Journal of Chromatography, 317 (1984) 483–492
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 433

DETERMINATION OF TENOXICAM, AND THE ISOLATION, IDENTIFICATION AND DETERMINATION OF Ro 17-6661, ITS MAJOR METABOLITE, IN HUMAN URINE

DENNIS DELL*, RAYMOND JOLY, WALTER MEISTER, WOLF ARNOLD, CHARLES PARTOS and BEATRIX GULDIMANN
Biological-Pharmaceutical Research Department, F. Hoffmann-La Roche & Co. Ltd., Basle (Switzerland)

SUMMARY

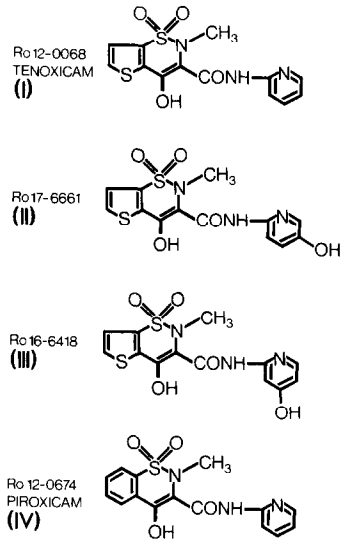
A high-performance liquid chromatographic method for the determination of tenoxicam (Ro 12-0068, I) and the hydroxy metabolite Ro 17-6661 (II) in human urine has been developed. The parent drug and metabolite were extracted from acidified urine by means of an Extrelut column with chloroform. The evaporated eluate was analysed on a C_{18} reversed-phase column with methanol–phosphate buffer as the mobile phase and UV detection at 371 nm. The detection limit for both compounds in a 1-ml sample was 50 ng/ml (C.V. 7%). The inter- and intra-assay precision up to 20 μg/ml was 3–6%. The method was applied to the analysis of I and II in the urine of a human subject who had received a 40-mg oral dose of the drug. Approximately 36% of the dose was eliminated in the urine as II, and less than 0.5% as unchanged I. After enzymatic hydrolysis of the urine, an extra 2% of the dose was found as II. Compound II was isolated from human urine by preparative thin-layer chromatography and identified by comparison of its mass and proton resonance spectra with those of an authentic specimen.

INTRODUCTION

Tenoxicam (Ro 12-0068, I) is a non-steroidal anti-inflammatory compound currently undergoing clinical trials. The major metabolites in dog urine, namely the 5′-hydroxy derivative (Ro 17-6661, II) and the thienoisothiazole, thienosaccharine (Ro 10-6559), have been isolated and identified[1]. In the rat, the major urinary metabolite is the acid resulting from cleavage of the amide bond of the N-methyl derivative of thienosaccharine (Ro 17-3034[2]).

Because of the long half-life of I in man (*ca.* 70 h), a conventional balance study with ^{14}C-labelled material is impracticable. It was considered necessary, therefore, to attempt to obtain a balance from data on urinary excretion. Since negligible amounts of I are found in human urine[3], it was necessary to identify the major metabolite and then develop an analytical method for its determination. Previous work[4] indicated that the hydroxypyridyl compound II was the major urinary metabolite in man.

0021-9673/84/$03.00



It was decided to develop a high-performance liquid chromatographic (HPLC) method for the analysis of I, II and any possible conjugates of these two compounds in urine. The method described is based upon the reversed-phase HPLC procedure for the determination of I in plasma first described by Pickup *et al.*[5], but with significant improvements in the sample preparation step. As part of the validation of this method, a healthy male volunteer took a single 40-mg dose of I and collected his urine over a period of 16 days in two daily fractions. This urine was then analysed for I and II both before and after enzymatic hydrolysis. The same urine was used for the isolation of identification of this metabolite.

EXPERIMENTAL

Reagents, solvents, and materials

Chloroform, Titrisol buffer, sodium hydroxide, potassium dihydrogen phosphate and methanol were all p.a. grade from E. Merck (Darmstadt, F.R.G.) and were used without further purification. Twice distilled water was used for the preparation of all standard solutions and buffer solutions. Extrelut cartridges were obtained from E. Merck; Art. No. 11,737 (capacity 20 ml) was used for the isolation of the metabolite, and Art. No. 15,371 (capacity 1 ml) for the quantitation. Suc d'Helix pomatia stabilized glucuronidase (10^5 Fishman units) and sulphatase (10^6 Roy units) was obtained from Pharmindustrie (Villeneuve La Garenne, France).

