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METHOD TO DETERMINE THE ENANTIOMERS OF IBUPROFEN FROM HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

By means of ethyl chloroformate, ibuprofen enantiomers were coupled to 4-methoxyaniline. The resulting amides were resolved from each other and from urinary constituents on a Pirkle column using an isocratic mobile phase with ultraviolet detection at 254 nm. Applicability of this method for the determination of inter-individual differences in urinary metabolic profiles of ibuprofen enantiomers is demonstrated. The chromatographic behavior of the corresponding amide derivatives of two ibuprofen metabolites is also described.

INTRODUCTION

The metabolism of ibuprofen (**1**), a commonly used α-methylarylacetic acid non-steroidal anti-inflammatory drug involves isomeric inversion of the pharmacologically inactive (*R*)-enantiomer to the active (*S*)-enantiomer in man [1–3]. Our interest in inter-individual differences in the metabolism of commonly used drugs prompted us to develop an assay for comparing urinary metabolic profiles of the ibuprofen enantiomers.

Several assays for the resolution of ibuprofen enantiomers from biological fluids have been reported which involve the formation of diastereomeric derivatives. A gas chromatographic (GC) method utilizes (*S*)-α-methylbenzylamide derivatives [2,4]. A recently reported high-performance liquid chromatographic (HPLC) method uses (*S*)-1-(naphthen-1-yl)-ethylamides [5], whereas an older procedure uses (*S*)-2-octanol ester derivatives [6]. Effective separation of diastereomers is achieved with these procedures but there is a risk of undetected

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error due to optically impure reagents, racemization of the reagents during derivatization, or unequal reaction rates in the formation of diastereomers [6–8].

Resolution of enantiomers by chromatography on chiral stationary phases avoids these errors [7,8]. Separation of ibuprofen enantiomers or enantiomeric derivatives can be effected on an α_1-acid glycoprotein column [9] or on columns developed by Pirkle [8,10,11], respectively. Herein we report an HPLC assay for the determination of ibuprofen enantiomers in human urine based on our previously reported methodology using the Pirkle (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine column [11].

Two major metabolites of ibuprofen have been isolated and identified as the hydroxylated metabolite, OH-ibuprofen (**2**), and the carboxylated metabolite, CO_2H-ibuprofen (**3**) [2,12–14], shown in Fig. 1. The OH-ibuprofen has one asymmetric centre and exists as a pair of enantiomers The CO_2H-ibuprofen has two asymmetric centres and exists as a mixture of four stereoisomers. It has been suggested that the oxidative metabolism of ibuprofen is a stereoselective process since the isolated metabolites show optical activity [2,12–14]. We are interested in studying inter-individual and inter-ethnic variability in these oxidative processes.

To the authors' knowledge, only the GC assay of Kaiser et al. [2] is reported for the separation and quantitation of these metabolite stereoisomers. An HPLC assay is reported for the quantitation of the metabolites, but it is non-stereoselective [15]. Due to the ability of the Pirkle column to separate derivatives of the ibuprofen enantiomers [11], we explored using it for a stereoselective HPLC assay of the major metabolites. Towards this end, the Pirkle column was only partially successful. Herein, we describe the chromatographic behavior of the 4-methoxyanilide derivatives of these metabolite stereoisomers and give preliminary results regarding an assay to compare human urinary profiles of these metabolites.

In man, negligible amounts of ibuprofen are excreted as the unchanged drug. An average of 14% of an administered dose is excreted as the ester glucuronide conjugate of ibuprofen [3,15]. The remainder is excreted as oxidative metabolites [13,14,15]. The major metabolites, OH-ibuprofen and CO_2H-ibuprofen, account for approximately 30 and 40% of the dose, respectively [13,15]. These metabolites are also conjugated, but the exact extent of conjugation is not clear [13,15]. In this work, the urine samples have been hydrolyzed with acid to ensure that the total drug and metabolites were assayed.

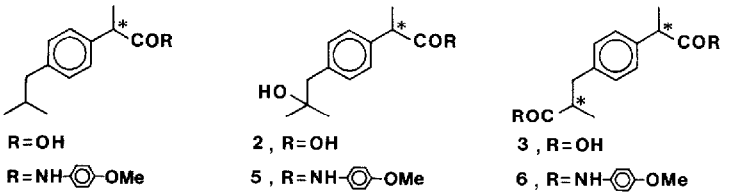

Fig. 1 Structures of ibuprofen (**1**), its major metabolites OH-ibuprofen (**2**) and CO_2H-ibuprofen (**3**) along with the corresponding 4-methoxyanilide derivatives, **4**–**6**, formed by reaction with ethyl chloroformate and 4-methoxyaniline. Asterisks denote asymmetric carbons.

