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Note

Measurement of ibuprofen in human whole blood by reversed-phase ion-paired high-performance liquid chromatography using a pH-stable polymeric column

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Ibuprofen, *R,S*-2-(4-isobutylphenyl)propionic acid, is an orally administered non-steroidal anti-inflammatory agent used extensively in the treatment of arthritis [1]. The toxicological properties of ibuprofen in humans and animals have been studied extensively [1]. The metabolism of the parent drug in humans and animals has been well established [2]. After oral administration, ibuprofen is completely absorbed and metabolized in two major metabolites, 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid and 2-[4-(2-carboxypropyl)phenyl]propionic acid. It has been reported that approximately 60% of the parent drug is converted into the above two metabolites [3].

Ibuprofen possesses a chiral center in the propionic acid moiety (Fig. 1). Commercially, the drug is sold and administered in the racemic form. An in-

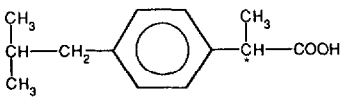

Fig. 1. Structure of ibuprofen. The asterisk indicates the chiral center.

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vivo chiral inversion of the inactive $R(-)$-enantiomer of ibuprofen to the active $S(+)$-enantiomer takes place during metabolism in humans and animals [4]. Because of the chiral inversion of ibuprofen, the concentration of the $S(+)$-enantiomer of the drug has been found to always be greater than that of the $R(-)$-enantiomer.

Paper chromatography [2], gas chromatography [5,6], and gas chromatography–mass spectrometry [7,8] have been used to determine ibuprofen and its metabolites in different sample matrices. Recently, high-performance liquid chromatography (HPLC) has been used extensively to measure ibuprofen and its metabolites in biological samples [9–15]. Most of the methods reported in the literature [5–15] require extensive labor-intensive sample work-up prior to chromatography. The chromatographic conditions of most of the methods are also complex.

This report describes a simple, selective and sensitive reversed-phase ion-paired HPLC method to determine the ingested parent drug (ibuprofen) in patients' whole blood sample. The pre-chromatography isolation of the drug was conducted by a simple one-step procedure. The alkaline mobile phase used in this method increased the selectivity of ibuprofen by decreasing the retention times of endogenous peaks which would otherwise elute with retention times similar to that of ibuprofen.

EXPERIMENTAL

Equipment

A Perkin-Elmer Series 410B solvent delivery system was used for mobile phase delivery (Perkin-Elmer, Norwalk, CT, U.S.A.). A Rheodyne 7275 sample injection valve equipped with a 200-μl loop was used for sample injection (Rheodyne, Cotati, CA, U.S.A.). A polymeric 15 cm$\times$4.1 mm reversed-phase (PRP-1) column, packed with 10-μm styrene divinylbenzene copolymeric particles was used throughout the method finally developed (Hamilton, Reno, NV, U.S.A.). A UV–visible detector (Spectroflow 783) from Applied Biosystem (Ramsey, NJ, U.S.A.) was used to monitor the parent drug. The signal from the UV–visible detector for the parent drug was recorded on a Houston Instruments (Austin, TX, U.S.A.) D5000 strip chart recorder. A Model 2200 Branson sonicator was used to degas the mobile phase (Branson Cleaning Equipment, Shelton, CT, U.S.A.). Deionized water was collected from a Milli-Q system (Millipore, Bedford, MA, U.S.A.). A vortex mixer from Scientific Industries (Bohemia, NY, U.S.A.) was used to vortex-mix the samples. A Gilson P-1000 digital pipette was used for all quantitative sampling (Gilson International, Middleton, WI, U.S.A.). The samples were centrifuged by an IEC centrifuge, Model HN (Damon, IEC Division, Needham Heights, MA, U.S.A.).

Materials

Acetonitrile and methanol (HPLC grade) were obtained from EM Science (Cherry Hill, NJ, U.S.A.). HPLC-grade isopropanol, *n*-propanol and ethanol were purchased from Aldrich (Milwaukee, WI, U.S.A.). Reagent-grade zinc sulfate was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Dibasic potassium phosphate (K_2HPO_4) was purchased from Mallinckrodt (St. Louis, MO, U.S.A.). The aged pooled whole blood was obtained from the Blood Center of Southeastern Wisconsin (Milwaukee, WI, U.S.A.). The borosilicate glass culture test tubes were purchased from American Scientific Products (McGaw Park, IL, U.S.A.). The gas-tight syringe and 0.45-μm Nylon-66 filter was purchased from Rainin (Woburn, MA, U.S.A.). Ibuprofen standard was purchased from Sigma (St. Louis, MO, U.S.A.).

Preparation of the stock solution

A stock solution of ibuprofen was prepared by dissolving 100 mg of ibuprofen in 50 ml of neat acetonitrile. The stock solution was stored at $-25\,^{\circ}C$ and was stable for at least six months. The stock solution was used to prepare the standard solutions of ibuprofen in whole blood and water (with appropriate dilutions) to construct the calibration curves.

