Journal of Chromatography, 226 (1981) 183—190
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1003

DETERMINATION OF IBUPROFEN IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND APPLICATION TO IBUPROFEN DISPOSITION

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(Received April 16th, 1981)

SUMMARY

A high-performance liquid chromatographic method for quantitation of ibuprofen from serum and application of this method to ibuprofen disposition in the dog is described. The drug was extracted from acidified plasma with dichloromethane. The internal standard used was a methanolic solution of 4-*n*-butylphenylacetic acid. A μBondapak C_{18} column was used for analysis; the mobile phase was methanol—water—glacial acetic acid (pH 3.4) (75 : 24 : 1, v/v). A wavelength of 272 nm was used to monitor ibuprofen and the internal standard.

Method sensitivity was 0.5 μg/ml serum using either 0.5 or 1.0 ml of sample, and no interference was found from endogenous compounds or other commonly used anti-inflammatory agents. The coefficients of variation of the method were 4.2% and 6.0% for samples containing 50.0 and 6.25 μg/ml of ibuprofen, respectively, and the calibration curve was linear for the range of 0.5 to 100 μg/ml. This method was demonstrated to be suitable for pharmacokinetic and/or biopharmaceutical studies of ibuprofen in man and the dog.

INTRODUCTION

Ibuprofen [DL-2-(*p*-isobutylphenyl)propionic acid, IBU] is a 2-phenylalkylcarboxylic acid derivative possessing potent anti-inflammatory, antipyretic and analgesic properties [1—3]. It is used extensively for the treatment of adult and juvenile rheumatoid arthritis [3—5], and additionally in the treatment of pain associated with dysmenorrhea [6] and for antipyresis [7].

IBU and its metabolites have been assayed in plasma by a variety of techniques including gas—liquid chromatography (GLC) with derivatization [8], colorimetric determination of a copper complex [9], GLC with electron-capture detection [10], and a combined gas chromatography—mass spectrometry

0378-4347/81/0000—0000/$02.50

method [11]. Several GLC methods have obviated the need for derivatization [12, 13] and hence have simplified the analysis of IBU. These methods, however, require 1.0—2.0 ml of plasma, and thereby render a pharmacokinetic or bioavailability study of IBU in children difficult.

Pitrè and Grandi [14] measured IBU in 1.0-ml samples of spiked canine plasma by utilizing high-performance liquid chromatography (HPLC). They did not, however, demonstrate appropriate utility or evaluate their method in a biological system. We developed a rapid, specific, and sensitive HPLC method for IBU in serum volumes of 0.5—1.0 ml. Application of this method to human serum and to IBU disposition in the dog is described. Suitability of the method for therapeutic drug monitoring is discussed.

MATERIALS AND METHODS

Standards

IBU and 4-*n*-butylphenylacetic acid, the internal standard (IS), were donated by Boots Pharmaceutical (Nottingham, Great Britain).

Preparation of sample

Ten micrograms of the IS were added to a serum sample (0.5 or 1.0 ml) contained in a 12.0-ml conical glass-stoppered tube. Hydrochloric acid (5 *M*, 0.2 ml) was added to precipitate serum proteins. Dichloromethane (3.0 ml) was added and the extraction was performed on a shaking board mixer for 10 min. The sample was centrifuged for 5 min at 1500 *g*, the clear upper aqueous layer removed by aspiration and the lower dichloromethane layer decanted into a 3.0-ml conical vial. The organic layer was evaporated to dryness under a gentle stream of nitrogen in a 37°C water bath. Evaporated samples were reconstituted with 40 μl of the HPLC eluent, mixed thoroughly and recentrifuged at 1500 *g* for 10 min. The clear supernatant fraction was transferred to a sample vial and capped in preparation for automated analysis.

Conditions of analysis

A Waters Assoc. (Milford, MA, U.S.A.) HPLC apparatus with the following instrumentation was used: M6000A Solvent Delivery System, Waters Intelligent Sample Processor (WISP), Data Module and M450 variable-wavelength UV detector. A μBondapak C_{18} column, particle size 10 μm (reversed-phase) preceeded by a guard frit and a guard column packed with C_{18}/Corasil bulk packing were also used (Waters Assoc.). The eluent (pH 3.4) was a mixture of 75% methanol (Waters Assoc.), 24% deionized water and 1% glacial acetic acid. All solutions were prefiltered with an FH-type filter (Millipore, Bedford, MA, U.S.A.), prewet with methanol.

The flow-rate was 1.5 ml/min at room temperature (20—22°C). The WISP and Data Module were programmed to inject 5.0—20.0 μl of the extracted samples. Both IBU and the internal standard were eluted for a period of 6 min. Absorbance detection at a wavelength of 272 nm and an instrument range setting of 0.01 were used to monitor both compounds.