Standard solutions and urine standards

Because of instability of I to light, all solutions were prepared in brown glass vessels. A stock solution of 10 mg of I and II in 100 ml methanol was prepared; this solution was then used to prepare urine standards in the concentration range 50–5000 ng/ml according to the dilution procedure in Table I. Both the stock solution and the urine standards were stable for at least 2 weeks at 0–4°C. The internal stan-

TABLE I

PREPARATION OF URINE STANDARDS FOR CALIBRATION

The stock solution (A) contained 10 mg of I and II per 100 ml methanol. Solution B was obtained by diluting 2.5 ml of A to 50 ml in control urine.

Solution B (ml)	*Control urine added (ml)*	*Concentration of I and II (ng/ml)*
8	12	2000
4	16	1000
2	18	500
1	24	200
0.5	49.5	50

dard (piroxicam, IV) was dissolved in methanol (10 mg per 100 ml). A 10-ml volume of this stock solution was diluted to 200 ml in Titrisol buffer (pH 4), and a further 2 ml were diluted to the same volume to give two solutions, containing 5000 and 1000 ng/ml of IV, respectively. The former solution was used for urine concentrations of II of more than 1000 ng/ml, and the latter for concentrations of less than 1000 ng/ml.

Sample preparation procedure

(a) *Free drug and metabolite.* Urine (1 ml) was mixed with Titrisol buffer pH 4 (1 ml) containing the appropriate quantity of internal standard (see above). An aliquot (1 ml) of this solution was applied to an Extrelut 1 column (capacity 1 ml of aqueous solution) and, after 10 min, chloroform (2 × 4 ml) was passed through the cartridge. The organic phase was evaporated to dryness, and the residue was taken up in the mobile phase (200 μl). A 175-μl volume of this solution was injected into the HPLC column.

(b) *Glucuronides of I and II.* Urine (5 ml) was adjusted to pH 4 with acetate buffer, and Suc d'Helix pomatia extract (50 μl) was added. After incubation at 37°C for 2 h, a further 50 μl of the enzyme preparation were added and the incubation continued overnight (18 h). After allowing the mixture to cool to room temperature, a 1-ml portion was taken and extracted as described in (a).

Chromatographic procedure

The HPLC system comprised an Altex 100 dual-piston pump (Beckman Instruments, Berkeley, CA, U.S.A.), Uvicon 725 UV detector (Kontron, Zürich, Switzerland), Rheodyne 7120 valve injector (Kontron), Spectra-Physics SP 4100 computing integrator (Spectra-Physics, Basle, Switzerland) and a μBondapak C_{18} column, 10 μm, 300 × 3.9 mm (Waters Associates, Brechbühler, Zürich, Switzerland). The mobile phase was of a 3:2 (v/v) mixture of phosphate buffer (0.1 *M*, pH 7.4) and methanol. The operating conditions were as follows: flow-rate, 1.1 ml/min; detection wavelength, 371 nm; volume injected (fixed loop), 175 μl. Under these conditions the retention times (minutes) were: II, 5.4; I, 7.9; IV, 10.8 (Fig. 1a).