Lee et al. [3] have reported urinary ratios of ibuprofen enantiomers in a four-subject study. We demonstrate the applicability of our method with the results of a study involving 43 subjects from two ethnic groups.

EXPERIMENTAL

Chemicals

The racemic ibuprofen, ethyl chloroformate, triethylamine, and 4-methoxyaniline were purchased from Aldrich (Milwaukee, WI, U.S.A.) and were used without purification. The preparation of partially resolved 91:9 (*S*)- to (*R*)-ibuprofen has been described [11]. Samples of (*S*)-(+)-ibuprofen (98%), OH-ibuprofen and CO_2H-ibuprofen were generously provided by Upjohn (Kalamazoo, MI, U.S.A.). Motrin® was supplied by Upjohn Canada. Derivatizing solutions were prepared in advance and could be stored at −20°C for up to six weeks without deterioration. Florisil (60–100 mesh) was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). HPLC-grade solvents, from Caledon Labs. (Georgetown, Canada), were degassed and then mixed prior to use.

Apparatus

Chromatography was performed on a Shimadzu system comprising an LC-6A pump, SCL-6A system controller, SIL-6A autoinjector, SPD-6A UV spectrophotometric detector, and C-R3A Chromatopac integrator. The column was a stainless-steel J.T. Baker packed Pirkle covalent column (25 cm × 4.6 mm I.D.) with an α-aminopropyl packing of 5-μm spherical particles modified with (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine. With a ternary mixture of 18:2:1 hexane–chloroform–2-propanol [16] at a flow-rate of 2.0 ml/min, the corrected retention time of the first eluted enantiomer, (*S*)-ibuprofen, is 2.6 min. The separation of the enantiomers is 1.19 and the resolution is 1.52. Mass spectra were recorded on a Searle Biospect mass spectrometer (Baltimore, MD, U.S.A.) operating in chemical ionization mode with methane as the reagent gas.

Urine samples

Human urine samples were obtained as pooled urine (0–12 h) after volunteers ingested a single 600-mg dose of Motrin (Upjohn). The study was approved by the Ethics Committee at the University of Toronto and informed consent was given by all volunteers. The urine was acidified to pH 2 with concentrated hydrochloric acid and was stored at −20°C until analysis. Rat urine was obtained following two 20 mg/kg doses by intubation, as suspension in 0.5% aqueous gum arabic, given at a 4-h interval to two male Sprague–Dawley rats (200 g). Food was withheld and a 0–24 h urine sample was collected using a metabolic cage. The urine was frozen at −20°C until analysis.

Conjugate hydrolysis and extraction of urine samples

To a 1.0-ml sample of thawed urine were added 100 μl of internal standard solution (1 mg/ml α-phenylpropionic acid in saturated aqueous sodium bicarbonate) and 1.0 ml of 6 *M* hydrochloric acid. The mixture was heated at 60–65°C for 30 min. The samples were extracted with 5 ml of 5% 2-propanol in methylene

chloride on a Genie-Vortex mixer for 30 s. The organic layer was transferred to a clean test tube and the solvent was evaporated in a water bath ($<40°C$) under a stream of nitrogen. Five drops of toluene were then added and evaporated in the same way to remove traces of 2-propanol and water.

Derivatization procedure

The samples were derivatized by the following modification of the Björkman procedure [17]. The residue was taken up in 400 μl of 50 m*M* triethylamine in methylene chloride solution. To this mixture were added in sequence with mixing at a 15-min interval 100 μl each of (1) a 60 m*M* solution of ethyl chloroformate in methylene chloride and (2) a 0.5 *M* solution of 4-methoxyaniline in methylene chloride. After 2 min, 1 ml of 1 *M* hydrochloric acid was added and the solution was mixed to quench the reaction. The aqueous layer was removed and the organic layer was washed with a second portion of 1 *M* hydrochloric acid to remove all traces of excess 4-methoxyaniline. The mixture was diluted with 3 ml of diethyl ether and mixed. The organic layer was then filtered through a plug of Florisil (0.4 g) and the Florisil was washed with 2 ml of diethyl ether. The combined organic solvent was evaporated as above, and the residue was taken up in 1.0 ml of a 1:1 mixture of hexane–2-propanol. This solution was transferred to a glass vial. The injection volume was 25 μl.