RESULTS

Chromatogram and elution characteristics

A typical chromatographic tracing is seen in Fig. 1. Variable injection volumes (5—20 μl) were used to ensure appropriate resolution characteristics for peak height determination. With a constant flow-rate (1.5 ml/min) and chart recording speed of 1 cm/min, IBU and the internal standard eluted at retention times of 4.4 and 3.97 min, respectively. Both compounds consistently produced symmetrical peaks with minimal tailing. Adequate separation was obtained during the elution of both compounds (α-value of 1.22 for IBU).

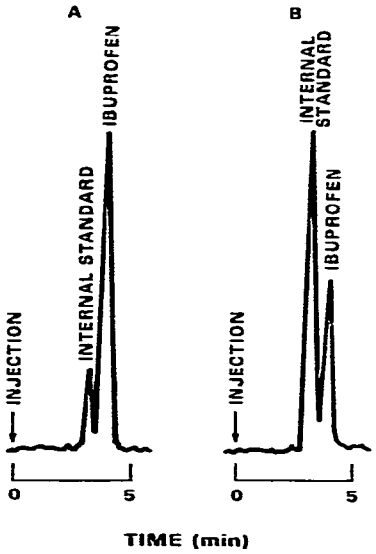

Fig. 1. Representative chromatographic tracings of 1.0-ml human serum, spiked with (A) 50 μg/ml and (B) 6.25 μg/ml ibuprofen. Detector sensitivity was changed in the recording of the tracings for qualitative purposes.

Standard curve and method sensitivity

Three standard curves were prepared using 1.0 ml of canine serum, and 0.5 and 1.0 ml of human serum. Correlation analysis was performed on each set of data obtained from the standard curves and the following tests were conducted: (1) a *t*-test; the null hypothesis (H_0) being that the slope of the regression line equals zero; (2) a correlation coefficient (r), H_0 being that x (IBU serum concentration in μg/ml) and y (the ratio of the peak heights calculated by dividing the IBU peak height by that of the internal standard) are independent; and (3) an analysis of variance, H_0 being that the regression of y on x is not linear. The p values for the t, r and analysis of variance were less than 0.005 in all cases, substantiating linearity of each standard curve to an IBU concentration of 100 μg/ml. Table I summarizes the results obtained from the linear regression analysis for each of the standard curves. The detection limit of 0.5 μg/ml and linearity through 100 μg/ml render the method suitable for monitoring IBU concentrations commonly found in man after therapeutic administration of the drug [2].

TABLE I

IBUPROFEN STANDARD CURVES IN CANINE AND HUMAN SERUM

Sample	n*	Equation	r**	F***
1.0 ml human serum	7	$y = 0.089 + 0.061x$	0.999§	2268.95§
1.0 ml canine serum	5	$y = 0.093 + 0.068x$	0.999§	4163.67§
0.5 ml human serum	1	$y = 0.120 + 0.07x$	0.999§	1955.82§

*n = Number of replicate samples analyzed at each of six concentration points in the construction of the standard curves.
**r = Correlation coefficient.
***F = Analysis of variance for linearity.
§$p < 0.005$.

Comparison of standard curves using a paired t-test revealed no statistically significant differences in the slopes or intercepts for dog vs. human serum, or for 1.0 vs. 0.5 ml of human serum. Rearrangement of the equation from the linear regression analysis of a standard curve permits calculation of IBU plasma concentrations from a corresponding peak height ratio determined from a given serum sample

Variability and stability

Serum samples containing 6.25 and 50.0 μg/ml of IBU, respectively, were extracted and prepared for analysis as described. Variability was assessed by analysis of seven extracted serum samples of both concentrations. The coefficients of variation for replication of the extraction were 4.2% and 6.0% for serum samples containing 50.0 and 6.25 μg/ml of IBU, respectively.

Sample stability in the automated sample processor (WISP) was assessed by analyzing extracted serum samples (n = 7), corresponding to IBU concentrations of 6.25 and 50.0 μg/ml, for a 10-h period. The average variation was 5.45% (range 47.5—58.2 μg/ml) for the 50.0 μg/ml standard and 6.27% (range 5.21—5.92 μg/ml) for the 6.25 μg/ml IBU standard. There was no trend indicative of compound instability while samples resided for 10 h in the WISP.

Variability in the automated sampling process was assessed by determining the reproducibility of absolute peak heights from ten 10-μl injections of the internal standard (which corresponded to a concentration of 100 μg/ml). Variability of automated sampling was minimal as revealed by a coefficient of variation of 3.3%.

