Journal of Chromatography, 430 (1988) 93–101
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4218

DETERMINATION OF IBUPROFEN AND ITS MAJOR METABOLITES IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received February 29th, 1988)

SUMMARY

A sensitive and selective high-performance liquid chromatographic assay for free and total ibuprofen and its major metabolites in human urine is described. Urine is acidified, drug and metabolites are extracted into hexane–propanol, back-extracted into sodium bicarbonate, neutralized and chromatographed. Ibufenac (4-isobutylphenylacetic acid) and 2-phenylpropionic acid were employed as internal standards. The extraction efficiencies were 94–100% for all compounds. The two metabolites and their internal standard were separated using an isocratic chromatographic system, followed by an abrupt step gradient to a second eluent for separation of ibuprofen and its internal standard with a total run time of 18 min. Detection was by a fixed-wavelength detector (214 nm). Sample-to-sample and day-to-day reproducibility studies yielded coefficients of variability of less than 9% for all compounds. The sensitivity was sufficient to determine 2.5 μg/ml free ibuprofen in 100 μl urine.

INTRODUCTION

Ibuprofen, 2-(4-isobutylphenyl)propionic acid, is an orally administered, non-steroidal anti-inflammatory agent used extensively in the treatment of arthritis [1]. The metabolism of ibuprofen in several animal species and in human has been reported [2, 3]. Previous workers identified the two major metabolites of ibuprofen as 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid (OH-ibuprofen) and 2-[4-(2-carboxypropyl)phenyl]propionic acid (COOH-ibuprofen). Methods presently available to measure the major metabolites of ibuprofen include thin-layer chromatography [3], gas chromatography [3], gas chromatography–mass spectrometry (GC–MS) [4, 5] and high-performance liquid chromatography (HPLC) [6, 7].

We have developed an analytical method for the determination of free and total

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ibuprofen and its major metabolites in human urine. It is characterized by sample isolation with quantitative recovery of ibuprofen and metabolites, employment of suitable internal standards (4-isobutylphenylacetic acid and 2-phenylpropionic acid) and HPLC separation–UV detection.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of two Model 6000A pumps, a Model 660 solvent programmer, WISP-710B automatic sampler, RCM-100 radial compression module, analytical column (10 cm × 0.5 cm NOVA-PAK C_{18} of 4 μm particle diameter), and Model 441 fixed-wavelength detector (operated at 214 nm) purchased from Waters Assoc. (Milford, MA, U.S.A.). A precolumn (5 cm × 0.4 cm) packed with Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.) was inserted between the automatic sampler and analytical column. A Hewlett-Packard (Avondale, PA, U.S.A.) Model 3354C laboratory automation system was used for chromatographic peak identification based on relative retention time, peak area integration, peak height measurement, peak shape characterization, and calculations derived from those measurements.

Materials

Acetonitrile (UV grade), hexane (UV grade), and 2-propanol were purchased from Burdick and Jackson (Muskegon, MI, U.S.A.). Reagent-grade water was prepared by passage through a MILLI-Q reagent grade water system (Millipore, Bedford, MA, U.S.A.). Phosphoric acid (85%), sodium phosphate (monobasic), ammonium sulfate, sodium bicarbonate, and hydrochloric acid were purchased from Fisher Scientific (Cleveland, OH, U.S.A.).

Ibuprofen, OH-ibuprofen, COOH-ibuprofen, and ibufenac (4-isobutylphenylacetic acid) were supplied by Upjohn (Kalamazoo, MI, U.S.A). 2-Phenylpropionic acid was purchased from Aldrich (Milwaukee, WI, U.S.A.). Standard solutions were prepared in 2-propanol.

