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Note

Rapid and simple determination of the major metabolites of ibuprofen in biological fluids by high-performance liquid chromatography

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Ibuprofen, a non-steroidal anti-inflammatory agent, has been widely used in the treatment of rheumatoid arthritis, relief of pain from dysmenorrhea and for the treatment of fever. The metabolism of ibuprofen in several animal species and in man has been reported [1]. Following oral administration, ibuprofen is completely absorbed and metabolized to two main metabolites, 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid (OH-ibuprofen) and 2-[4-(2-carboxypropyl)phenyl]propionic acid (COOH-ibuprofen) and its conjugates. These two metabolites and their respective conjugates account for approximately 60% of the metabolic end-products of ibuprofen in rats and humans [1]. Methods presently available to measure ibuprofen and its major metabolites include paper chromatography [2], gas—liquid chromatography (GLC) [3, 4], GLC—mass spectrometry [5, 6] and high-performance liquid chromatography (HPLC) [7—10]. All of these methods, however, require extensive sample work-up. Presently, only one HPLC assay [10] is available for quantitating ibuprofen metabolite levels in urine and none in plasma. We report, for the first time, a selective and sensitive isocratic HPLC method for determining the two major metabolites of ibuprofen in urine and plasma that requires no extraction procedure.

EXPERIMENTAL

Reagents

Ibuprofen was donated by Upjohn (Kalamazoo, MI, U.S.A.). OH-Ibuprofen and COOH-ibuprofen were kindly supplied by Boots (Nottingham, U.K.). The

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internal standard, sodium pentobarbital, was obtained from Abbott Labs. (North Chicago, IL, U.S.A.). Acetonitrile and 85% phosphoric acid (both HPLC grade) were obtained from Fisher Scientific (Itasca, IL, U.S.A.).

Instrumentation

A Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a variable-wavelength ultraviolet detector (Spectromonitor III, Laboratory Data Control, Riviera Beach, FL, U.S.A.) set at 196 nm and a sensitivity of 0.2 a.u.f.s. for urine and 0.1 a.u.f.s. for plasma, an IBM octadecyl column (250 mm × 4.5 mm I.D., 5 μm particle size) and a Brownlee Labs. (Santa Clara, CA, U.S.A.) RP-18 precolumn (30 mm × 4.6 mm I.D., 10 μm particle size) were used at ambient temperature. The mobile phase was comprised of 300 ml acetonitrile, 700 ml Milli-Q water, 0.5 ml acetone and 0.5 ml phosphoric acid. The flow-rates were 1.3 ml/min for urine and 1.6 ml/min for plasma. The detector output was recorded on a dual-channel recorder (Linear Instruments, Irvine, CA, U.S.A.) set at a chart speed of 6 cm/h. Injections were made through a Rheodyne Model 7125 injection valve equipped with a 100-μl sample loop.

Analysis of urine samples

Urine samples were processed by transferring 5 μl urine into a 500-μl polypropylene microcentrifuge tube and 10 μl of a 178 mg/l sodium pentobarbital solution in acetonitrile. After the sample was vortex-mixed for 5 s, it was centrifuged for 2 min at 12 800 *g* using an Eppendorf microcentrifuge (Model 5412), and 5 μl of the clear solution were injected onto the column. In order to determine free and conjugated metabolites in urine, all samples were assayed twice. Free drug and metabolites were assayed as described above, while total metabolites (free + conjugated) were determined following alkaline hydrolysis. Hydrolysis was performed by incubating 1 ml urine with 0.5 ml of 1 *M* sodium hydroxide for 20 min at ambient temperature. Immediately following the hydrolysis procedure, 5 μl urine were assayed as described above.

Analysis of plasma samples

To assay plasma samples for ibuprofen metabolites, a procedure similar to that described above was used except that 20 μl of plasma was deproteinized with 40 μl of a 17.8 mg/l sodium pentobarbital solution in acetonitrile and 20 μl of the supernatant were injected onto the column.