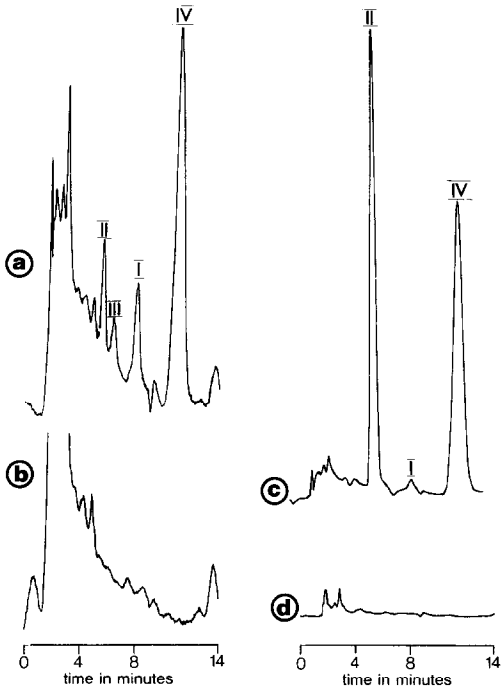


Fig. 1. Chromatograms of (a) urine sample containing 100 ng/ml each of I, II and III, and 1000 ng/ml IV; (b) corresponding blank urine; (c) 8–24-h urine from a human subject following a 40 mg oral dose of I; the peaks correspond to 3.2 μg/ml and 100 ng/ml of II and I respectively; (d) corresponding blank urine.

Calibration and calculation

Together with the unknown samples, the corresponding calibration samples, each consisting of five urine standards containing appropriate concentrations of the two compounds to be analysed, were processed as described above. The calibration curves were obtained by least squares regression of the peak height ratios I/IV and II/IV against the respective concentrations. These curves were then used to interpolate the concentrations of the parent compound and metabolite from the respective peak height ratios obtained from the unknown samples. All data processing and calculations were carried out by the Spectra-Physics computing integrator SP 4100 and Minifile 4100 D.

Isolation of the major metabolite from human urine

Pooled samples of urine from the study described above were concentrated and extracted as follows. A 20-ml volume of concentrated urine was adjusted to pH 5 and applied to an Extrelut column. After allowing 10–15 min for the aqueous solution to be absorbed by the Kieselguhr, the metabolite was extracted from the aqueous phase by passing 40 ml of ethyl acetate through the column. The extraction solvent was then evaporated to dryness under vacuum.

Aliquots of the collected urines or extracts thereof were applied to thin-layer chromatographic (TLC) plates together with the corresponding reference materials.

For all analytical thin-layer chromatograms, either precoated thick layer silica gel plates (Merck silica gel F 254, thickness 2 mm), or Merck silica gel 60 for column chromatography, particle size, 0.063–0.200 mm (70–230 mesh ASTM), were chosen. The eluting solvents were as follows (volume proportions): (1) chloroform–acetone–methanol–acetic acid (70:20:10:2); (2) chloroform–acetone–methanol–acetic acid (70:10:20:2); (3) chloroform–acetone–methanol–acetic acid (50:10:40:2); (4) chloroform–methanol–acetic acid (90:5:5).

The metabolic zone, with an R_F value corresponding to the 5′-hydroxy derivative previously identified in animal urines, was scraped off and eluted with the appropriate solvents (ethyl acetate and ethyl acetate–methanol, 1:1). The structural identification was carried out on the basis of the chromatographic behaviour, mass spectral fragmentation pattern and the proton resonance spectral data.

Mass spectrometry

Mass spectra were obtained on a Micro-Mass 7070/F instrument, equipped with a DS 2050 data system (Vacuum Generators Ltd., Altrincham, U.K.) by using direct introduction, ionization energy 70 eV and an ion source temperature of 250°C.

Nuclear magnetic resonance spectrometry

The ^{1}H NMR spectra were recorded with a Fourier transform spectrometer (Bruker-Spectrospin) at 400 or 270 MHz.

RESULTS AND DISCUSSION

Sample preparation and recovery

Recent experience has shown that the use of Extrelut columns for sample extraction can offer certain important advantages in comparison to the conventional procedure in which the aqueous phase is mixed with the organic phase by means of a mechanical shaking device. These advantages include time saving, improved extraction efficiency and convenience[6,7].

In this method, several solvents were investigated in conjunction with Extrelut, including methylene chloride, *n*-butyl chloride, isopropyl acetate, ethyl acetate and chloroform. The latter was chosen because it gave the most efficient extraction and least contamination with endogenous interfering components. The conventional extraction method was compared with the Extrelut method by using the same volume of isopropyl acetate in both. The recovery by Extrelut was 98%, compared with 80–85% for extraction by tumbling (head-over-head extractor). In addition, the Extrelut method was quicker, saving about 1 h in the processing of sixteen samples.