Sample preparation

Free ibuprofen and metabolites. To a 14 × 100 mm polypropylene tube was added 100 μl of urine, 25 μl of 4 *M* hydrochloric acid, 50 μl of saturated ammonium sulfate, 25 μl of ibufenac solution (50 μg/ml), 25 μl of 2-phenylpropionic acid solution (1000 μg/ml), 0.5 ml of 2-propanol, and 3 ml of hexane. The tube was vortexed for 10 s, centrifuged at 1200 *g* for 2 min and placed in a dry ice–ethanol bath for 15 min. The organic phase was poured into a second test tube to which 100 μl of 0.1 *M* sodium bicarbonate was added. This tube was vortexed, centrifuged at 1200 *g* for 5 min, and placed again in a dry ice–ethanol bath. This organic phase received a second 100 μl of 0.1 *M* sodium bicarbonate and the extraction was repeated. The resultant two frozen sodium bicarbonate fractions were thawed, combined, and neutralized with 20 μl of 1 *M* hydrochloric acid. A 50-μl aliquot of this extract was injected into the HPLC system. Quantification standards were generated by aliquoting appropriate volumes of ibuprofen, OH-ibuprofen, and

COOH-ibuprofen into tubes, evaporating the 2-propanol to dryness, and reconstituting in 100 μl drug-free urine. Standard curves were generated to accommodate the concentration ranges of ibuprofen, OH-ibuprofen, and COOH-ibuprofen found in experimental samples.

Total (free + conjugated) ibuprofen and metabolites. To a 14 × 100 mm polypropylene tube was added 100 μl of urine and 50 μl of 1 *M* sodium hydroxide. The specimen remained for 20 min at room temperature, was neutralized with 4 *M* hydrochloric acid (25 μl), and prepared by the procedure described above for free ibuprofen and metabolites. However, the concentrations of the internal standards were altered to reflect different constituent concentration: ibufenac concentration was changed to 500 μg/ml and 2-phenylpropionic acid concentration was changed to 1500 μg/ml.

Chromatography

The chromatographic procedure consisted of a step gradient. Eluent A was prepared by dissolving 1 ml concentrated phosphoric acid in 800 ml reagent-grade water. This was filtered through a 0.45-μm pore diameter membrane and added to 200 ml filtered acetonitrile with thorough mixing. Eluent B was prepared analogously; it contained 1 ml concentrated phosphoric acid, 34.5 g sodium phosphate (monobasic), 400 ml water, and 600 ml acetonitrile. At each injection, eluent A was being pumped through the HPLC system at a flow-rate of 2.5 ml/min. After 9 min, the solvent programmer changed the chromatographic eluent abruptly from 100% eluent A to 100% eluent B. After an additional 9 min, at the conclusion of the chromatographic run, there was an equilibration delay of 5 min where 100% eluent A was pumped to permit reequilibration of the HPLC system prior to the subsequent injection.

Quantification

Standard curves of OH-ibuprofen/2-phenylpropionic acid, COOH-ibuprofen/2-phenylpropionic acid, and ibuprofen/ibufenac peak-height ratios versus concentration were established daily. The ibuprofen, OH-ibuprofen, and COOH-ibuprofen concentrations in experimental samples were interpolated from a least-squares regression line through standard data points. All standards and experimental samples were analyzed in duplicate.

Recovery and reproducibility studies

The recovery of ibuprofen and its metabolites from the sample isolation procedure was demonstrated by external standardization. The procedure required 36 tubes. To each of tubes 1–6 were added 100 μl 2-phenylpropionic acid (100 μg/ml); tubes 7–12, 100 μl OH-ibuprofen (200 μg/ml); tubes 13–18, 100 μl COOH-ibuprofen (200 μg/ml); tubes 19–24, 100 μl ibuprofen (200 μg/ml); tubes 25–30, 100 μl ibufenac (200 μg/ml); and tubes 31–36, 100 μl 2-propanol. Each tube next received 100 μl drug-free urine and was subjected to the preparation procedure described under *Free ibuprofen and metabolites*. To the resulting sodium bicarbonate solutions were added external standards: tubes 1–6, 100 μl OH-ibuprofen; tubes 7–18, 100 μl 2-phenylpropionic acid; tubes 19–24, 100 μl ibufenac; tubes 25–

30, 100 μl ibuprofen; and tubes 31–36, 100 μl of these 5 solutions. To tubes 1–30, 400 μl of 2-propanol was added, and then to all tubes, 300 μl of sodium bicarbonate, 500 μl of eluent A and 50 μl of 1 *M* hydrochloric acid. Each sample was injected (50 μl) into the HPLC system and the peak-height ratios were determined. The ratio of the mean of the peak-height ratios from each group of tubes divided by the mean of the peak-height ratios of standards (tubes 31–36) × 100% expresses the recovery of, respectively, 2-phenylpropionic acid, OH-ibuprofen, COOH-ibuprofen, ibuprofen, and ibufenac.