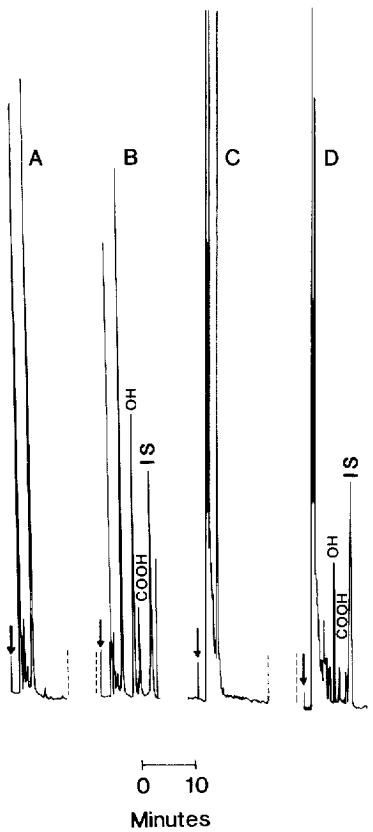
Calibration curves

Blank urine was spiked with OH-ibuprofen and COOH-ibuprofen in the ranges 10—200 mg/l and 5—100 mg/l, respectively. Blank plasma was spiked with OH-ibuprofen and COOH-ibuprofen in the range 5—50 mg/l. The urine and plasma samples were subjected to the preparation procedures described above and were chromatographed in the normal manner. Peak-height ratios of OH-ibuprofen and COOH-ibuprofen to the internal standard, pentobarbital, were used to construct the standard curves. All standard curves were calculated by a least-squares linear regression analysis of peak-height ratios versus drug concentration. The peak-height ratios of unknown samples were compared to the standard curve in urine or plasma.

RESULTS AND DISCUSSION

Fig. 1 includes chromatograms of blank urine and plasma and a typical urine (0—24 h) and plasma (30 min) sample from a rat given 20 mg/kg ibuprofen intravenously. No interfering peaks from endogenous compounds were observed when blank urine was subjected to the assay. The retention times of OH-ibuprofen, COOH-ibuprofen and pentobarbital were 8.7, 10.4 and 14.3 min in urine and 7.9, 9.1 and 12.5 min in plasma, respectively. Ibuprofen does not interfere with the assay.

The standard curves based on peak-height ratios of the metabolites to the internal standard (pentobarbital) were all linear and highly reproducible. The inter-day variability of the assay was assessed by performing nine standard curves using five different concentrations over a range of 5- 50 mg/l in plasma



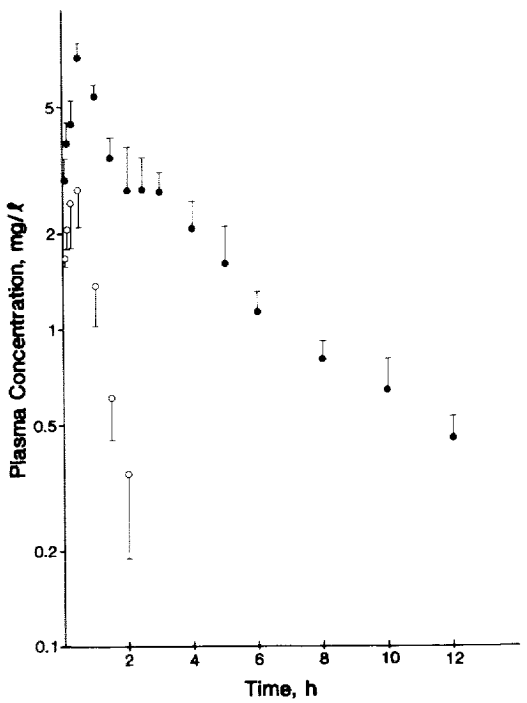


Fig. 1. Chromatograms of ibuprofen metabolites in urine and plasma. (A) Blank rat urine, showing no interfering peaks; (B) rat urine collected over 24 h following intravenous injection of 20 mg/kg ibuprofen; (C) blank rat plasma; (D) rat plasma 30 min following intravenous administration of 20 mg/kg ibuprofen. Arrow indicates when injection was made. Range = 0.2 a.u.f.s. for urine and 0.1 a.u.f.s. for plasma. Peaks: OH = OH-ibuprofen; COOH = COOH-ibuprofen; IS = pentobarbital, internal standard.

Fig. 2. Plasma concentration versus time curves of OH-ibuprofen (•) and COOH-ibuprofen (○) following 20 mg/kg ibuprofen administered intravenously to four male rats. Bars indicate standard deviation.

