard solutions of ximoprofen, and its keto and hydroxy analogues (analytical standards provided by Laboratoires Jacques Logeais, Paris,

France) were each prepared individually in methanol at concentrations of 10, 100 and 1000 μg/ml; a stock solution of the internal standard, 4-chloro-2-methylphenoxyacetic acid (MCPA; Riedel-de Haën, Seelze, F.R.G.), was prepared in methanol at a concentration of 10 μg/ml.

Sample preparation procedure

Extraction procedure. Samples (0.1 ml) of urine were transferred to screw-capped extraction tubes, and internal standard (1.5 μg) was added followed by MTBE (5 ml) and citrate buffer (1 ml, 0.05 *M*, pH 5) in that order (to achieve prompt extraction into the organic phase, thereby minimising any chemical degradation of ximoprofen to its keto analogue). The contents of the tubes were mixed on a rotary mixer (50 cpm, 10 min), then centrifuged (*ca.* 1600 *g*, 10 min) at room temperature. The organic phase was transferred to a second extraction tube containing disodium hydrogen orthophosphate (1 ml, 0.05 *M* pH 9.1). The tube contents were rotary-mixed (50 cpm, 10 min), centrifuged (*ca.* 1600 *g*, 10 min) and the organic phase was discarded. MTBE (5 ml) was added and the pH of the aqueous phase adjusted to *ca.* 5 by addition of phosphoric acid (0.15 ml, 1 *M*). The contents of the tube were again mixed (50 cpm, 10 min) and centrifuged (*ca.* 1600 *g*, 10 min) at room temperature. The organic phase was transferred to a tapered glass centrifuge tube and evaporated to dryness under a stream of dry nitrogen at *ca.* 40°C. The residue was reconstituted in mobile phase (0.1 ml) and aliquots (40 μl) were subjected to liquid chromatography.

Enzymic hydrolysis. Urine samples (0.1 ml) were transferred to screw-capped extraction tubes and incubated (37°C, 3 h) with β-glucuronidase suspension (0.1 ml, 2 mg/ml, pH 6.8). At the end of the incubation period, internal standard was added to the samples, which were then taken through the extraction procedure previously described.

Calibration procedure

Aliquots of control human urine (pH adjusted to 7.0, to prevent degradation of ximoprofen to its keto analogue) were spiked with amounts of each of ximoprofen and its keto and hydroxy analogues to give concentrations of each equivalent to 0, 1, 2.5, 5, 7.5, 10, 20 and 30 μg/ml. The "bulk" standards were mixed to ensure adequate dispersal of the compounds, then sub-sampled (0.1-ml aliquots) and stored at *ca.* −80°C until required for analysis, at which time they were thawed and submitted to the extraction procedure described previously.

Instrumentation

The liquid chromatograph consisted of a Spectra-Physics SP 8770 pump (Spectra-Physics, St. Albans, U.K.) coupled to a Spectro Monitor® D UV absorbance detector (LDC Analytical, Stone, U.K.) operated at a wavelength of 220 nm. Injection was via a WISP™ 710B autosampler [Millipore (U.K.), Watford, U.K.]. Chromatograms were recorded on a Trilab 3000 computing integrator

(Trivector International, Sandy, U.K.) which automatically calculated peak heights and peak-height ratios.

Chromatography

The column used for the analysis was constructed of stainless steel (12.5 cm × 0.49 cm I.D.) and packed with Spherisorb 5-ODS2 (mean particle diameter 5 μm) (Hichrom, Reading, U.K.). A pre-column (5 cm × 0.2 cm I.D.) dry-packed with Pellicular ODS (particle diameter 37–53 μm) (Whatman, Maidstone, U.K.) was installed in front of the analytical column to protect it from contamination. Chromatography was performed in the reversed-phase mode, using a mobile phase of phosphate buffer (pH 7.4)–acetonitrile (84:16, v/v). The mobile phase was filtered through glass fibre paper under vacuum prior to use and was passed through the column at a flow-rate of 1.2 ml/min.

