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Validation of a liquid chromatographic method for the determination of ibuprofen in human plasma

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Abstract

A simple, rapid method of determining the ibuprofen concentration in small volumes of human plasma (50 μl) by HPLC was developed. The sample was prepared for injection using a solid-phase extraction method, with naproxen as the internal standard. A 96-well extraction plate was used, easing sample preparation and allowing the simultaneous extraction of multiple plasma samples directly into the HPLC injection vials. Samples were stable at room temperature for at least 48 h prior to injection. The HPLC method used an ultraviolet detector with a 5-min run time and measured concentrations across the range typically seen with the clinical use of this drug. The calibration curve was linear across the concentration range of 0.78–100 μg/ml with a limit of quantitation (LOQ) of 1.56 μg/ml. The coefficient of variation for intra-day and inter-day precision was 6% or less with accuracies within 2% of the nominal values for low (4.5 μg/ml), medium (40 μg/ml) and high (85 μg/ml) ibuprofen concentrations. For ibuprofen concentrations at the LOQ, the intra-day and inter-day precision and accuracy were within 10 and 15%, respectively. Recovery was 87% or greater for ibuprofen. This method was used to analyze plasma samples for unknown ibuprofen concentrations in bioequivalence and limited food effect studies of different formulations of ibuprofen. Thus, this method has been fully validated and used in the analysis of unknown plasma samples for ibuprofen.

Keywords: Ibuprofen

1. Introduction

Ibuprofen, a propionic acid derivative, was the first non-steroidal antiinflammatory drug to be commonly used for the treatment of pain and inflammation [1]. Because this is a commonly used drug, there are frequently studies in which ibuprofen concentrations must be determined.

We report a method for measuring ibuprofen concentrations in human plasma using a simple solid-phase extraction technique and a rapid HPLC method with naproxen used as the internal standard. The advantages of this method over frequently reported methods include the ease of sample preparation, the small amount of plasma sample required (50 μl), stability of the prepared sample prior to injection for at least 48 h at room temperature, and an HPLC

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analysis using an ultraviolet detector with a run time of only 5 min. Limitations to other reported methods include solvent evaporation in the extraction process [7–9], requirement of larger plasma volumes [6–9] or no validation in human plasma [10].

Also, this method was developed using a 96-well extraction plate further easing sample preparation by reducing the number of tube–tube sample transfers and allowing the simultaneous extraction of multiple plasma samples directly into the HPLC injection vials.

This method has been validated according to the criteria established by the Journal of Chromatography B [2] and the United States Center for Drug Evaluation and Research [3]. Included with this report, as proof of applicability, are the results of the analysis of human plasma samples for ibuprofen performed for bioequivalence studies supporting abbreviated new drug applications of new formulations of ibuprofen to the United States Food and Drug Administration (US FDA).

2. Experimental

2.1. Chemicals and reagents

Ibuprofen and the internal standard, naproxen, where both from United States Pharmacopeia primary reference standards (Ibuprofen: lot 1, catalog number 335508; Naproxen: lot 1, catalog number 457301). Methanol and acetonitrile were both HPLC grade and were obtained from Fisher (Houston, TX, USA). Phosphoric acid was purchased from Sigma (St Louis, MO, USA). Water used in this study was obtained using a Millipore water purification system (Bedford, MA, USA). Drug-free human plasma was obtained from Biochemed Pharmacologicals (Winchester, VA, USA). The sample for injection onto the HPLC was extracted using a Waters 96-well Oasis HLB 1 cc (30 mg) extraction plate (Waters Associates, Milford, MA, USA).

2.2. Apparatus

The chromatographic HPLC system included a Waters LC Module 1 with Waters Millenium 32 Chromatography Manager and a variable wavelength ultraviolet detector set at 220 nm (Waters, Milford, MA, USA). Analytical columns were Waters Symmetry C_{18} 5 μm 4.6×150 mm cartridge column with a Waters Symmetry C_{18} 5 μm 3.9×20 mm pre-column guard column. The mobile phase was 40% water (pH adjusted to 2.6 with phosphoric acid) and 60% acetonitrile. The mobile phase was degassed by continuous helium sparging at a rate of 30 ml/min. The mobile phase flow-rate was 2 ml/min at a column temperature of 40 °C.