A representative human urine specimen was analyzed in replicate groups of six on three separate days to determine the sample-to-sample and day-to-day reproducibility.

RESULTS AND DISCUSSION

This method for the determination of the major metabolites of ibuprofen has the following features: (1) sample size: the amount of urine/analysis is 100 μl; (2) sample isolation: we have developed an extraction–back extraction procedure with quantitative recovery resulting in minimal chromatographic background; (3) internal standards: we employed two internal standards, one for ibuprofen metabolites and the other for ibuprofen; (4) ibuprofen: in addition to isocratic determination of the two major metabolites of ibuprofen, by changing the eluent, ibuprofen could also be quantified; (5) precision and sensitivity: studies were undertaken to document the sample-to-sample and day-to-day precision; this method possesses sufficient sensitivity to permit quantification of free ibuprofen in urine; (6) chromatographic peak characterization: the quality of the chromatographic procedure was examined. In summary, this method has good sensitivity (2.5 μg/ml ibuprofen), the chromatographic time is 18 min with a 5-min reequilibration, and the reproducibility, precision, and chromatographic characteristics are excellent.

The sample isolation has two components. First, the compounds are extracted from the aqueous phase into an organic phase (hexane–2-propanol). The addition of saturated ammonium sulfate to urine increases the extraction efficiency for the metabolites of ibuprofen. The extraction efficiencies for OH-ibuprofen, COOH-ibuprofen, and ibuprofen were 73 ± 6, 64 ± 6, and 95 ± 1%, respectively, without ammonium sulfate, whereas they were 95 ± 7, 98 ± 9, and 95 ± 2%, respectively, with ammonium sulfate. Second, the compounds are back-extracted from the organic phase into an aqueous phase. Sodium bicarbonate (0.1 *M*) was used to neutralize the organic phase thus permitting back-extraction into an aqueous phase and resulted in a markedly decreased chromatographic background. With acidification, the bicarbonate solution could be directly injected and thus an evaporation step was not needed. This procedure proved satisfactory for ibuprofen, its major metabolites, and the internal standard solutions (Table I). No other reports determined recoveries from sample isolation by means of their respective methods.

The HPLC determination of the major metabolites of ibuprofen has been described using either an isocratic or a gradient elution technique. An isocratic HPLC

TABLE 1

EXTRACTION EFFICIENCIES OF IBUPROFEN, ITS MAJOR METABOLITES AND INTERNAL STANDARDS FROM HUMAN URINE

Compound	Extraction (mean ± C.V., $n=6$) (%)
OH-ibuprofen	94 ± 5
COOH-ibuprofen	99 ± 2
2-Phenylpropionic acid	100 ± 4
Ibuprofen	95 ± 5
Ibufenac	95 ± 9