Using the method described and a concentration of 1 μg/ml for both I and II, the recovery was investigated over the range pH 1–11. The results are presented in Fig. 2. While I could be extracted over a wide pH range (pH 1–9, pK_a 1.09 and 5.27), II showed a maximum recovery at pH 1 and 4. The sharp drop in extractibility of II above pH 5 is presumably due to the greater acidity conferred on this metabolite by the hydroxyl group in the pyridine ring. From these data, pH 4 was chosen for the routine extraction of both substances.

The recovery of the two substances was established as follows. Compounds I and II were added to control urine to give samples in the concentration range 500–

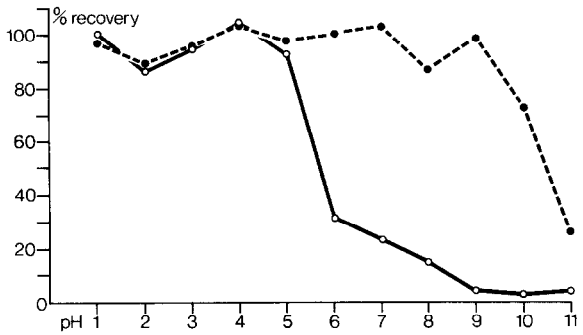


Fig. 2. Recovery of I (●---●) and II (○—○) from urine over the range pH 1–11.

5000 ng/ml. These samples were processed as described above, each concentration in duplicate, except that the internal standard was not added. While these samples were analysed, another series was prepared in mobile phase over the same concentration range, which was analysed directly. The peak heights obtained from this latter analysis provided the 100% values which could be compared with the corresponding peak heights obtained from the extracted samples, thus enabling the recovery to be calculated (Table II). The overall recoveries were 104 ± 9% and 97.8 ± 8% for I and II, respectively. The presence of other extractives can inhibit any possible glass adsorption of an analyte, thus giving rise to false recovery figures from the above experiment. This was checked as follows: two solutions containing 50 and 500 ng/ml of both substances were prepared in the mobile phase; a portion of each of these solutions was analysed directly by the HPLC method. An equal volume was also added to the evaporated extract of 1 ml of control urine and similarly analysed. Any glass adsorption is ruled out by the data in Table III which show that, within experimental error, the same responses were obtained for the pure solutions and the reconstituted extract solutions.

TABLE II

RECOVERY OF I AND II FROM HUMAN URINE

Concentration (ng/ml)	*I*		*II*	
	Recovery (%)	*C.V. (%) (n = 2)*	*Recovery (%)*	*C.V. (%) (n = 2)*
500	117	2.4	109	2.4
1000	105	2.6	95	2.2
2000	96	1.7	93	3.2
5000	100	1.3	94	1.5
Overall recovery	104	9	98	8

Selectivity

The chromatograms from extracted control urine (Fig. 1b and d) indicate the degree of specificity of the method with respect to endogenous substances. Three other possible metabolites, namely the 4′-hydroxy derivative of tenoxicam (III), the

TABLE III

POSSIBLE INFLUENCE OF GLASS ADSORPTION ON RECOVERY

	(i) I and II dissolved in mobile phase		*(ii) I and II dissolved in mobile phase and added to evaporated extracts of control urine*	
	50 ng/ml	*500 ng/ml*	*50 ng/ml*	*500 ng/ml*
I found* (ng/ml), $n = 2$	50.0 + 7	500 + 3	46.5 + 2	472 + 3
II found* (ng/ml)	50.0 + 12	500 + 0.3	53.3 + 15	507 + 4

* In the case of (i) the mean peak heights were taken as the "100%" values for comparison with (ii).

thienosaccharine Ro 10-6559 and the acid Ro 17-3034, were chromatographed under these conditions; III is separated from the peaks of interest (Fig. 1a), and the latter two compounds have no UV absorption at the wavelength used.

Linearity and limit of detection

A urine sample spiked with 50 ng/ml of I and II gave a signal-to-noise ratio of about 10:1 for both compounds when a 1-ml sample was used. At this concentration, an intra-assay precision (five replicate samples analysed on the same day) of R.S.D. = 6 and 7% for II and I, respectively, was obtained. According to the expected concentrations in the unknown urine samples, two calibration ranges were used, either 200–20,000 or 50–1000 ng/ml. Within both of these ranges, a linear correlation between peak height ratios and concentration was obtained.