2.3. Standard solutions

The ibuprofen stock solution was prepared as a 5 mg/ml solution in methanol. Standards for the calibration curves and quality control samples were then prepared using serial dilutions of this stock solution in drug-free human plasma. The standard solution of naproxen, the internal standard, was 0.1 mg/ml in methanol with 20 μl of this solution added to the plasma sample prior to extraction. To test for interfering peaks, human plasma was also spiked with caffeine, aspirin and acetaminophen at concentrations of 100 μg/ml and then extracted and analyzed.

2.4. Sample preparation

Samples were prepared for injection onto the HPLC column using a solid-phase extraction method with the Oasis HLB extraction plate. The plate wells were initially washed with 1 ml of methanol followed by 1 ml of water. Prior to being applied to the extraction plate, the plasma sample was centrifuged for 1 min at 10 000 rpm to pellet debris. Then 50 μl of plasma, 250 μl of dilute phosphoric acid (4% phosphoric acid by volume in water) and 20 μl of the internal standard solution were combined in a disposable polypropylene tube and mixed using a vortex. After mixing, this sample was applied to the extraction plate and drawn through using a vacuum. The plate was then washed with 1 ml of 5% methanol in water. The eluate was collected into injection tubes using 0.5 ml of methanol under vacuum and 25 μl was injected into the HPLC system.

2.5. Initial method validation

Calibration curves were developed using standards of ibuprofen in human plasma with nine concentrations varying between 0.78 and 100 μg/ml (3.8–484.8 μ*M*). Zero concentration solutions used in the calibration curves included a blank of plasma only and a blank of plasma plus internal standard.

The calibration curves were generated by plotting the ratio of the peak area of the ibuprofen and internal standard against the ibuprofen concentration in solution. The calibration curve was based on a simple linear model relating the ibuprofen concentration to the HPLC response. Linear regression techniques were used to assess the calibration curves with the determination of the correlation coefficient.

The limit of quantitation (LOQ) was defined as the ibuprofen concentration at which: (1) the ibuprofen peak was clearly identifiable and discrete, (2) the HPLC response was at least five times the response for the plasma blank and (3) a concentration at which there was reproducible precision (% coefficient of variation of less than 20%) and accuracy (determined concentration being within 20% variation of the nominal concentration).

Quality control (QC) solutions were at concentrations of 1.56, 4.5, 40 and 85 μg/ml of ibuprofen in human plasma representing, respectively, the limit of quantitation (LOQ) and low, medium and high concentration quality controls. The intra- and inter-day accuracy and precision were determined for each of these concentrations. Accuracy and precision were determined using six replicate preparations of plasma samples at these concentrations on three separate days using three separate sources of human plasma.

Recovery of ibuprofen was determined by comparing the response of six preparations at each of the QC sample concentrations to the response of the pure authentic standard. The recovery of naproxen was determined by comparing the mean response for the 48 internal standard injections for one study day (day 3 of the precision and accuracy analysis) to the detector response for the pure authentic standard.

Analyte stability was assessed for all phases of the storage and analytical process by determining the accuracy and precision of the measured concentration compared to the nominal concentration. Stock solution stability was determined for stock solutions stored at 4 °C for 15 days. Short term room temperature stability was assessed for low (3.5 μg/ml) and high (100 μg/ml) standards for thawed aliquots of ibuprofen in human plasma at room temperature for 24 h. Freeze–thaw stability was evaluated for these same low and high concentrations of ibuprofen in human plasma for three cycles of thawing at room temperature followed by re-freezing to −70 °C for 24 h. Post-preparative (or autosampler) stability was determined using low and high concentrations extracted and left at room temperature on the autosampler for 48 h prior to injection on the HPLC column. Long term stability was assessed using plasma ibuprofen samples at concentrations of 10, 30, 50 and 100 μg/ml stored at −70 °C for 30 months.

2.6. Proof of applicability

To demonstrate the applicability of this method, the authors report the results of the analysis of plasma samples for ibuprofen carried out in conjunction with studies evaluating the bioequivalence and limited food effects of new 200- and 800-mg formulations of ibuprofen tablets. The new formulations used (test products) were IBU®200 and IBU®800, Ibuprofen 200 and 800 mg film coated tablets by BASF (Shreveport, LA, USA). The reference listed products to which these were compared were Motrin®IB (Nuprin®) 200 mg tablets and Motrin® 800 mg tablets by McNeil Consumer Products (Fort Washington, PA, USA). These studies were designed, and the pharmacokinetic and statistical analysis performed, using methods described in guidance documents provided by the US FDA [4,5].

