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Note

Determination of ibuprofen in human plasma by high-performance liquid chromatography

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Ibuprofen, D,L-2-(4-isobutylphenyl)propionic acid, is an anti-inflammatory, antipyretic, analgesic drug widely used in the treatment of arthritis. Many analytical procedures for the determination of ibuprofen have been reported including methods employing gas chromatography [1,2], gas chromatography–mass spectroscopy [3], and high-performance liquid chromatography (HPLC) [4–13]. Recently we became involved in a pharmacokinetic study with cystic fibrosis children requiring the ability to analyze a large number of samples of limited volume. We evaluated those methods referenced [1–13], carefully examined our needs, and developed a new procedure for the determination of ibuprofen in human plasma which is rapid, convenient, and precise.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of a Model 6000A pump, WISP-710B automatic sampler, RCM-100 radial compression module, analytical column (10 cm×0.5 cm Radial-PAK C_{18} of 10 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 measure-

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ment, peak-shape characterization, and calculations derived from those measurements.

Materials

Acetonitrile (UV grade), methanol, hexane (UV grade), and 2-propanol were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Reagent-grade water was prepared using a Milli-Q reagent-grade water system (Millipore, Bedford, MA, U.S.A.). Phosphoric acid (85%) and hydrochloric acid were purchased from Fisher Scientific (Cleveland, OH, U.S.A.).

Ibuprofen and ibufenac (4-isobutylphenylacetic acid) were generously supplied by Upjohn (Kalamazoo, MI, U.S.A.). Standard solutions of 200 μg/ml in methanol were prepared. All procedural solutions were made as dilutions, in methanol, of these stock solutions.

Sample preparation

To a 12 mm × 75 mm glass tube was added 50 μl of plasma, 10 μl of 1 *M* hydrochloric acid, 25 μl of ibufenac in methanol (50 μg/ml for high-level samples and 10 μg/ml for low-level samples), and 1 ml of hexane–2-propanol (85:15, v/v). The tube was vortexed for 2 s, the precipitate allowed to settle (10 min), and the supernatant poured into a second test tube. This supernatant was evaporated to dryness under a stream of compressed air, the residue reconstituted in 100 μl of the HPLC eluent, and 50 μl were injected into the HPLC system. Quantification standards were generated by aliquoting appropriate volumes of ibuprofen to 12 mm × 75 mm glass tubes, evaporating the methanol to dryness, and reconstituting in 50 μl blood bank plasma. Standard curves were generated (5–50 μg/ml for high-level standards and 1–10 μg/ml for low-level standards) to accommodate the concentration range of ibuprofen found in experimental samples.

Chromatography

The chromatography eluent was prepared by dissolving 4 ml concentrated phosphoric acid in 600 ml reagent-grade water and 400 ml acetonitrile. The eluent was pumped through the HPLC system at a flow-rate of 3.0 ml/min.

Quantification

Standard curves of ibuprofen/inbufenac peak-height ratios versus concentration were established daily. The ibuprofen concentration in experimental samples was 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 from the sample isolation procedure was demonstrated by external standardization. To six of twelve sample tubes were added 50 μl of ibuprofen (50 μg/ml in methanol) and the contents evaporated to dryness. To each of the twelve tubes were added 50 μl of blood bank plasma and 10 μl of 1 *M* hydrochloric acid followed by vortexing. To each was next added 25 μl methanol and 1 ml hexane–2-propanol (85:15, v/v). After vortexing the contents were

allowed settle for 10 min. The supernatant from each of tubes 1–6 was poured into an analogous tube containing 50 μl of ibufenac external standard (50 μg/ml); the contents from tubes 7–12 were poured into analogous tubes containing 50 μl of ibuprofen (50 μg/ml) and 50 μl of ibufenac external standard (50 μg/ml). The contents of all twelve tubes were evaporated to dryness under a stream of compressed air, the residue was reconstituted in 100 μl of the HPLC eluent, 50 μl were injected into the HPLC system, and ibuprofen/ibufenac peak-height ratios were determined. The ratio of the mean of the peak-height ratios from tubes 1–6 divided by the mean of the peak-height ratios of tubes 7–12 $\times 100\%$ expresses the recovery of ibuprofen. The recovery of ibufenac was determined in an analogous manner.

Two representative human plasma specimens (one within the 5–50 μg/ml standard curve and the second within the 1–10 μg/ml standard curve) were 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 human plasma ibuprofen analysis has the following features: (1) Sample size: the amount of required plasma/analysis is 50 μl. (2) Extraction: we have developed an extraction procedure which is extremely rapid. There is no centrifugation; the upper organic layer containing drug is simply poured from the sample tube. (3) Internal standard: we employed an extremely close chemical analogue of ibuprofen, ibufenac, for internal standardization. (4) Chromatographic peak characterization: this permitted a precise quality check of the chromatographic procedure leading to increased confidence in the procedure as a whole. To conclude, this method has good sensitivity (1 μg/ml), the chromatographic run time is brief (8 min), and the precision and chromatogrpahic characteristics are excellent.