assay for the two major metabolites has been reported for plasma and urine [7]. Due to the more highly polar nature of the two metabolites relative to ibuprofen, ibuprofen is retained on the column. The drawback to this method is that the metabolites of ibuprofen can be determined, but not ibuprofen. A gradient system has been employed to determine ibuprofen and its metabolites in plasma and urine [6]. This procedure uses methylene chloride extraction and methylprednisolone as the internal standard (we previously have shown that the recovery of ibuprofen during methylene chloride extraction of plasma is 68.6 ± 7.9% [8]). We elected to use an isocratic system to separate the metabolites with an appropriate internal standard and then a step gradient to achieve a new eluent whereby ibuprofen and its internal standard are eluted. Thus we overcame the problem of retaining ibuprofen on the column as in the method of Shah and Jung [7] with the need to purge the system to clean the column. Unlike the method reported previously which utilizes only one internal standard solution for ibuprofen and its major metabolites [6], we used two internal standard solutions. The urine samples contained ibuprofen metabolites in higher concentration than ibuprofen (the range of free OH-ibuprofen concentration was 50–500 μg/ml, total OH-ibuprofen was 100–1000 μg/ml, free and total COOH-ibuprofen was 150–1500 μg/ml, free ibuprofen was 2.5–25 μg/ml, and total ibuprofen was 25–250 μg/ml) and so we used different internal standards and different concentrations in accordance with published guidelines on selection and concentration of internal standards [9, 10]: ibufenac (50 and 500 μg/ml) for ibuprofen and 2-phenylpropionic acid (1000 and 1500 μg/ml) for metabolites. This method could be used to detect very low free ibuprofen concentrations (2.5 μg/ml). Considering the small amount of unconjugated ibuprofen found in urine, we selected 100 μl of urine for the free procedure to enhance sensitivity. Furthermore, all values reported herein are derived from patient urine samples whereas the previous studies used standard solutions in water to validate these methods [6, 7].

Fig. 1A is a chromatogram of 100 μl blank urine carried through the analytical procedure for total ibuprofen and its metabolites replacing internal standard solutions with 2-propanol. No interfering peaks were observed when blank urine was subjected to the assay. Fig. 1B is a chromatogram of 100 μl of urine from a patient receiving ibuprofen carried through the analytical procedure of uncon-

jugated ibuprofen and its metabolites and Fig. 1C is that same urine processed for total ibuprofen and metabolites.

The reproducibility of the procedure was determined by analyzing in replicates of six on three separate days a sample of urine from a human subject taking ibuprofen (Table II). The standard curves for the three days were linear; the slopes and y-intercepts were consistent. The sample-to-sample and day-to-day variability study values are shown in Table II and are excellent for both free and total compounds. The highest coefficient of variation (C.V.) observed was less than 9%.

In recent years there has been considerable interest in the characterization of experimental chromatographic peaks. It is proposed that these parameters of peaks be referred to collectively as chromatographic figures of merit (CFOM) [11]. We developed the computer software for routine calculation of chromatographic column and peak descriptors and have employed them with great success in the past [12]. These are very helpful not only for chromatographic development but also