Extraction effectiveness and reproducibility

Ten urine samples (1.0 ml) were each spiked with 100 μg of racemic ibuprofen and the complete assay was conducted. Duplicate samples of 100 μg of racemic ibuprofen from a 1 mg/ml methylene chloride solution were evaporated under nitrogen and derivatized by the above procedure. The absolute peak areas of these samples were compared to determine extraction effectiveness.

Standard curves and reproducibility

To blank urine samples (1.0 ml) were added 12.5, 25, 50, 100, or 200 μg of racemic ibuprofen and 100 μg of internal standard by using appropriate volumes of 1.0 mg/ml stock solutions of each compound in saturated aqueous sodium bicarbonate. These were analysed by the above complete assay, and standard curves were drawn using peak-height ratios of the appropriate ibuprofen peak with the first internal standard peak.

Preparation and characterization of metabolite derivatives

The OH-ibuprofen (500 μg) and CO_2H-ibuprofen (500 μg) were derivatized separately with the following modification of the above procedure. After the addition of the 4-methoxyaniline solution, the samples were allowed to stand for 15 min before quenching with 1 *M* hydrochloric acid. Mass spectral analysis of the products yielded the following data: OH-ibuprofen: m/z 328 (MH^+), 311 ($M^+ - H_2O$); CO_2H-ibuprofen: m/z 447 (MH^+).

RESULTS

Chromatograms

With a ternary mixture of hexane–chloroform–2-propanol (18:2:1) [16] at a flow-rate of 2.0 ml/min, the corrected retention time of the first eluted enantiomer, (*S*)-ibuprofen, is 2.6 min. The separation of the enantiomers is 1.19 and the resolution is 1.52. Typical chromatograms of blank and spiked urines and urine from a volunteer who had ingested ibuprofen are shown in Fig. 2. The specificity of the assay for the parent drug enantiomers was confirmed by the assay of five urine samples.

From numerous arylacetic acid and α-methylarylacetic acid analogues of ibuprofen tested, α-methylphenylacetic acid was chosen as the most suitable internal standard. Since this compound contains a chiral centre, it gives a double peak. The corrected retention time of the first peak is 5.2 min.

Extraction yield

Recovery of racemic ibuprofen from spiked urine (100 μg/ml) which had undergone the entire assay was determined by comparison with the average of duplicate samples of 100 μg which were subjected only to derivatization. A variety of extraction solvents were compared including 5% 2-propanol in each of the following solvents: toluene, ethyl acetate, diethyl ether, and methylene chloride. The latter gave the cleanest and most efficient extraction. The effectiveness and reproducibility for racemic ibuprofen extraction and derivatization was $95.4 \pm 4.9\%$ ($n=8$).

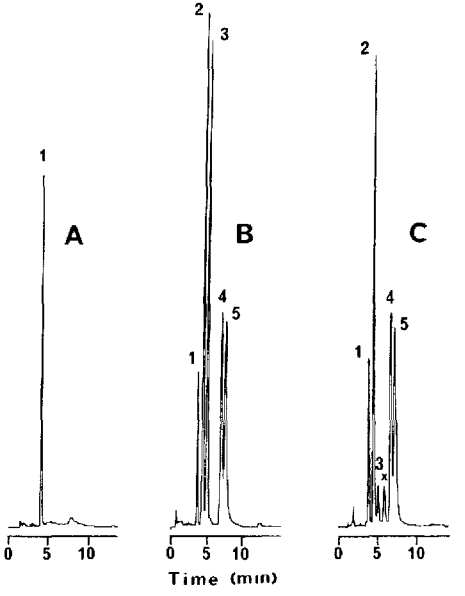

Fig. 2. Typical chromatograms of derivatized ibuprofen and internal standard. (A) Blank human urine. Peak 1 = urethane caused by condensation of excess chloroformate and 4-methoxyaniline. (B) Spiked human urine. Peaks: 2 = (*S*)-ibuprofen, 3 = (*R*)-ibuprofen; 4 and 5 = (*S*)- and (*R*)-enantiomers of internal standard, α-phenylpropionic acid. (C) Pooled human urine (0–12 h) after ingestion of a 600-mg dose of racemic ibuprofen. Peak X: unidentified metabolite. All chromatograms were run with hexane–chloroform–2-propanol (18 2:1) as mobile phase at 2 0 ml/min with UV detection at 254 nm.