TABLE I

INTER-DAY VARIABILITY OF OH-IBUPROFEN AND COOH-IBUPROFEN IN PLASMA AND URINE

Biological fluid	Concentration (mg/l)	Coefficient of variation (%)	
		OH-Ibuprofen	COOH-Ibuprofen
Plasma	5.0	6.0	10.1
	10.0	1.9	3.8
	20.0	3.9	3.7
	40.0	0.6	2.0
Urine	10.0	3.7	7.7
	50.0	1.5	1.4
	100.0	3.2	2.8
	200.0	3.7	3.5

and 10—200 mg/l in urine made at different times over a period of two weeks. The inter-day variabilities of the assay were found to be 4.1 and 3% for OH-ibuprofen and 7.1 and 7.3 for COOH-ibuprofen in plasma and urine, respectively. The intra-day variability of the assay was determined by replicate ($n = 5$) determinations of four different concentrations. The intra-day variabilities of the assay in plasma (5—40 mg/l) and urine (10—200 mg/l) were found to range between 0.6 and 3.9% for OH-ibuprofen and between 1.4 and 10% for COOH-ibuprofen. These results are presented in Table I.

The accuracy of the method was determined in plasma and urine containing OH-ibuprofen and COOH-ibuprofen in concentrations of 5, 10 and 20 mg/l for plasma and 50, 75, 100 and 200 mg/l for urine, prepared by one person and assayed by another who had no knowledge of the concentrations. The relative accuracy of the method as applied to plasma and urine ranged from 1.4 to 8.3% and 1.9 to 8.0%, respectively. Analytical recoveries comparing spiked urine and plasma with aqueous standards over a concentration range of 10—100 mg/l and 5—40 mg/l, respectively, showed essentially complete recovery of both metabolites of ibuprofen.

The applicability of the assays reported herein has been demonstrated in rats given 20 mg/kg ibuprofen intravenously. Fig. 2 presents the mean plasma concentration—time curves for OH-ibuprofen and COOH-ibuprofen obtained from four rats. Urinary excretion data from the same study showed that 68 and 10% of the administered dose was excreted as unconjugated OH-ibuprofen and COOH-ibuprofen, respectively, while 71 and 10% could be accounted for as total (free + conjugated) ibuprofen metabolites. Thus, in the rat, there appears to be very little conjugation of the metabolites prior to urinary excretion. In addition to its applicability to biological fluids from the rat, the method described has been used to determine the concentrations of ibuprofen metabolites following a single 400-mg oral dose of ibuprofen to a normal human subject. These results are presented in Fig. 3. Essentially no interference from endogenous compounds has been observed in chromatograms from human urine and plasma.

Unlike the method reported previously [10] which utilizes gradient-elution

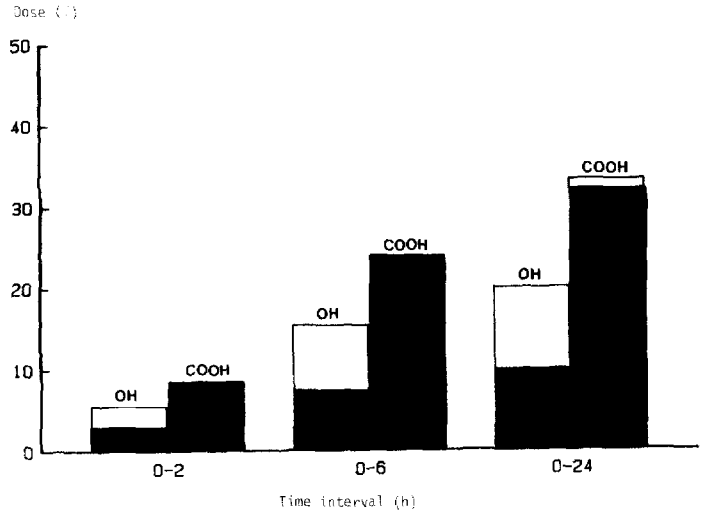


Fig. 3. Cumulative percentage of OH-ibuprofen and COOH-ibuprofen excreted in the urine after a 400-mg dose of ibuprofen to a single male subject. The larger bars represent the total percentage (free + conjugated) excreted in the urine collection interval. The smaller, black bars, represent the percentage of free metabolite excreted in the same interval.

HPLC and a complex methylene chloride extraction procedure, the isocratic HPLC method described herein requires only a simple dilution of the urine with acetonitrile and, thus, results in higher analytical recoveries of the metabolites and less inter- and intra-day variation. In addition, the use of a variable-wavelength detector set at 196 nm, as compared to 220 nm, increases the sensitivity of the assay significantly (approximately four-fold) so that only 5 μl or less of urine are required to determine ibuprofen metabolites accurately to a practical limit of 1 mg/l in urine and 0.2 mg/l in plasma. As a result of the small volumes of urine injected onto the column, column performance and life is significantly prolonged. In addition, the method described here can also quantitate the metabolites of ibuprofen in as little as 10 μl of plasma.

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