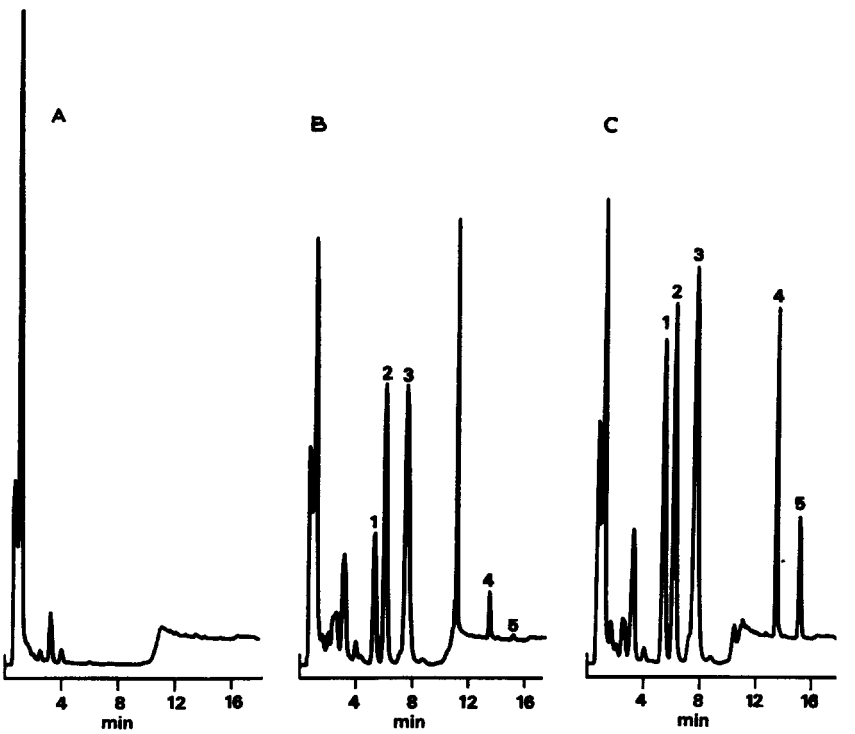

Fig. 1. Chromatograms of (A) 100 μl drug-free urine carried through the analytical procedure for total ibuprofen and its metabolites, (B) 100 μl of urine collected during 0–8 h from a patient receiving 300 mg oral ibuprofen and processed for free ibuprofen and its metabolites, and (C) 100 μl of the same urine processed for total ibuprofen and its metabolites. The absorbance detector was operated at 214 nm. The injection volume was 50 μl. The eluent flow-rate was 2.5 ml/min. Full scale absorbance was 0.2 absorbance units. Peaks: 1 = OH-ibuprofen; 2 = 2-phenylpropionic acid; 3 = COOH-ibuprofen; 4 = ibufenac; 5 = ibuprofen. Quantification: free OH-ibuprofen, 112.1 μg/ml; free COOH-ibuprofen, 311.5 μg/ml; free ibuprofen, 3.1 μg/ml; total OH-ibuprofen, 273.7 μg/ml; total COOH-ibuprofen, 434.8 μg/ml; total ibuprofen, 145.6 μg/ml.

TABLE II

REPRODUCIBILITY STUDY

Compound	Concentration (mean ± S.D.) (μg/ml)				C.V. (%)
	Day 1 ($n=6$)	Day 2 ($n=6$)	Day 3 ($n=6$)	Mean ($n=18$)	
Free OH-ibuprofen	111 ± 5	110 ± 8	116 ± 3	112 ± 6	5
Free COOH-ibuprofen	327 ± 12	299 ± 23	323 ± 10	316 ± 19	6
Free ibuprofen	3.5 ± 0.4	3.5 ± 0.2	3.2 ± 0.2	3.4 ± 0.3	8
Total OH-ibuprofen	263 ± 11	270 ± 13	268 ± 12	267 ± 12	4
Total COOH-ibuprofen	425 ± 17	407 ± 22	425 ± 17	419 ± 20	5
Total ibuprofen	144 ± 6	149 ± 16	153 ± 7	149 ± 11	7

TABLE III

SELECTED CHROMATOGRAPHIC FIGURES OF MERIT FOR IBUPROFEN AND ITS MAJOR METABOLITES DURING ANALYSIS OF PATIENT URINE SAMPLES MADE DURING A SINGLE CHROMATOGRAPHIC RUN CONSISTING OF A FIVE-POINT STANDARD CURVE AND NINETEEN SAMPLES PERFORMED IN DUPLICATE

Compound	Internal standard variability	Peak symmetry (τ/σ_G)	Relative system efficiency
Free OH-ibuprofen	–	1.11 ± 0.08	0.44 ± 0.04
2-Phenylpropionic acid	6%	1.36 ± 0.09	0.34 ± 0.03
Free COOH-ibuprofen	–	1.46 ± 0.35	0.33 ± 0.10
Ibefunac	8%	1.19 ± 0.12	0.41 ± 0.05
Free ibuprofen	–	1.28 ± 0.43	0.40 ± 0.12
Total OH-ibuprofen	–	1.20 ± 0.13	0.41 ± 0.05
2-Phenylpropionic acid	6%	1.44 ± 0.09	0.32 ± 0.03
Total COOH-ibuprofen	–	1.59 ± 0.42	0.30 ± 0.10
Ibufenac	11%	1.34 ± 0.15	0.35 ± 0.05
Total ibuprofen	–	1.10 ± 0.10	0.45 ± 0.05

in pharmacokinetic studies where day-to-day precision must be maintained. The CFOMs most useful to chromatographers include the ratio τ/σ_G (a fundamental index of peak asymmetry; values between 1 and 2 are common for experimental chromatographic peaks and indicate a slight to moderate amount of peak asymmetry), N_{sys} (efficiency expressed in theoretical plates), N_{max} (theoretical maximum efficiency) and RSE (N_{sys}/N_{max}, relative system efficiency). In Table III the CFOMs are reported for nineteen urine samples obtained in a clinical study (to be reported separately) plus a five-point standard curve analyzed for free and total metabolites using the described method. The symmetries and efficiencies reported imply minimal chromatographic interference of the peaks of interest. The small variability of the height of the internal standards implies consistency throughout the entire procedure.

