Journal of Chromatography, 232 (1982) 335—343
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1391

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF IBUPROFEN AND ITS MAJOR METABOLITES IN BIOLOGICAL FLUIDS

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(First received February 16th, 1982; revised manuscript received June 21st, 1982)

SUMMARY

A sensitive and selective high-performance liquid-chromatographic assay for ibuprofen and its major metabolites in biological fluids is described. To ensure good chromatographic separation the drug and metabolites were run on a gradient elution system and detected with a variable wavelength detector set at 220 nm. A second, more rapid, isocratic system is also described for the detection of only ibuprofen.

INTRODUCTION

Ibuprofen [2-(4-isobutylphenyl)propionic acid] is an orally administered, non-steroidal anti-inflammatory agent used extensively in the treatment of arthritis. The literature concerned with the biochemical and toxicological studies carried out in man and animals has been reviewed [1]. The metabolism of ibuprofen in man and several animal species is documented [2]. Major metabolites in man are 2-[4-(2-hydroxy-2-methylpropyl)phenyl]-propionic acid (OH-ibuprofen) and 2-[4-(2-carboxypropyl)phenyl]propionic acid (COOH-ibuprofen). Among the techniques employed to quantify ibuprofen and its metabolites in biological samples are paper chromatography [3], gas—liquid chromatography (GLC) with prior derivatization [4], GLC with electron capture detection [5], GLC—mass spectrometry combinations [6] and high-performance liquid chromatography (HPLC) [7—10]. To the authors' knowledge all the HPLC assays reported for ibuprofen measure only the parent compound. The ability to quantify metabolite levels in urine and plasma can greatly aid in drug metabolism and pharmacokinetic studies. It was therefore considered appropriate to develop an HPLC assay capable of detection of

0378-4347/82/0000—0000/$02.75

ibuprofen and its major metabolites (Assay I). A second, more rapid assay for ibuprofen alone was also developed (Assay II).

MATERIALS AND METHODS

Standards

Ibuprofen, OH-ibuprofen, COOH-ibuprofen, and methyl prednisolone were provided by the Upjohn Company (Kalamazoo, MI, U.S.A.). Tolmetin was provided by McNeil Laboratories (Fort Washington, PA, U.S.A.)

HPLC apparatus for the simultaneous determination of ibuprofen, OH-ibuprofen and COOH-ibuprofen in biological samples, Assay I

Due to the more highly polar nature of the two metabolites relative to the parent compound, isocratic elution was found to be unsatisfactory and a gradient system had to be employed. The apparatus used consisted of two Waters Model 6000A pumps (Waters Assoc., Milford, MA, U.S.A.) controlled by a Model 660 solvent programmer, a Waters variable wavelength detector set at 220 nm and an Omniscribe chart recorder (Houston Instruments). Separation was achieved with a prepacked Whatman column (Whatman, Clifton, NJ, U.S.A.) (25 cm × 4.5 mm I.D., Partisil 10 ODS-3 packing). The eluents delivered by the two pumps were of the following compositions. Eluent A: acetonitrile—water (28:72). To each liter of eluent was added 500 μl of phosphoric acid and 500 μl of acetone. Eluent B: acetonitrile—0.05 *M* monobasic potassium phosphate (50:50). The delivery rate of the mobile phase was 2 ml min^{-1} and a typical run was performed in the following manner. The column was allowed to come to equilibrium with eluent A. At time zero the sample was loaded onto the column through a Waters loop injector. After 8 min the solvent programmer was switched from these initial conditions to the "run" mode. Over the next 6 min (i.e. 8—14 min after the injection) the percentage of eluent A in the eluting solvent was reduced from 100% to 0% in a linear manner with respect to time while the percentage of eluent B increased from 0% to 100%. Thus, from 14 min after the injection to the termination of the run the eluting solvent was 100% B. Fig. 1c graphically represents the change in eluent composition with respect to time.

HPLC apparatus for the determination of ibuprofen in biological samples, Assay II

The basic HPLC equipment used for Assay II was similar in all respects to that used in Assay I. However, due to the isocratic nature of the assay, the solvent delivery system consisted of a single pump. The mobile phase was methanol—water (70:30) with 1 ml of phosphoric acid added to each liter of eluent. The mobile phase was delivered at a flow-rate of 2.5 ml min^{-1}.