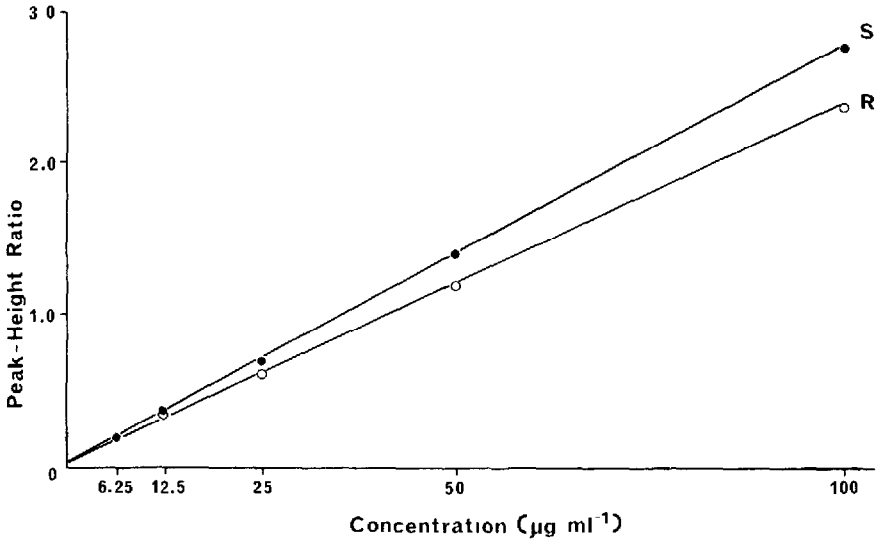

Fig. 3 Calibration curves of the (*S*)- and (*R*)-enantiomers of ibuprofen following the complete assay procedure. Peak-height ratios were determined by comparing the appropriate ibuprofen peak with the first internal standard peak.

Standard curves and precision

Fig. 3 summarizes the results of inter-day standard curve reproducibility for the complete assay of human urine spiked with racemic ibuprofen in the concentration range 12.5–200 μg/ml (6.25–100 μg/ml for each enantiomer). For both enantiomers the coefficients of correlation for the five-point standard curves exceeded 0.999 on three different days. Average equations of the lines were: (*S*)-enantiomer: $y=0.0276x+0.020$ (slope coefficient of variation, C.V. = 7.4%); (*R*)-enantiomer: $y=0.0234x+0.034$ (slope C.V. = 6.6%). Small interference peaks corresponded to a maximum of 1.2 and 2.2 μg/ml for the (*S*)- and (*R*)-enantiomers, respectively.

Applicability to a population study

For the population study, human subjects ingested 600 mg of racemic ibuprofen and 0–12 h pooled urine was collected. Table I shows the intra-day reproducibility for duplicate samples of three volunteers. Further analysis of urine samples from 43 volunteers of Caucasian and Oriental origin are summarized in Table II.

Derivatized ibuprofen metabolites

Solutions of each metabolite of ibuprofen were derivatized. Mass spectral analysis showed that OH-ibuprofen formed amide **5** and CO_2H-ibuprofen formed bisamide **6**. The best mobile phase for eluting these compounds in a short run without interfering peaks from human urine was hexane–chloroform–2-propanol (6:1:1). Typical chromatograms are shown in Fig. 4. The two enantiomers of the OH-ibuprofen were only partially resolved ($k'_1=3.66$, $\alpha=1.06$, $R_s=0.31$). The

TABLE I

INTRA-DAY REPRODUCIBILITY IN DUPLICATE ANALYSES

Dose: oral 600-mg ibuprofen (*S*/*R* 50.50). Urine. 0–12 h collection.