The stability of frozen aliquots of human serum containing 50.0 and 6.25 μg/ml of IBU was examined by analysis of extracted samples (n = 6) of each concentration for ten days. The variations ($\bar{x}$ ± S.E.) for the samples were 5.46 ± 1.24% and 10.22 ± 2.35%, respectively. There was no trend indicative of compound instability as a result of freezing and thawing for at least ten days.

Selectivity

Table II contains the resolution characteristics for IBU, structurally and non-structurally related analgesic compounds, and substances which could be found concomitantly with IBU in plasma. The only compound evaluated which

TABLE II

RESOLUTION CHARACTERISTICS OF IBUPROFEN, OTHER ANALGESIC COMPOUNDS, AND POTENTIAL INTERFERING SUBSTANCES

Each substance was prepared as a 20% methanol in water solution and injected without prior extraction. Detection was evaluated at 272 nm during a 10-min elution period. The separation factor, α, was calculated with regard to the internal standard (4-*n*-butylphenylacetic acid) according to $\alpha = (V_2 - V_0)/(V_1 - V_0)$, where V_0 is the void volume, V_1 is the internal standard elution volume, and V_2 is the elution volume of the compound of interest. Retention times (in minutes) were used instead of volumes for the calculation of α. The concentration of the drug solutions is not intended to represent those found at steady state upon therapeutic administration of the respective agents.

Drug	Concentration (μg/ml)	Retention time (min)	α
4-*n*-Butylphenylacetic acid	100	4.09	—
Ibuprofen	100	4.52	1.22
Indomethacin	125	4.31	1.11
Phenylbutazone	125	3.55	0.72
Tolmetin Sodium	250	3.12	0.49
Fenoprofen Calcium	250	3.68	0.78
Sulindac	200	3.29	0.58
Salicylic acid	250	Not detectable	
Salicylamide	250	2.49	0.16
Salicyluric acid	250	2.42	0.13
Gentisic acid	250	6.02	2.01
Acetaminophen	25	2.36	0.09
Phenacetin	250	2.68	0.26
Codeine sulfate	250	2.75	0.30
Caffeine	250	2.60	0.22
Penicillin G	250	Not detectable	
Sodium Phenobarbital	250	2.51	0.17

demonstrated a potential for interfering with IBU analysis was indomethacin. Since these compounds were analyzed in a non-extracted solution of methanol and water, analyses of serum samples containing these compounds may yield somewhat different values, and each should be evaluated if the IBU method described herein is adapted to routine clinical use.

Method selectivity was assessed by HPLC recycling of the IBU peak from an extracted serum sample containing 100 μg/ml of IBU. A 1-h recycling period (equivalent to six cycles) revealed that the peak could not be split into two or more components, and this confirmed the presence of the single compound, IBU. Comparison of IBU plasma concentrations assayed by a standard GLC technique in dogs [15] revealed that our method was sufficiently specific, from both a biochemical and biological perspective.

Extraction efficiency

Known amounts of IBU in methanol (corresponding to representative serum concentrations of 6.25 and 50.0 μg/ml), and similar tubes evaporated to dryness and reconstituted with 40 μl of the eluent were compared to serum samples containing identical concentrations of IBU. Five serum samples of each

concentration were extracted, and aliquots of each were analyzed as previously described. Since IBU and the internal standard behaved in a similar fashion quantitatively (as determined by peak height comparison), the ratios of IBU: internal standard peak heights were used for comparison of extraction efficiency. The extraction efficiencies from 1.0 ml of serum were 82.5% and 100.0% for IBU concentrations corresponding to 50.0 μg/ml and 6.25 μg/ml, respectively. Evaporation did not account for a significant loss of compound as the mean recoveries from evaporated samples corresponding to IBU concentrations of 50.0 μg/ml and 6.25 μg/ml were 100.8% and 96.7%, respectively.

Application — serum levels of IBU in the dog

To demonstrate the utility of the analytical method, a male mongrel dog was administered 400 mg (13.8 mg/kg) of sodium IBU* as a single intravenous injection over a 5-min period. A peak serum concentration of 111.47 μg/ml was observed 10.9 min after termination of the injection. ESTRIP [16] analysis of the plasma concentration versus time points (Fig. 2) revealed that a bi-exponential equation best described IBU disposition in this animal. IBU disposition was modeled using an open, two-compartment pharmacokinetic model. Pharmacokinetic parameters calculated from the data analysis are described in Table III. The serum elimination half-life of IBU determined in the application of our method correlates closely to that extrapolated from the study of IBU plasma levels in dogs utilizing a colorimetric method for analysis [9], as well as that from a study of IBU bioavailability in dogs employing a GLC analysis method [15].