Under the conditions described, ximoprofen chromatographed as two peaks, presumably corresponding to its oxime geometric isomers, with retention times of *ca.* 4.2 min and *ca.* 6.4 min (Fig. 2). For the purposes of measurement of xi-

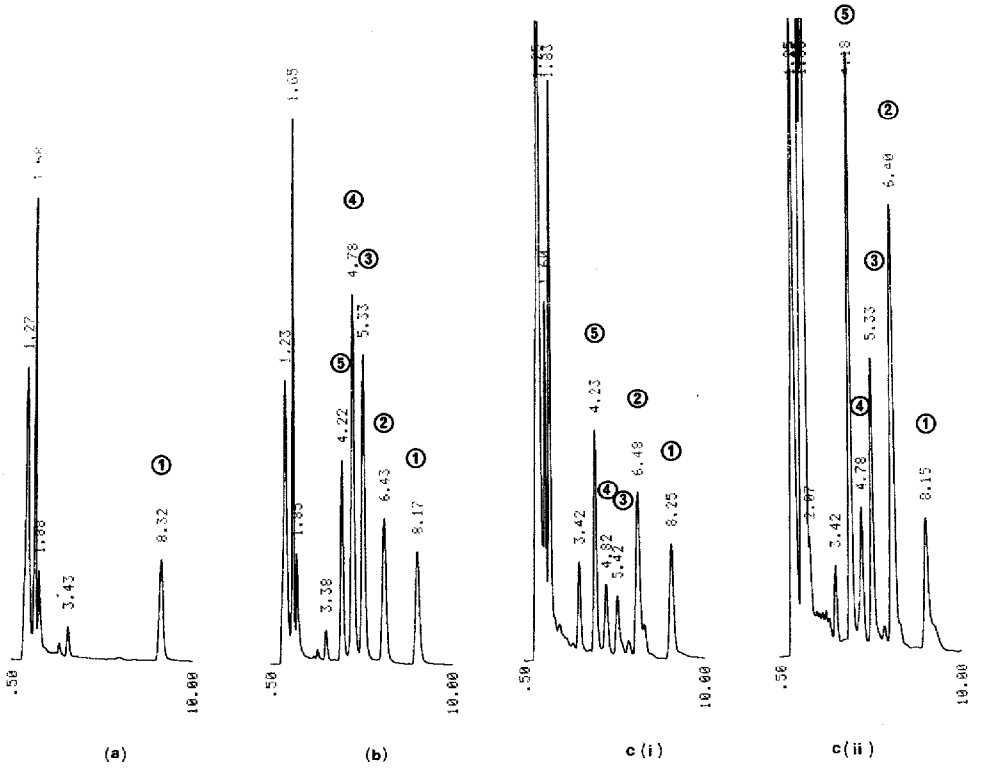

Fig. 2. Chromatograms of (a) control urine plus internal standard, (b) urine calibration standard (10 μg/ml), (c) post-dose urine, (i) prior to enzymic hydrolysis and (ii) post-enzymic hydrolysis. Peaks: 1 = internal standard; 2 and 5 = ximoprofen geometric isomers; 3 = keto analogue; 4 = hydroxy analogue. Conditions for chromatography as in text.

moprofen concentrations, the peak heights were summed together: there was no evidence that the relative proportions of ximoprofen geometric isomers excreted in urine were different to that of the analytical standard. The keto and hydroxy analogues of ximoprofen and the internal standard each chromatographed as single peaks with retention times of *ca.* 5.3, 4.8 and 8.2 min, respectively (Fig. 2).

Studies in human subjects

The method of analysis was applied to urine samples obtained after ximoprofen was administered to two healthy adult males under conditions described elsewhere [6]. Subjects were fasted for 8 h prior to drug administration and for 4 h afterwards: water was available *ad libitum* throughout this period. Ximoprofen (30 mg) was administered as an intravenous infusion in isotonic saline (2 mg/min, 15-min infusion). Urine was collected during 12 h before dosing and at 0–3, 3–6, 6–12, 12–24 and 24–36 h thereafter. Upon collection, the volume and pH of the urine were recorded and sub-samples (20 ml) were frozen immediately; the pH of the remaining urine was adjusted to 7 and sub-samples were again frozen immediately. All urine samples were stored at *ca.* −80°C until required for analysis, at which time they were thawed, sub-sampled and taken through (i) the extraction procedure (for determination of unconjugated drug-related material) and (ii) the enzymic hydrolysis and extraction procedure [for determination of total (unconjugated plus enzyme-hydrolysed) drug-related material], with the minimum sample handling time. Samples containing ximoprofen at concentrations in excess of the highest calibration standard were suitably diluted with distilled water prior to sub-sampling and reanalysis.