Subject	(*S*)-Ibuprofen (μg/ml)		(*R*)-Ibuprofen (μg/ml)	
	Individual values	Mean ± S E.	Individual values	Mean ± S.E.
1	75.5, 77.1	76.3 ± 0.8	5.7, 6.3	6.0 ± 0.3
2	88.4, 77.7	83.1 ± 5.4	7.3, 6.7	7.0 ± 0.3
3	162.0, 146.4	154.2 ± 7.8	7.6, 8.2	7.9 ± 0.3

TABLE II

URINARY RECOVERY OF IBUPROFEN IN 43 HEALTHY SUBJECTS

Dose: oral 600-mg ibuprofen (*S*/*R*, 50·50). Urine: 0–12 h collection.

Ethnic group	Number of subjects	(*S*)-Ibuprofen (mean ± S.D) (mg)	Percentage of dose	$(S \times 100)/(S+R)$ (%)
Caucasians	31	35 4 ± 8.6	5.90 ± 1.43	91.6 ± 1.84
Orientals	12	34.2 ± 10.6	5.70 ± 1.76	91.7 ± 2.22

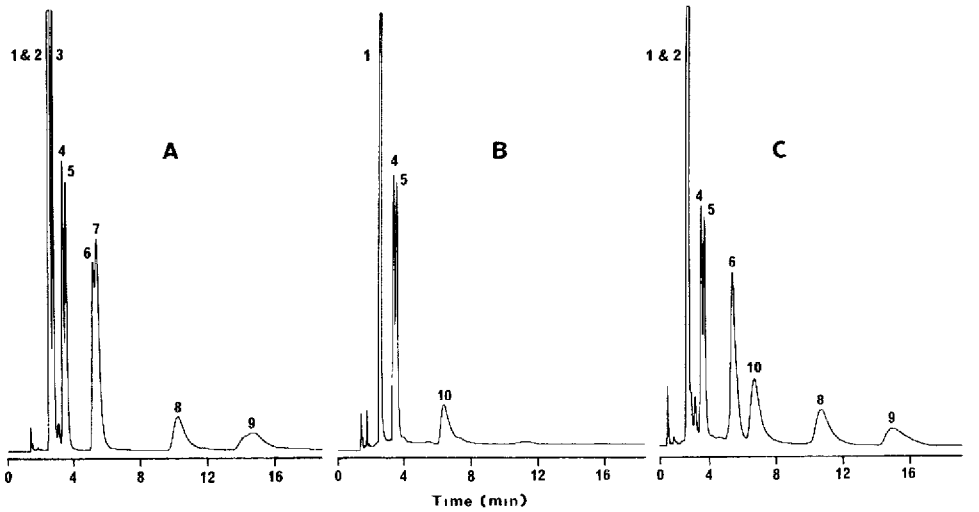

Fig. 4. Typical chromatograms of the derivatized ibuprofen metabolites and internal standard. (A) Standard test sample of racemic derivatives. Peaks: 1–3 = urethane by-product and ibuprofen enantiomers; 4 and 5 = (*S*)- and (*R*)-enantiomers of internal standard, α-phenylpropionic acid; 6 and 7 = (*S*)- and (*R*)-enantiomers of OH-ibuprofen, respectively; 8 and 9 = diastereomers of CO_2H-ibuprofen. (B) Blank human urine. Peak 10 = endogenous metabolites. (C) Human urine pooled for 12 h after ingestion of a 600-mg dose of racemic ibuprofen. All chromatograms were run with hexane–chloroform–2-propanol (6:1 1) at 2.0 ml/min with UV detection at 254 nm.

four diastereomers of the CO_2H-ibuprofen were separated into two peaks of equal area ($k'_1 = 8.58$, $\alpha = 1.52$, $R_s = 2.06$).

DISCUSSION

We have reported the behaviour of five ibuprofen amides on a Pirkle column and have proposed a stereoelectronic model to explain the enantiomer separation and order of elution [11]. For the purpose of assay development we chose to use the 4-methoxyanilide derivative. With this derivative, the ibuprofen amide enantiomers exhibited good separation ($\alpha = 1.21$) and the best resolution ($R_s = 2.46$) using, as mobile phase, hexane–2-propanol (97:3). In addition, the UV spectrum of this derivative indicated that detection at 254 nm would be sensitive ($E_{254} = 16\,300$ l mol^{-1} cm^{-1}). The corrected retention time of the first eluted enantiomer was 14.2 min. Using a 91:9 mixture of ibuprofen enantiomers we showed that the (*S*)-enantiomer elutes first and that ibuprofen does not undergo isomerization during derivatization using ethyl chloroformate, following a procedure of Björkman [17].