Chromatographic conditions

The mobile phase of the chromatographic system consisted of acetonitrile (solvent A)–0.01 *M* K_2HPO_4 and 0.005 *M* tetrabutylammonium hydroxide (solvent B) (23:77, v/v). Solvent A and solvent B were pre-mixed before chromatography. The nominal pH of the mobile phase mixture was adjusted to 11.6 with dilute sodium hydroxide. The flow-rate of the mobile phase was 1.5 ml/min. The analytical column and the mobile phase were operated at ambient temperature ($25 \pm 2\,^{\circ}C$). The parent drug was monitored with a UV–visible absorbance detector at a wavelength of 220 nm and 0.10 to 0.005 a.u.f.s.

Purification of zinc sulfate and cleaning of borosilicate culture tubes

Purification of zinc sulfate ($ZnSO_4$) and cleaning of borosilicate culture tubes were done by using procedures described elsewhere [16].

Pre-chromatographic isolation of ibuprofen from whole blood

Isolation of ibuprofen from whole blood before chromatography was conducted by adding 0.25 ml of acetonitrile to 1.0 ml of whole blood. This solution was vortex-mixed for 2 min and centrifuged for 4 min at 2000 *g*. The supernatant was transferred into a fresh borosilicate culture tube, 20 mg of purified $ZnSO_4$ and 50 mg of K_2HPO_4 were added and the contents vortex-mixed for 1 min and centrifuged for 3 min at 2000 *g*. The supernatant was transferred into another fresh borosilicate culture tube with a Pasteur pipette. This solution

was filtered through a 0.45-μm Nylon-66 membrane with a gas-tight syringe. An aliquot (100 μl) of the filtered solution was injected into the HPLC system.

Construction of the calibration curves in whole blood and water

Two calibration curves of ibuprofen were constructed: one in whole blood and one in water. The standard solutions of ibuprofen in whole blood and water were treated identically as described in the *Pre-chromatographic isolation of ibuprofen from whole blood* section. Six solutions of ibuprofen (both in whole blood and water) were prepared at concentrations of approximately 200, 1000, 5000, 10 000, 15 000 and 20 000 ng/ml. A straight calibration curve of ibuprofen peak height versus concentration was constructed to quantify ibuprofen concentrations in patents' whole blood samples.

RESULTS AND DISCUSSION

A typical chromatogram of a human whole blood sample with no ibuprofen (blank) is shown in Fig. 2. Fig. 3 is a representative chromatogram of a whole blood sample from a patient who has orally ingested 200 mg of ibuprofen in tablet form. Inspection of Fig. 2 reveals that no chromatographic peak from the control whole blood elutes with the same retention time as ibuprofen (Fig. 3). The ibuprofen concentration determined in Fig. 3 was 2.30 μg/ml.

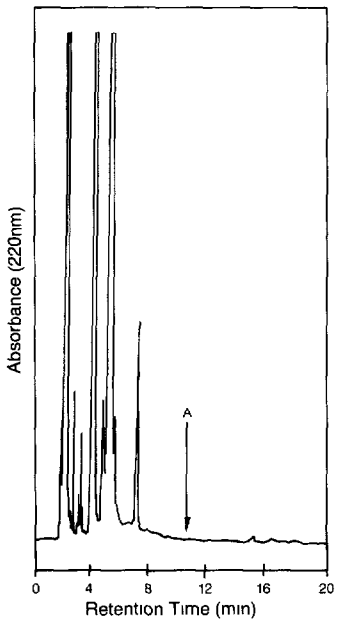

Fig. 2. Chromatogram of control whole blood (with no ibuprofen). "A" indicates the retention time of ibuprofen The detector was set at 220 nm and 0.05 a.u.f.s.

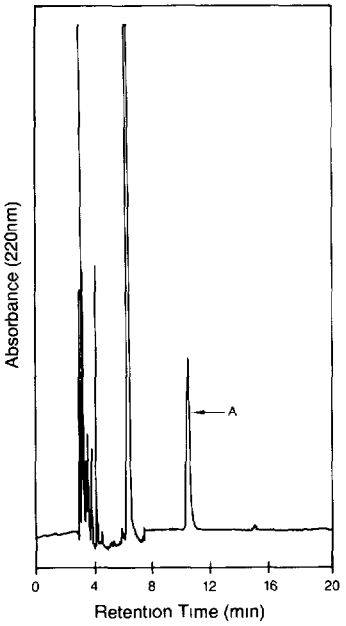

Fig. 3. Chromatogram of a patient's whole blood containing ingested ibuprofen "A" indicates the ibuprofen peak. The detector was set at 220 nm and 0.05 a.u.f.s. The ibuprofen quantitated was 2.30 μg/ml.

A standard calibration curve constructed by plotting peak height against concentration of ibuprofen was used to quantify ibuprofen in patients' whole blood samples. Two calibration curves of ibuprofen were constructed: one in whole blood and another in water. These calibration curves were constructed to determine if some ibuprofen co-precipitates with the whole blood endogenous substances. The slope of the calibration curve of ibuprofen in whole blood was 11% lower than the slope of the calibration curve of ibuprofen in water. This is a clear indication of the co-precipitation of ibuprofen with the whole blood endogenous substances during the sample clean-up procedure. Therefore, a calibration curve of ibuprofen in whole blood must be used to determine ibuprofen concentrations in patients' whole blood samples. The calibration curves of ibuprofen (in whole blood and water) were linear from 200 to 20 000 ng/ml with a correlation coefficient of 0.990 or higher.