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each of three concentrations over the calibration range was repeated on six occasions. The within-day precision of the assay for the measurement of ximoprofen, as determined by the coefficients of variation of replicate concentration measurements at concentrations of 1, 10 and 30 μg/ml, was <1, ± 10 and $\pm 3\%$, that of its keto analogue at the same concentrations was ± 7, ± 12 and $\pm 5\%$, and that of the hydroxy analogue ± 5, ± 10 and $\pm 3\%$ (Table I).

Linearity and accuracy of measurement

Daily calibration lines for the measurement of ximoprofen and its keto and hydroxy analogues in urine were each constructed over the concentration range 0–30 μg/ml. Plots of peak-height ratio against concentration, for each of the three compounds, were linear ($y = a + bx$, where y is the peak-height ratio and x is the analyte concentration) over the concentration range 0–30 μg/ml; however, in the case of ximoprofen, greater accuracy of measurement was achieved by use of low

(0–10 μg/ml) and high (0–30 μg/ml) range calibration lines using unweighted data; peak-height ratios of ximoprofen were determined by summation of the peak-height ratios of the individual isomers. The accuracy of measurement as the mean percentage deviation from the expected value was ± 10% or less over the calibration range for each compound (Table I).

TABLE I

WITHIN-DAY PRECISION MEASUREMENTS OF XIMOPROFEN AND ITS KETO AND HYDROXY ANALOGUES IN URINE

Compound	Concentration added (μg/ml)	Concentration determined (mean ± S.D., $n=6$) (μg/ml)		Coefficient of variation (%)	Mean relative error of measurement (%)
Ximoprofen	1	1.1	–	–	+10.0
	10	10.4	1.0	10	+ 4.0
	30	30.0	0.8	3	0
Keto analogue	1	1.0	0.07	7	0
	10	10.1	1.2	12	+ 1.0
	30	29.5	1.5	5	− 1.7
Hydroxy analogue	1	1.1	0.05	5	+10.0
	10	10.2	1.0	10	+ 2.0
	30	30.0	1.0	3	0

Recovery

The recoveries of ximoprofen and its keto and hydroxy analogues from urine (each at a concentration of 10 μg/ml) and of internal standard (at a concentration of 15 μg/ml) were determined by comparison of peak-height ratio measurements of standards taken through the extraction procedure with those of equivalent standards injected into the chromatograph without prior extraction. The mean (± S.D.) recovery of ximoprofen from urine at a concentration of 10 μg/ml was 72 ± 3% ($n=6$), that of the keto analogue (10 μg/ml) was 95 ± 4% ($n=6$) and that of the hydroxy analogue (10 μg/ml) was 80 ± 2% ($n=6$). The mean (± S.D.) recovery of internal standard, at a concentration of 15 μg/ml, was 80 ± 3% ($n=6$).

Selectivity and limits of reliable determination

No peaks with retention times similar to those of ximoprofen, its keto or hydroxy analogues or internal standard were present in extracts of pre-dose (control) urine. No peak with the same retention time as the internal standard was present in extracts of post-dose urine analysed without the addition of internal standard, indicating the absence of metabolite interference with the internal standard used.

Ximoprofen, and its keto and hydroxy analogues could be adequately detected at concentrations of 0.2 μg/ml in standards prepared in control human urine, *i.e.* for each compound the signal-to-background interference ratio exceeded 2:1 at this concentration. The amount of interfering endogenous material varied considerably in post-dose (test) urines but was generally higher than that in the control urine used for calibration purposes; the degree of interference increased after enzymic hydrolysis. The limit of reliable determination of each compound in urine was therefore set at 1 μg/ml, in each case the lowest datum point on the calibration line. Representative chromatograms of ximoprofen and its metabolites in urine are illustrated in Fig. 2.

Studies in human subjects

The analytical method described was successfully applied to the analysis of urine samples generated after administration of single intravenous doses of 30 mg ximoprofen to two healthy male volunteers. During 36 h after dose administration, *ca.* 10–15% of the dose was excreted as unconjugated ximoprofen, *ca.* 30–45% was excreted as conjugated ximoprofen, *ca.* 10–15% of the dose as the conjugated keto analogue, but less than 5% as the unconjugated keto or as the conjugated or unconjugated hydroxy analogue. 70–80% of the dose was accounted for as these drug-related compounds in urine (Table II) which were almost completely excreted in the first 12 h after dosing.