To adapt this work to a viable assay procedure we studied various mobile phases to yield a shorter run time. A ternary mixture of hexane–chloroform–2-propanol (18:2:1) [16] results in a run time of approximately 10 min for derivatives of ibuprofen and the internal standard (Fig. 2). Another mobile phase which gives comparable results is a ternary mixture of hexane–ethyl acetate–2-propanol (16:2:1). Using either of these isocratic mobile phases, it takes about 10 min for the run and an additional hour to elute polar ibuprofen metabolites. This time can be shortened by 20 min by increasing the flow-rate to 4.0 ml/min after elution of the parent drug and internal standard. The use of mobile phases more polar than these results in coalescence of the urethane by-product and the (*S*)-ibuprofen derivative peaks. This urethane by-product is formed by condensation of excess ethyl chloroformate and 4-methoxyaniline [11]. Since ethyl chloroformate is destroyed by water, it is reassuring to see this peak because it demonstrates that there was excess reagent present during derivatization.

Initially we planned to develop an assay which would allow detection and quantitation of the stereoisomers of the two major ibuprofen metabolites [2,12–14]. Due to our success in separating ibuprofen enantiomers on the Pirkle column, we anticipated that the two OH-ibuprofen enantiomers could be resolved and that the two enantiomeric pairs of CO_2H-ibuprofen diastereomers could be separated.

Initial studies showed that the above mobile phases would not be suitable for quantitation of the ibuprofen metabolites. The OH metabolite derivatives co-eluted with endogenous urinary compounds and the CO_2H metabolite derivatives gave very broad peaks. For the following qualitative studies, the mobile phase hexane–chloroform–2-propanol (6:1:1) was used.

Chromatography of the OH-ibuprofen derivative **5** gave rise to two partially resolved peaks (Fig. 4). Presumably the hydroxyl group in this compound causes the poor separation. Possibly, competitive intermolecular hydrogen bonding of the hydroxyl group occurs with the chiral stationary phase. This would alter the drug–chiral stationary phase complex proposed earlier [11], thereby affecting the

enantiomer separation. The four stereoisomers of the CO_2H-ibuprofen derivative **6** resulted in two peaks of equal area. The presence of the second amide in this compound must perturb the chiral recognition process, such that presumably the two diastereomeric pairs are separated, but not the enantiomers.

Analysis of urine from a rat which had ingested pure (*S*)-ibuprofen allowed us to determine which stereoisomers caused these peaks. Since the (*S*)-ibuprofen is not isomerized [1,3] only the (*S*)-enantiomer of OH-ibuprofen and the (*S,S*)- and (*S,R*)-diastereomers of CO_2H-ibuprofen would be produced by the rat. For the OH-ibuprofen derivative, the retention time and a co-injection study proved that the first peak is due to the (*S*)-enantiomer. For the CO_2H-metabolite, visual comparison of the chromatogram with the chromatograms of blank rat urine and the standard test mixture suggested that the (*S,S*)- and (*S,R*)-diastereomers of the CO_2H-ibuprofen derivative elute in separate peaks. The chromatogram of a human urine sample from a volunteer who had ingested racemic ibuprofen shows only one OH-ibuprofen peak but shows two peaks for the CO_2H-ibuprofen stereoisomers (Fig. 4).

The applicability of this assay for quantitative analysis of the parent drug enantiomers was demonstrated by conducting a population study. Table I, showing the duplicate analyses of three subjects, demonstrates the consistency of the assay. The results of a 43 subject population study are summarized in Table II. No ethnic differences were observed in the average amount or ratio of ibuprofen enantiomers.

CONCLUSIONS

We have described the HPLC behavior of the stereoisomers of the OH-ibuprofen and CO_2H-ibuprofen metabolite derivatives of ibuprofen on a Pirkle-type column. These results indicate that, for ibuprofen, the separation and resolution of stereoisomers are better for the parent drug than for its metabolites. However, despite this limitation, there are few HPLC assays available for determining total ibuprofen metabolite quantities in human urine samples. This method could easily be developed for that purpose.

A viable assay for the resolution and quantitation of ibuprofen amide enantiomers from human urine has been described. This assay has been successfully applied to a preliminary population study of the enantiomer ratio of excreted ibuprofen.

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