Studies were undertaken to document the precision of this method. The recovery of ibuprofen from human plasma using the described extraction procedure was $84 \pm 3\%$ ($n=6$) and the recovery of ibufenac was $85 \pm 4\%$ ($n=6$). Two human plasma samples were prepared in replicates of six on three separate days and carried through the analytical procedure. The standard curves for the three days ($\pm$S.D.) were linear and described as follows: 5–50 μg/ml: slope $= 0.028 \pm 0.003$; y-intercept $= 0.005 \pm 0.034$; linear regression coefficient $= 0.997 \pm 0.001$; 1–10 μg/ml: slope $= 0.153 \pm 0.003$; y-intercept $= -0.011 \pm 0.007$; linear regression coefficient $= 0.99 \pm 0.01$. The intercept values were 3 and 7% of the peak-height ratios obtained for the standard curve low points of 5 and 1 μg/ml, respectively. The sample-to-sample variability study values (day 2) were 15.6 ± 0.4 and 4.5 ± 0.2 μg/ml ($\pm$S.D., $n=6$). The day-to-day variability study for these same plasma samples over the three days gave values of 16 ± 1 and 4.6 ± 0.2 μg/ml.

The methods referenced employ extraction [1–8], solid-phase extraction [9], and precipitation [10–12]. Our method combines precipitation and extraction. The internal standard solution is in methanol. After acidification, this methanol solution is added, causing the protein to precipitate. Instead of centrifugation, we

then add an extraction solvent. After vortexing, the previously precipitated protein adheres to the glass sample tube, allowing the organic layer, containing drug and internal standard, to be quickly and easily poured into a second tube, leaving the protein, etc. behind. After evaporation of this organic phase, the residue is reconstituted and injected. Although perhaps slightly more time-consuming than simple precipitation, this procedure is considerably more rapid than normal liquid–liquid extractions and more thorough than simple precipitation.

The selection of an appropriate internal standard is often a difficult choice. Of the methods cited, about half [1–4,7,8,13] selected internal standards containing structural features similar to ibuprofen. We [13] and Heikkinen [2] used ibufenac as the internal standard. Its selection and the concentration used were consistent with accepted ideas on internal standardization [14,15].

In describing an HPLC analytical procedure, it is helpful to report functions associated with peak and column performance characteristics. Using the method of Foley and Dorsey [16,17] for calculation of "Chromatographic Figures Of Merit" (CFOM), we developed the computer software for routine calculation of chromatographic column and peak descriptors [18]. We feel that chromatographic descriptors are invaluable for pharmacokinetic studies, where day-to-day precision must be maintained. The symmetry functions are used to help ensure that the chromatographic column has not undergone any noticeable degradation and that the peaks of interest remain free of chromatographic interference. Retention times are monitored to ensure their variability remains insignificant, allowing standardization by peak-height ratios to be performed. Internal standard peak-height variability is an indicator of overall precision from sample addition to injection. Since this peak height should be identical in all samples, any significant variation is an indication of some inherent problem. In summary, CFOM can help ensure that developed analyses remain "routine". The CFOM 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 or "skewedness"), N_{SYS} (efficiency of the separation, including chromatographic and non-chromatographic contributions to band broadening, expressed as theoretical plates), N_{MAX} (theoretical maximum separation efficiency in the absence of band broadening contributors), and RSE (relative system efficiency, N_{SYS}/N_{MAX}). Table I displays CFOM data generated during the three-day quality control study. The values for τ/σ_G and RSE are indicative of a high degree of peak symmetry for ibuprofen and ibufenac and high overall chromatographic system efficiency. The small ibufenac internal standard variability, despite the fact that these analyses were performed on three separate days using three different batches of eluent, was an indication of the excellent precision of the entire analytical procedure. Variability within a single day was as low as 6%. Subsequently, we have reduced the autosampler injection volume and now typically show same-day internal standard peak-height variability of 3%.