Sample preparation for Assay I

Assay I was primarily used to assay urine samples for unchanged drug and metabolites. A simple clean-up extraction was required before urine samples could be chromatographed. A 1-ml sample of urine or diluted urine was added to a screw-topped test tube. A 100-μl aliquot of internal standard solution

(methylprednisolone 1 mg/ml) was added followed by 1 ml of 1.5 *M* hydrochloric acid to reduce the pH below 1. Finally, 500 μl of water and 10 ml of methylene chloride were added and the tubes were capped and shaken for 20 min. The tubes were then gently centrifuged (250 *g*, 3 min, Sorvall RC3 centrifuge). The lower organic phase was transferred to a clean, dry test tube and was evaporated to dryness under a stream of prepurified nitrogen at 40°C. Samples were reconstituted with 200 μl of methanol and 10—30 μl of this were injected onto the column.

To enable free and conjugated metabolites to be assayed in the urine, all samples were assayed twice. Free drug and metabolites were assayed as described above. Total drug and metabolites (free plus conjugated) were assayed following alkaline hydrolysis. Hydrolysis was carried out by incubating 1 ml of urine or diluted urine with 500 μl of 1 *M* sodium hydroxide solution for 20 min at room temperature. Following this period the extraction was carried out as described above.

Sample preparation for Assay II

Assay II was primarily used to assay plasma samples for unchanged drug. Plasma samples were subjected to a clean-up extraction before being chromatographed. To 1 ml of plasma in a screw-topped test tube were added 100 μl of internal standard solution (tolmetin, 100 μg/ml internal standard solution). The samples was acidified with 500 μl of 1 *M* hydrochloric acid and 10 ml of methylene chloride were added. The tubes were capped, shaken for 10 min and then centrifuged at 1000 *g* for 5 min to ensure complete phase separation. The lower, organic layer was transferred to a clean, dry test tube and was evaporated to dryness under a stream of prepurified nitrogen at 40°C. Just prior to analysis the residue was redissolved in 200 μl of Assay II eluent and 10—30 μl of this sample were loaded onto the column.

Calibration procedure for Assay I

Blank urine was spiked with ibuprofen, OH-ibuprofen and COOH-ibuprofen in the range 5—200 μg ml^{-1}. The urine samples were subjected to the preparation procedures described above and were chromatographed in the normal manner. Peak heights of ibuprofen, metabolites and internal standard were measured from the resultant chromatograms. A peak height ratio (peak height of compound divided by peak height of internal standard) versus concentration curve was constructed for the parent drug and metabolites. The peak height ratios of unknown samples were compared to this standard curve, corrections being made for any dilutions involved.

Calibration procedure for Assay II

Blank plasma was spiked with ibuprofen over the range 1—140 μg/ml. Plasma prepared in this way was subjected to the normal extraction and chromatographic procedures. From the chromatograms obtained the ibuprofen:tolmetin peak height ratio was calculated and a calibration curve relating this ratio to the plasma concentration of ibuprofen was constructed. Unknown samples were quantified by reference to this standard curve.

RESULTS AND DISCUSSION

A typical HPLC trace for Assay I is shown in Fig. 1a. The relative order of peak retention and retention times were: OH-ibuprofen (8.0 min), COOH-ibuprofen (10.2 min), methylprednisolone (13.6 min) and ibuprofen (21.8 min). No interfering peaks were observed when blank urine was subjected to the assay (Fig. 1b). Calibration data (compound peak height ratio versus compound concentration, $\mu g\ ml^{-1}$) were best fitted by the power curve described by the equation

$$\ln Y = S \ln X + \ln D \quad (1)$$

where X and Y are concentration and peak height ratio, respectively. The relationship between X and Y was actually linear since S was essentially equal to unity. However, the use of eqn. 1 gave the lowest coefficient of variation of inversely estimated concentrations, apparently as a result of the different weighting of the points. The results of inversely estimating ibuprofen and metabolite concentrations from the calibration data generated from standards prepared over several months are shown in Table I. No systematic bias was observable over the concentration range studied. The intra-day reproducibility of Assay I is good, as is shown in Table II. Five independently prepared samples at three concentrations were run for each compound. The highest coefficient of variation (C.V.) observed was less than 14%, two other