Purification of $ZnSO_4$ and cleaning of borosilicate culture test tubes are critical for this method in order to obtain good reproducible chromatographic results. If $ZnSO_4$ and borosilicate tubes are used (without purification and cleaning) in the pre-chromatographic isolation of ibuprofen from whole blood, then huge interfering peaks overlap the ibuprofen peak in random occasions.

The addition of acetonitrile in the whole blood during sample preparation

precipitated significant amounts of proteins and other endogenous substances. The addition of $ZnSO_4$ in the supernatant of the above sample precipitated additional endogenous substances which were not affected by acetonitrile. The addition of dibasic potassium phosphate with the zinc sulfate minimized the co-precipitation of ibuprofen with the endogenous substances. If dibasic potassium phosphate is not used in the sample clean-up procedure, then the recovery of ibuprofen shows irreproducible results and declines more than 50%.

Investigation of analytical columns having different dimensions and packed with different types of reversed-phase stationary phases was conducted to find the one which gives maximum selectivity with minimum loss of sensitivity for the parent drug. To ensure complete and effective ion pairing of the ibuprofen molecule with tetrabutylammonium hydroxide, it was necessary to use an alkaline mobile phase with high pH. The nominal pH (11.6) of the mobile phase used in this experiment was required to increase the selectivity of ibuprofen. The silica-based octylsilane (C_8) and octadecylsilane (C_{18}) columns gave good column efficiencies, but they were very unstable due to the dissolution of silica back bone in the alkaline mobile phase. The column efficiency of the polymeric reversed-phase column (PRP-1) was lower than the silica bonded-phase column. However, the polymeric reversed-phase column (PRP-1) was very stable under the chromatographic conditions used in this experiment. The selectivity and efficiency of the PRP-1 column was adequate to obtain baseline resolution of the ibuprofen peak from the peaks of the endogenous substances of the whole blood.

The reproducibility and accuracy of the assay was determined by analyzing standard ibuprofen samples of whole blood spiked with aliquots of stock solution of ibuprofen. The data for the same-day and between-day analyses has been summarized in Table I and II. The reproducibility of the method (R.S.D.) ranged from 1.1 to 2.0% for the same-day analyses and from 1.2 to 3.3% for the between-day analyses. The accuracy of the method varied from 95 to 107% both for the same-day and between-day analyses.

TABLE I

REPRODUCIBILITY AND ACCURACY OF IBUPROFEN FOR SAME-DAY ANALYSIS

Actual concentration (ng/ml)	Concentration determined (mean ± S.D., $n=6$) (ng/ml)	Relative standard deviation (%)	Assay accuracy (%)
200	190 ± 3.8	2.0	95
1000	960 ± 19	1.9	96
5000	5200 ± 80	1.5	104
10000	10600 ± 200	1.9	106
30000	31500 ± 350	1.1	105

TABLE II

REPRODUCIBILITY AND ACCURACY OF IBUPROFEN FOR BETWEEN-DAY ANALYSIS

Between-day analyses were conducted every other day for seven days.

Actual concentration (ng/ml)	Concentration determined (mean ± S.D., $n=6$) (ng/ml)	Relative standard deviation (%)	Assay accuracy (%)
200	210 ± 7.0	3.3	105
1000	950 ± 23	2.4	95
5000	5250 ± 150	2.9	105
10000	10700 ± 170	1.6	107
30000	28900 ± 350	1.2	96

An experiment was conducted to test if ion pairing of the ibuprofen (anion form) with the tetrabutylammonium (cation) was the retention mechanism of the ibuprofen molecule under the chromatographic conditions used in this experiment. Sodium chloride was added in the mobile phase instead of tetrabutylammonium hydroxide and all other chromatographic conditions were kept constant. The ibuprofen molecule eluted with a capacity factor (k') of less than 1. This indicates that the retention of the ibuprofen molecule without tetrabutylammonium hydroxide is weak and elutes with a retention time similar to an unretained molecule. Therefore, it can be concluded that the retention time of the ibuprofen anion was due to the formation of an ion pair with the tetrabutylammonium molecule (the cation) in the mobile phase which has a strong hydrophobic site to interact with the PRP-1 stationary phase.

CONCLUSION

The reversed-phase ion-pairing HPLC method described in this report to determine the concentration of the parent drug in human whole blood is sensitive and simpler than most of the methods reported in the literature. This method requires only a simple one-step sample clean-up before chromatography and, thus, results in good analytical recovery of the parent drug. The variations for the same-day and between-day analyses were also minimal, even without using an internal standard. The elimination of the evaporation step (of the extracting organic solvent) from the procedure reduced the time of the sample preparation and also improved the precision and accuracy of the method. Aspirin, cimetidine, theophylline, ranitidine, and acetaminophen were tested for interference with ibuprofen and were all found to be negative.

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