Precision

The precision of this method for both substances was evaluated over the concentration range 200–5000 ng/ml. The overall intra-assay precision, determined by analysing each concentration five times on the same day, was found to be 2.8 and 5.5% for I and II, respectively. For the inter-assay precision, obtained by analysing the samples over a period of 5 days (using a separate calibration for each day), the corresponding figures were 3.5 and 3.9%. The precision at each concentration may be found in Table IV.

Stability

Solutions of both substances were prepared in control urine at concentrations of 200, 1000, 2000 and 5000 ng/ml in dark brown vessels. One portion of these urines was stored at room temperature for 24 h under normal laboratory lighting conditions and then analysed. The other portion was frozen, stored at −20°C for 3 months and then analysed. With each set of stored samples an equal number of freshly spiked samples were analysed to provide the 100% values. The procedure and subsequent calculations were carried out according to our established method[8]. The data from these stability determinations are presented in Tables V and VI, respectively. The room temperature data for II reflect, probably, more the relatively low precision of

TABLE IV

PRECISION (n = 5)

Concentration added (ng/ml)	*Intra-assay precision*			*Inter-assay precision*		
	Concn. found (ng/ml)	*C.V. (%)*	*Diff. between found and added concentrations (%)*	*Concn. found (ng/ml)*	*C.V. (%)*	*Diff. between found and added concentrations (%)*
I						
200	191.4	1.3	−4.3	203.3	7.0	+1.7
500	501.0	1.9	+0.2	502.4	3.7	+0.5
1000	1022	7.4	+2.2	986.6	2.9	−1.3
2000	2072	1.9	+3.6	2018	2.5	+0.9
5000	4970	1.7	−0.6	4970	1.1	−0.6
II						
200	197.0	5.1	−1.5	192.2	6.8	−4
500	480.4	3.0	−4.0	501.9	2.8	+0.4
1000	988.0	7.6	−1.2	984.0	5.1	−1.6
2000	2085	5.2	+4.3	2049	3.9	+2.5
5000	5048	8.3	+1.0	4980	0.9	−0.4

TABLE V

STABILITY OF I IN HUMAN URINE

Sample	*Concentration (ng/ml)*	*No. of replicates*	*Change of concentration after storage (%)*	*90% confidence interval (%)*
Control*	200	5		
24 h/25°C	199	5	−0.5	−7.5 to 7.8
3 months/−20°C	183	5	−8.4	−10.3 to −6.4
Control*	1000	5		
24h/25°C	989	5	−1.1	−3.0 to 0.9
3 months/−20°C	975	5	−2.3	−6.5 to 2.0
Control*	2000	5		
3 months/−20°C	2037	5	+1.8	−5.1 to 9.6
Control*	5000	5		
24 h/25°C	4887	5	−2.2	−4.7 to 0.3

* Freshly prepared.

these particular measurements rather than any serious instability. As far as I is concerned, it is recommended that urine samples kept at −20°C should not be stored longer than 3 months before being analysed.

Application

A healthy 46 year old male subject took two 20-mg tablets of I. Urine was collected during 0–8- and 8–24-h periods just before the dose and for 16 days after the dose. After measuring the volume and pH, the urine was stored at −20°C until required for analysis.

TABLE VI

STABILITY OF II IN HUMAN URINE

Sample	*Concentration (ng/ml)*	*No. of replicates (n)*	*Change of concentration after storage (%)*	*90% confidence interval (%)*
Control*	200	5		
24 h/25°C	204	5	+2.0	−3.7 to 8.4
3 months/−20°C	204	5	+2.0	−0.8 to 5.2
Control*	1000	5		
24 h/25°C	939	5	−6.1	−7.6 to −4.5
3 months/−20°C	992	5	−0.8	−3.5 to 2.0
Control*	2000	5		
3 months/−20°C	2013	5	+0.7	−5.0 to 7.0
Control*	5000	5		
24 h/25°C	4626	5	−7.4	−12.3 to −2.2

* Freshly prepared.