Fig. 1A is a chromatogram of 50 μl blood bank plasma carried through the analytical procedure, replacing the ibufenac internal standard solution with methanol. Fig. 1B is a chromatogram of 50 μl of plasma from a patient receiving

TABLE I

CHROMATOGRAPHIC PEAK DESCRIPTORS AND CHROMATOGRAPHIC FIGURES OF MERIT (CFOM) FOR IBUPROFEN AND IBUFENAC ACCUMULATED DURING THREE SEPARATE DAYS AS A PART OF THE QUALITY CONTROL STUDY

Values expressed as mean ± standard deviation

	High-level standards and samples (5–50 μg/ml)		Low-level standards and samples (1–10 μg/ml)	
	Ibufenac	Ibuprofen	Ibufenac	Ibuprofen
Number of injections	48	48	48	48
Number of injections suitable for calculation	48	47*	47*	43*
Retention time (min)	3.98 ± 0.09	5.7 ± 0.1	3.91 ± 0.09	5.8 ± 0.2
Peak-height variability of ibufenac internal standard**	11.5	–	10	–
Peak symmetry (τ/σ_G)	0.79 ± 0.08	0.79 ± 0.09	0.7 ± 0.1	0.6 ± 0.2
Efficiency (N_{SYS})	1975 ± 76	2112 ± 110	2028 ± 122	1976 ± 295
Relative system efficiency (RSE; N_{SYS}/N_{MAX})	0.62 ± 0.05	0.49 ± 0.05	0.7 ± 0.1	0.7 ± 0.1

*In a few cases, peaks were rejected for CFOM analysis. See ref. 18 for details.
**Expressed as ± the percentage standard deviation

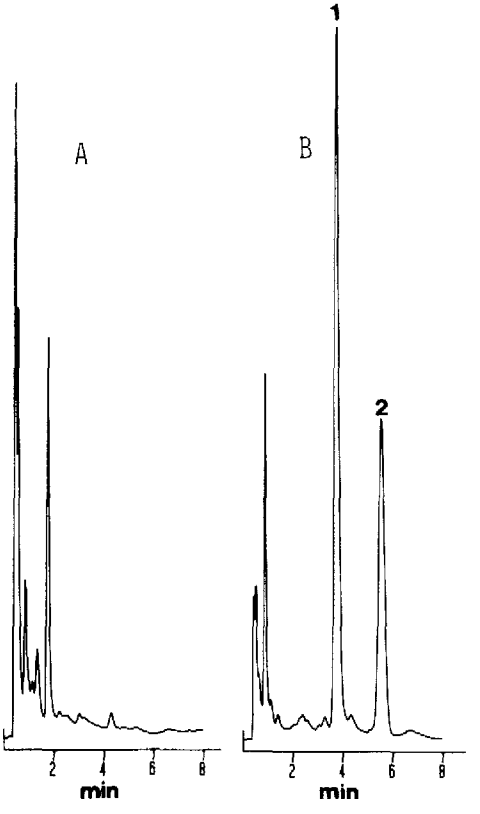

Fig. 1. Chromatograms of (A) 50 μl drug-free plasma carried through the analytical procedure and (B) 50 μl of plasma from a patient receiving ibuprofen and prepared as described in the text. Full scale absorbance is 0.025 absorbance units. Peaks: 1 = ibufenac, internal standard; 2 = ibuprofen. Ibuprofen quantification: 16.8 μg/ml.

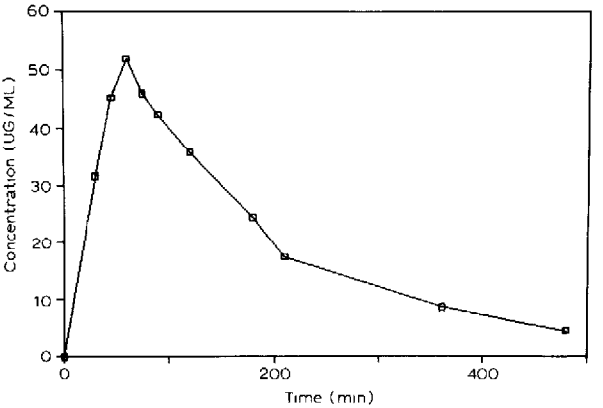

Fig. 2. Plasma ibuprofen concentration versus time curve following oral ingestion of 400 mg ibuprofen in a normal volunteer.

ibuprofen carried through the analytical procedure. The ibuprofen concentration is 16.8 μl/ml. The CFOM for ibufenac and ibuprofen are, respectively: τ/σ_G: 0.86 and 0.47; N_{SYS}: 2032 and 2242; RSE: 0.57 and 0.85. As is clear from this chromatogram, sensitivity is not a problem for normal patient samples. We investigated reducing the volume of plasma required for analysis from the stated 50 μl to 5 μl and found the results to be completely satisfactory. However, we did not anticipate needing a micro sample assay. In order to pipet a viscous fluid like plasma at volumes less than 50 μl one should probably use positive displacement pipetting devices, which are not as convenient or inexpensive as conventional pipetting devices. Therefore, we chose to use 50 μl of plasma/analysis in our routine procedure. Of the methods cited, only one uses less than 50 μl [10].

Fig. 2 is the ibuprofen plasma concentration versus time curve generated using this procedure for ibuprofen concentration determination for a normal volunteer following a morning 400-mg oral dose of ibuprofen.

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