Five metabolites of ibuprofen have been observed in human urine using a GC–MS method [4, 5] with the major metabolites being OH-ibuprofen and COOH-

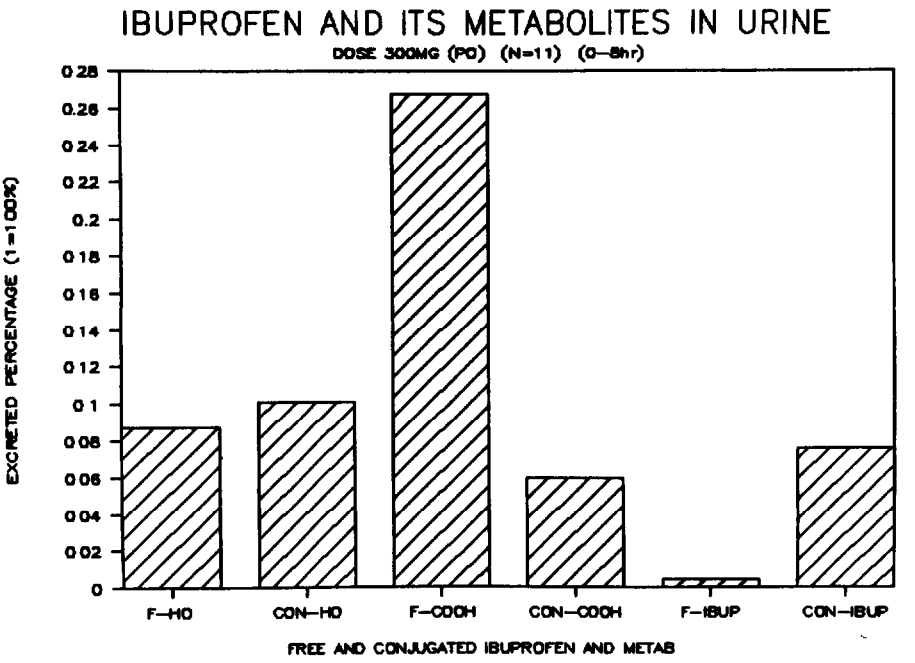

Fig. 2. Urinary free and conjugated ibuprofen and its major metabolites from eleven children with cystic fibrosis after a 300-mg oral dose of ibuprofen (0–8 h).

ibuprofen. These two metabolites and their respective conjugates accounted for approximately 60% of the metabolic end-products of ibuprofen in rats and humans [3]. It was reported that there was no unconjugated ibuprofen or conjugated ibuprofen in human urine after three oral doses of 200 mg, given 6 h apart, to three human volunteers during 0–24 h [3]. Lockwood and Wagner [6] reported no unconjugated ibuprofen excreted in the urine samples 0–12 h after a 400-mg dose of ibuprofen in fifteen adults but about 13% of the dose was excreted as conjugated ibuprofen. In our studies, we found that children with cystic fibrosis ($n=11$) administered 300-mg ibuprofen oral tablets excreted unconjugated ibuprofen as 0.5% of the dose and conjugated ibuprofen as 8.1% of the dose in the urine (0–8 h, Fig. 2). We believe the detection of unconjugated ibuprofen in urine resulted from the increased precision of the method described herein compared to other methods [3, 6]. However, the excretion of unconjugated ibuprofen in children may result from a different handling of ibuprofen in children compared to adults.

ACKNOWLEDGEMENTS

This work was supported by funds provided by the Veterans Administration Medical Research Program and the National Cystic Fibrosis Foundation. We thank Drs. M. Konstan and P. Davis for the urine samples from the clinical study and Dr. K.-Y. Tserng for helpful suggestions.

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