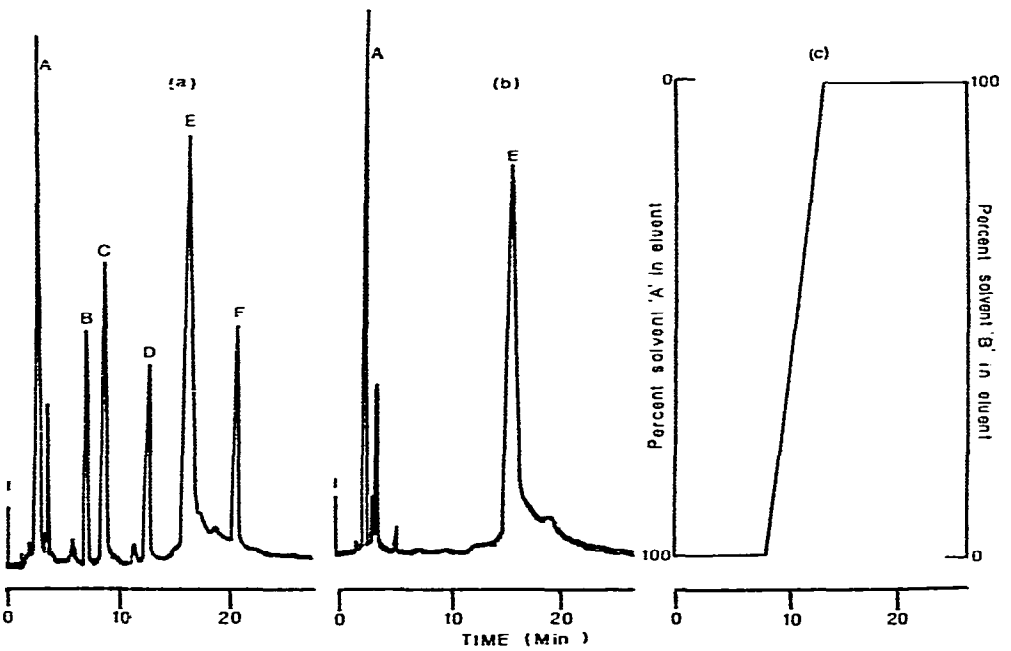

Fig. 1. (a) A typical chromatogram of ibuprofen and its metabolites extracted from urine. I = Injection; A = solvent front and highly polar contaminants; B = OH-ibuprofen; C = COOH-ibuprofen; D = methylprednisolone; E = low polar contaminants; F = ibuprofen. Blank urine (b) shows no interfering peaks. The change in eluent composition as a function of time is shown in part c.

TABLE I

PERCENTAGE THEORETICAL IBUPROFEN, OH- AND COOH-METABOLITE CONCENTRATIONS OBTAINED FROM THE URINARY CALIBRATION CURVES (ASSAY I)

Data presented in this table was collected over several months. Each inversely estimated concentration is the mean of at least 15 independently prepared calibration points assayed over this time span.

Ibuprofen concentration (μg/ml)	7.0	14.0	28.0	56.1	84.1	112.2	140.2
Mean (%)	102.2	98.9	100.6	105.5	98.6	93.7	107.6
Bias (%)	2.2	−1.1	0.6	5.5	−1.4	−7.3	7.6
C.V. (%)	13.6	15.1	14.6	11.6	10.2	10.5	8.3
OH-metabolite concentration (μg/ml)	9.1	18.3	36.6	73.2	109.7	146.3	184
Mean (%)	99.6	102.8	104.9	104	98.7	92.2	106.7
Bias (%)	−0.4	2.8	4.9	4	−1.3	−7.8	6.7
C.V. (%)	16.4	19.7	16.8	12.2	10.6	13.1	9.7
COOH-metabolite concentration (μg/ml)	9.3	18.6	37.2	74.5	111.7	149.0	186
Mean (%)	100.5	98.1	110.8	100.0	97.3	97.2	104.4
Bias (%)	0.5	−1.9	10.8	0	−2.7	−2.8	4.4
C.V. (%)	18.6	14.5	20.7	9.01	11.0	17.7	10.4

C.V. values were just greater than 10% while the remaining six were less than 10%. Urine spiked with ibuprofen and metabolites at 30 μg/ml was divided into 1-ml aliquots and frozen. On each assay day one urine aliquot was thawed and assayed. The stability of frozen samples and the inter-day reproducibility is shown in Table III. No trend towards sample degradation is seen but, as may have been expected over such a protracted period of time, slightly larger coefficients of variation are observed.

The lower limit of detection of Assay II as described for ibuprofen and its metabolites (around 5 μg ml^{-1}) was sufficient to enable detection of 1.0% of a 400-mg dose of ibuprofen in 1 l of urine. For the purposes for which the assay was used this proved to be adequate. If greater sensitivity is desired, extraction from a larger urine volume, or a decrease in internal standard and injection of a larger volume of the final extract would prove satisfactory.