The samples were analysed both before and after enzymatic deconjugation. The data are presented in Fig. 3. About 36% of the dose was excreted in the urine as free II, and a further 2% could be attributed to a glucuronide of this substance. Not more than 0.5% was excreted as unchanged I. In Fig. 1c the chromatogram of the 8–24-h urine extract shows 100 ng/ml (0.2% of the dose) of I and 3.9 μg/ml (4.9% of the dose) of II.

Maintenance of the chromatographic system

After the injection of about 2000 urine extracts the column usually needed to be regenerated by flushing, in succession, with 75-ml portions of water, methanol, chloroform and finally methanol. After analysing a further 1000 urine extracts, a new column was required.

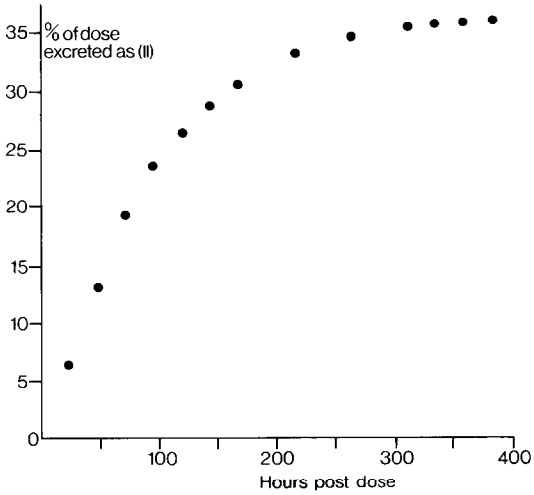


Fig. 3. Excretion of free II in urine following a 40-mg (oral) dose.

Isolation and identification of II from human urine after a single oral administration of I

From pooled human urine, a compound could be isolated by extraction and repetitive chromatography which was identical in its chromatographic behaviour [TLC (Table VII) and HPLC] with the previously identified 5'-hydroxy derivative of I. The mass spectrum showed characteristic peaks at *m/z* 353 (molecular ion), 137, 110, 94 and 64. This spectrum was compared with that of a synthetic sample, and the same peaks, including that corresponding to the molecular ion at *m/z* 353, were found in both spectra.

TABLE VII

THIN-LAYER CHROMATOGRAPHY OF I AND II

R_F values obtained on Merck silica gel plates, thickness 0.25 mm; UV detection at 254 nm. Solvent systems: A, chloroform–acetone–methanol–acetic acid (70:10:20:2, v/v/v/v); B, chloroform–acetone–methanol–acetic acid (50:10:40:2, v/v/v/v); C, chloroform–methanol–acetic acid (90:5:5, v/v/v).

	Solvent		
	A	*B*	*C*
I	0.71	0.75	0.67
II	0.64	0.74	0.47

The ^1H NMR spectrum of II isolated from human urine (Roche reference No. NMR 180,579; 20 μg in 0.27 ml [$^2H_6$]dimethyl sulphoxide) was very similar to that of the synthesized reference compound. In addition, a nearly equal amount of authentic II was added to the solution of isolated urinary metabolite and the resulting ^1H NMR spectrum indicated one compound only and not a mixture.

Full details of the NMR and mass spectra will be reported in ref. 1.

REFERENCES

1 R. Joly, W. Arnold and W. Meister, personal communication.
2 M. Tateishi, S. Ichihara, Y. Tsuyuki, H. Tomisawa, H. Fukazawa, N. Nakayama and R. Joly, *Xenobiotica*, submitted for publication.
3 R. Heintz, T. W. Güntert, J. F. Enrico, U. C. Dubach and F. S. Jeunet, *Eur. J. Rheum. Inflam.*, submitted for publication.
4 M. Tateishi, personal communication.
5 M. E. Pickup, J. R. Lowe and D. B. Galloway, *J. Chromatogr.*, 225 (1981) 493.
6 J. Breiter, R. Helger and H. Lang, *Forensic Sci.*, 7 (1976) 131.
7 E. G. Clarke, *Isolation and Identification of Drugs*, The Pharmaceutical Press, London, 1969.
8 U. Timm, M. Wall and D. Dell, *J. Pharm. Sci.*, submitted for publication.