Ximoprofen in control urine was stable at pH 7 (24 h, 37°C), but underwent degradation to its keto analogue at pH 5 (half-life at 37°C *ca.* 2.5 h). Storage of post-dose urines at *ca.* −80°C, together with minimisation of sample handling times during the present study, appeared to have successfully limited the degradation of ximoprofen to its keto analogue; differences in the measured urinary

TABLE II

EXCRETION OF UNCONJUGATED AND TOTAL (UNCONJUGATED PLUS ENZYME-HYDROLYSED) XIMOPROFEN AND ITS KETO AND HYDROXY ANALOGUES DURING 36 h AFTER ADMINISTRATION OF SINGLE INTRAVENOUS INFUSION DOSES OF 30 mg XIMOPROFEN

Compound	Dose recovered in urine (%)					
	Unconjugated		Conjugated		Total	
	Subject 1	Subject 2	Subject 1	Subject 2	Subject 1	Subject 2
Ximoprofen	10.4	13.5	44.2	33.4	54.6	46.9
Keto analogue	3.2	4.7	17.4	12.1	20.6	16.8
Hydroxy analogue	1.3	1.9	3.8	4.1	5.1	6.0
Total	14.9	20.2	65.4	49.4	80.3	69.6

TABLE III

CONCENTRATIONS OF UNCONJUGATED AND TOTAL (UNCONJUGATED PLUS ENZYME-HYDROLYSED) XIMOPROFEN AND ITS KETO AND HYDROXYANALOGUES EXCRETED IN THE URINE OF TWO HUMAN VOLUNTEERS AFTER ADMINISTRATION OF SINGLE INTRAVENOUS INFUSION DOSES OF 30 mg XIMOPROFEN

NS = No sample voided; ND = not detected (< 1 μg/ml); U = pH unadjusted.

Time (h)	Urine concentration (μg/ml)							
	Unconjugated				Total			
	Subject 1		Subject 2		Subject 1		Subject 2	
	U	pH 7	U	pH 7	U	pH 7	U	pH 7
Ximoprofen								
0–3	NS	NS	NS	NS	NS	NS	NS	NS
3–6	19	19	28	27	99	94	94	81
6–12	2	2	2	2	5	6	9	8
12–24	ND	ND	ND	ND	1	ND	ND	ND
24–36	ND	ND	ND	ND	ND	ND	ND	ND
Keto analogue								
0–3	NS	NS	NS	NS	NS	NS	NS	NS
3–6	6	6	10	11	39	34	27	19
6–12	ND	ND	ND	ND	1	2	11	7
12–24	ND	ND	ND	ND	ND	ND	ND	ND
24–36	ND	ND	ND	ND	ND	ND	ND	ND
Hydroxy analogue								
0–3	NS	NS	NS	NS	NS	NS	NS	NS
3–6	3	3	4	4	7	7	7	7
6–12	ND	ND	ND	ND	2	2	5	4
12–24	ND	ND	ND	ND	ND	ND	ND	ND
24–36	ND	ND	ND	ND	ND	ND	ND	ND

concentrations of unconjugated ximoprofen and the keto analogue between samples stored and analysed at the pH at which they were voided (pH range 5.4–7.0) and those "stabilised" at pH 7 were found to be negligible (Table III).

Glucuronic acid conjugates of other propionic acid non-steroidal anti-inflammatory drugs have been reported to be unstable at body temperature, rapidly liberating the aglycone; they may also be subject to intramolecular rearrangements at pH values of 7 or greater, which render them resistant to enzymic hydrolysis [7]. Although no reference standards of the glucuronic acid conjugates of ximoprofen or its metabolites were available during the course of the present study, excretion of much of the urinary drug-related compounds as conjugated material precludes significant instability of these compounds under the handling

conditions used; values obtained would represent limiting minima for the "true" amounts excreted in urine (Table II). Measured concentrations of total (unconjugated plus enzyme-hydrolysed) drug-related components in urine stored at pH 7 were only slightly lower than those measured in urine stored at the voided pH, indicating that rearrangement of the conjugates to hydrolysis-resistant forms was also minimal.

The analytical method described was suitable for the assay of samples subjected to strong acid (5 *M* hydrochloric acid, 3 h, 100°C) or strong alkaline (5 *M* sodium hydroxide, 3 h, 100°C) hydrolysis; *i.e.* after neutralisation, samples could be successfully subjected to the extraction and chromatographic procedures described, without additional interference with the assay. However, recovery of drug-related material from standards was reduced by the hydrolysis procedures (*ca.* 70% recovery using strong acid hydrolysis and *ca.* 50% recovery using strong alkaline hydrolysis): thus enzyme hydrolysis was considered the technique of choice for estimation of drug-related conjugates of ximoprofen and its metabolites in urine.

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