A typical HPLC trace from Assay II is shown in Fig. 2a. The relative order of peak retention and retention times were tolmetin 3.2 min, and ibuprofen 7.7 min. No interfering peaks in blank plasma were observed (Fig. 2b). Calibration data were again fitted by a power curve described by eqn. 1. A summary of the calibration data obtained from 22 pooled calibration curves is summarized in Table IV. Again no systematic bias was noted over the concentration range studied. The intra-day reproducibility of Assay II is good as assessed by assaying six independently prepared plasma samples at three different concentrations (Table V). The coefficients of variation observed (all less than 10%) are of the same order as those reported by Kearns and

TABLE II

THE INTRA-DAY REPRODUCIBILITY OF ASSAY I

Each peak height ratio was obtained from an independently prepared calibration sample; all samples were assayed on the same day.

OH-metabolite concentration (μg/ml)	183	73.2	9.1
Peak height ratio	2.49	1.22	0.186
	2.87	1.30	0.182
	2.65	1.15	0.194
	2.88	1.20	0.179
	3.25	1.10	0.179
Mean	2.83	1.20	0.184
C.V. (%)	10.1	6.5	3.4
COOH-metabolite concentration (μg/ml)	186	74.5	9.3
Peak height ratio	2.08	0.876	0.150
	2.78	0.885	0.136
	2.09	0.886	0.137
	2.58	0.906	0.137
	2.18	0.808	0.117
Mean	2.34	0.872	0.135
C.V. (%)	13.5	4.3	8.7
Ibuprofen concentration (μg/ml)	140	56.1	7.0
Peak height ratio	2.83	1.19	0.134
	2.45	1.28	0.162
	2.74	1.09	0.158
	2.55	1.05	0.158
	2.95	0.999	0.147
Mean	2.70	1.121	0.152
C.V. (%)	7.4	10.4	7.5

TABLE III

INTER-DAY REPRODUCIBILITY OF ASSAY I AS INDICATED BY QUALITY CONTROL SAMPLES

	OH-Ibuprofen	COOH-Ibuprofen	Ibuprofen
Theory (μg/ml)	36.6	37.2	28.0
Range (μg/ml)	31.8—41.3	28.9—42.0	21.9—37.6
Mean (μg/ml)	36.5	36.8	28.2
S.D. (%)	3.1	4.0	4.9
C.V. (%)	8.4	10.8	17.3

Wilson [10] although the range of concentrations reported here is much greater. Plasma spiked with ibuprofen at 30 μg/ml was divided into 1-ml aliquots and frozen. On each assay day one of these quality control samples was thawed and assayed. The results are presented in Table VI. The low

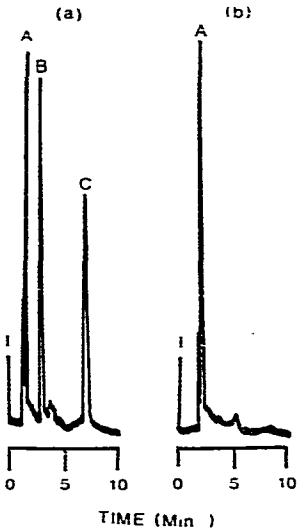

Fig. 2. (a) A typical chromatogram of ibuprofen extracted from plasma. I = Injection; A = solvent front and highly polar contaminants; B = tolmetin; C = ibuprofen. Blank plasma (b) showed no interfering peaks.

TABLE IV

PERCENTAGE OF THEORETICAL IBUPROFEN CONCENTRATION ESTIMATED FROM STANDARD CURVES FROM ASSAY II

Data summarized in this table were collected over several months. Each inversely estimated mean is the result of at least 22 independently prepared calibration points assayed over this time span.

Ibuprofen concentration (μg/ml)	1.4	2.8	7.0	14.0	42.1	105	140
Mean (%)	106	95.7	105.9	100.3	98.1	101.5	100.4
Bias (%)	6	−4.3	5.9	0.3	−1.9	1.5	0.4
C.V. (%)	21.1	13.9	14.8	13.6	7.4	11.3	5.8

TABLE V

THE INTRA-DAY REPRODUCIBILITY OF ASSAY I

Each peak height ratio was obtained from independently prepared samples; all samples were assayed on the same day.