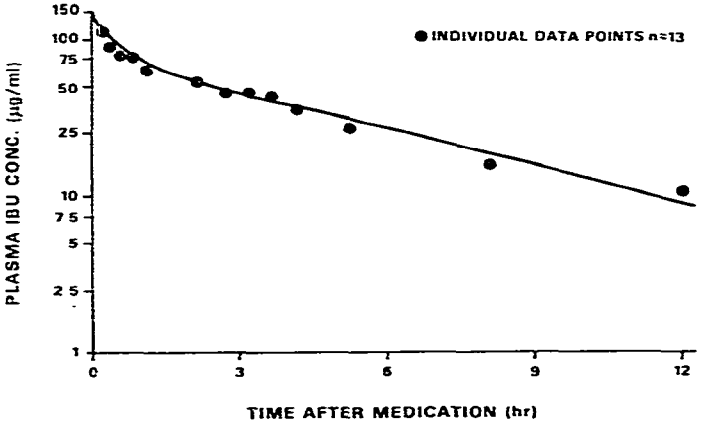

Fig. 2. Plasma concentrations of IBU versus time in the dog following a 400-mg (13.8 mg/kg) intravenous injection of sodium IBU. Biexponential curve fit is from estimates generated by ESTRIP [16] analysis of the data.

*Sodium IBU for intravenous use was kindly supplied by the Upjohn Company, Kalamazoo, MI, U.S.A.

TABLE III

PHARMACOKINETIC PARAMETERS CALCULATED FROM A 400-mg (13.8 mg/kg) INTRAVENOUS DOSE OF SODIUM IBUPROFEN IN A DOG

α (h^{-1})	$T\frac{1}{2}\alpha$ (h)	β (h^{-1})	$T\frac{1}{2}\beta$ (h)	Vd_{area} (l/kg)	Plasma clearance (ml/kg/min)	$AUC^{0\rightarrow\infty}$ * [(μg/ml) · h]
3.35	0.207	0.182	3.81	0.18	0.502	457.827

*$AUC^{0\rightarrow\infty}$ represents the area under the plasma level vs. time curve from time zero to infinity as calculated by the trapezoidal rule to time t, plus the $AUC^{t\rightarrow\infty}$ as calculated by the concentration at time t/β.

DISCUSSION

The HPLC method described herein greatly simplifies sample handling procedures and avoids derivatization as required by previous GLC methods [8—11]. Despite the recent development of GLC methods which obviate derivatization of IBU [12, 13], the identification of interfering peaks associated with certain GLC support packings [17] introduces possible errors with this method of analysis. The larger plasma volumes (i.e., > 1.0 ml) necessary for previously reported methods [8—14] could also limit their application to detailed pharmacokinetic or bioavailability studies in pediatric populations.

The method described has demonstrated appropriate sensitivity (range of linearity = 0.5—100.0 μg/ml) with as little as 0.5 ml of serum. The simple extraction procedure, isocratic composition of the solvent system, rapid elution characteristics and application to automated analysis systems make it well suited for the study of IBU disposition and possible therapeutic monitoring of this drug. This is emphasized by findings for extraction efficiency, stability, variability, selectivity and a demonstrated lack of potential interfering substances. These are advantages to previously published methods for the analysis of IBU [8—14, 17].

In addition to demonstrating the application of our method to the study of IBU disposition in the dog, we have also shown that the method is qualitatively and quantitatively identical when applied to human serum. This is in contrast to a previously reported HPLC method illustrating only the detectability of IBU from canine serum [14].

While hemolysis did not appreciably alter the chromatogram of the extracted sample, comparison of the results determined from a standard curve prepared in serum from hemolyzed samples were significantly lower than those obtained from the analysis of non-hemolyzed samples. We have not at this time determined the etiology of this disparity in resultant IBU concentrations from hemolyzed samples. The reliability of IBU determination from hemolyzed specimens is questionable and results should not be reported for such samples, or used for data analysis.

CONCLUSION

We have developed a specific HPLC method for the determination of IBU

from human serum and have demonstrated its utility in a limited study of IBU disposition. Suitable application of this method to pharmacokinetic and bioavailability studies of IBU in both adults and children is apparent. We are currently evaluating IBU disposition in man using this method and are investigating its application to microanalytical procedures for the determination of free vs. protein-bound IBU in plasma, and for the determination of IBU in other biological fluids.

ACKNOWLEDGEMENTS

The technical assistance of Mrs. Sue Greene, Mrs. Deborah Lane and Mr. Philip Burns is appreciated. The editorial assistance of Dr. R. Don Brown and the skillful secretarial assistance of Mrs. Janis Doyle, Mrs. Carole Webb and Ms. Susie Fessler is gratefully acknowledged. We also thank The Boots Company and the UpJohn Company for supplying us with ibuprofen and 4-*n*-butylphenylacetic acid.

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