Ibuprofen concentration (μg/ml)	140	14	2.8
Peak height ratio	10.53	1.39	0.253
	10.20	1.36	0.264
	10.11	1.38	0.258
	11.36	1.30	0.273
	11.48	1.17	0.281
	10.97	1.11	0.279
Mean	10.77	1.28	0.268
C.V. (%)	5.4	9.2	4.3

TABLE VI

INTER-DAY REPRODUCIBILITY OF ASSAY II AS INDICATED BY QUALITY CONTROL SAMPLES

Theory (μg/ml)	30
Range (μg/ml)	25.9—34.4
Mean (μg/ml)	29.1
S.D. (%)	3.3
C.V. (%)	11.3

coefficient of variation (11.3%) indicates good stability of frozen plasma samples and reproducibility of the assay over several months.

The applicability of the assays reported in a clinical study has been demonstrated. Fig. 3a presents the mean plasma concentration—time curves obtained

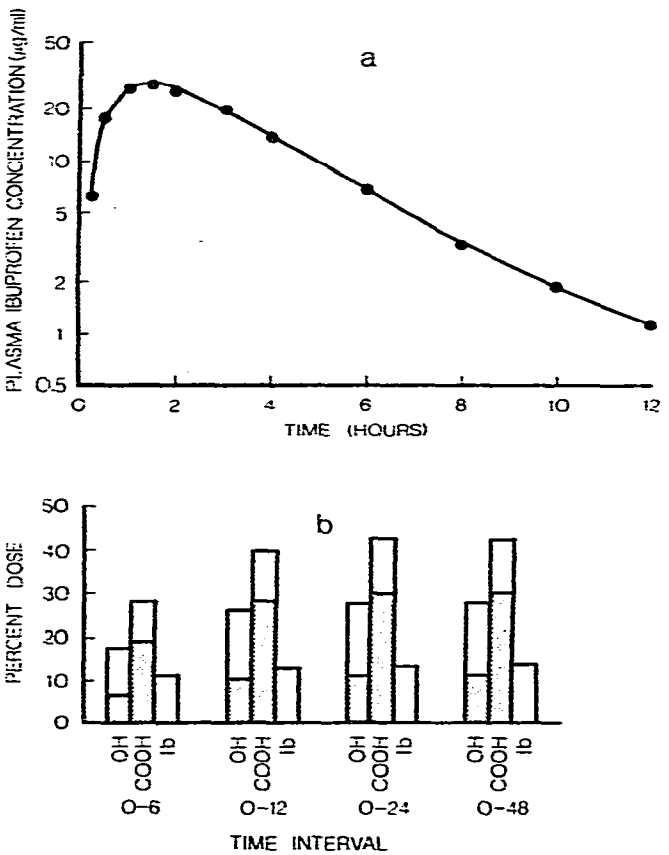

Fig. 3. A demonstration of the applicability of Assays I and II to pharmacokinetic studies. (a) Plasma concentration—time curve of ibuprofen after oral administration of a 400-mg tablet. (Each point is the mean of 15 subjects.) (b) Cumulative percentage of ibuprofen and its two major metabolites excreted in the urine after a 400-mg dose of ibuprofen. The larger bars represent the total percentage excreted in the measuring period. The smaller, shaded bars represent the percentage of free drug or metabolite excreted in the same interval. (All values are the means of 15 subjects.)

from 15 subjects after a 400-mg dose of ibuprofen orally. Assay II as reported here was ideally suited for following these plasma profiles since the concentration range encountered over the study was 110—1 μg/ml. It was generally considered to be unnecessary to lower the assay limits; however, since only about 1/10th of the reconstituted sample was injected on column the theoretical limits of the assay as described could easily be reduced to 0.1 μg/ml, thus making this assay comparable to or better than that of Ali et al. [9].

Urinary excretion data from the same study are presented in Fig. 3b to demonstrate the applicability of Assay I. Urinary recovery as assayed by this new HPLC method is in good agreement with literature values. It should be noted that Assay I is applicable to plasma samples but that the concentrations of metabolite present are nearly always at the limits of detection.

CONCLUSION

Assays have been developed to quantify ibuprofen or ibuprofen and its major metabolites in biological fluids. Adequate sensitivity and reproducibility of calibration data have been demonstrated. The applicability of the described assay methods has been shown. In future papers the applications of these assays to biological samples (plasma and urine) obtained in a four-phase clinical study involving ibuprofen will be discussed.

ACKNOWLEDGEMENT

This research was supported by the Medical Bioavailability Unit of the Upjohn Company, Kalamazoo, MI, U.S.A.

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