Bioequivalence studies were randomized, cross-over clinical trials in healthy, fasting adults in which subjects received one 200-mg dose of ibuprofen as either the test product or the reference listed product. After a washout period, they were then crossed over to receive the other formulation. Plasma samples were collected pre-dose and then at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, and 12 h post-dose with each of these formulations. From these data, the peak concentration (C_{max}) and the area under the concentration versus time curve for the study interval ($AUC_{0\text{-}t}$) and extrapolated to infinity ($AUC_{0\text{-}inf}$) were determined. The log of the ratio of test to

reference values for these parameters was determined for each subject [5]. The antilog of the averages of the logged data was then compared and bioequivalence defined as a ratio of the test to reference product being 80–125% [5]. This study was then repeated with the 800-mg tablet formulation.

Limited food effects studies were also carried out for 200- and 800-mg formulations. In summary, subjects were given the test and reference products after a meal in a random crossover trial with plasma sampling performed at similar times as previously described. A comparable food effect was defined as a percent variation within ±20% for the ratio of the mean value for these same pharmacokinetic parameters for the two formulations in fed subjects.

Different ranges of concentrations and different QC sample concentrations were used in the studies of 200- and 800-mg doses because of the anticipated differences in plasma concentrations following these doses. Thus, in the studies using ibuprofen doses of 200 mg, the range of calibration curve concentrations were from 0.78 to 75 μg/ml, while range of calibration curve standards in the studies of 800-mg doses was 0.78–100 μg/ml. The concentrations for QC samples in the 200-mg dose studies were 4.5, 30 and 65 μg/ml. The QC sample concentrations in the 800-mg dose studies were 4.5, 40 and 85 μg/ml.

3. Results

3.1. Assay validation

Standards of ibuprofen and naproxen were injected onto the HPLC column and found to elute at 3.1 and 1.7 min, respectively (Figure 1). Plasma obtained from six different individuals was tested for interference and showed no interfering peaks at these retention times. Plasma spiked with caffeine, aspirin and acetaminophen did not have any interfering peaks with ibuprofen and naproxen (Figure 1).

The concentration–response relationship for the calibration curve was described by a simple non-weighted regression analysis. The correlation coefficient of the calibration curve over the tested concentration range (0.78–100 μg/ml) was 0.999 on each of the 3 days on which calibration curves were run. The LOQ was consistently identified to be at 1.56 μg/ml using the defined criteria for acceptance.

The intra-day and inter-day precision (Table 1) were well within the limits for acceptance with coefficients of variation of less than 10% for the medium and high ibuprofen QC samples and 20% for the low QC sample and the sample at the LOQ [2]. Also, there was an acceptable level of accuracy across the range of QC sample concentrations tested for both intra-day and inter-day analysis (Table 1). The percent recovery for ibuprofen was consistently greater than 85% at all QC concentrations tested with a mean percent recovery of 93%. The mean percent recovery for naproxen was 79%.

Acceptable analyte stability was demonstrated for all phases of storage and processing. The accuracies of stock solutions of ibuprofen and naproxen stored at 4 °C for 15 days were 98 and 101%, respectively. The accuracy of low (3.5 μg/ml) and high (100 μg/ml) concentrations of ibuprofen in human plasma for 24-h room temperature stability (108 and 111%), three cycle freeze–thaw stability (100 and 103%), and 48-h post-preparative stability (88 and 99%) was also acceptable. Finally, the accuracy of plasma samples after 30 months of storage at −70 °C was 94, 100, 95 and 99% for ibuprofen concentrations of 10, 30, 50 and 100 μg/ml, respectively. The coefficient of variation for each of the stability experiments varied between 1 and 6%.

3.2. Proof of applicability

The calibration curves and QC samples in the bioequivalence and limited food effects studies for these formulations of ibuprofen demonstrated a high level of reproducibility. The correlation coefficients for the calibration curves for the studies of 200-mg ibuprofen tablets varied between 0.997 and 0.999 and varied between 0.998 and 0.999 for the calibration curves in the studies of 800-mg tablets. The limit of quantitation was consistently 1.56 μg/ml in these studies. In all there were 328 QC samples for all runs in all studies, and these demonstrated a high degree of precision and accuracy (Table 2). Of these QC samples, only one was outside the defined range of acceptance of ±15% of the nominal concentration. Thus, 99.7% of all QC samples in these studies met acceptance criteria.

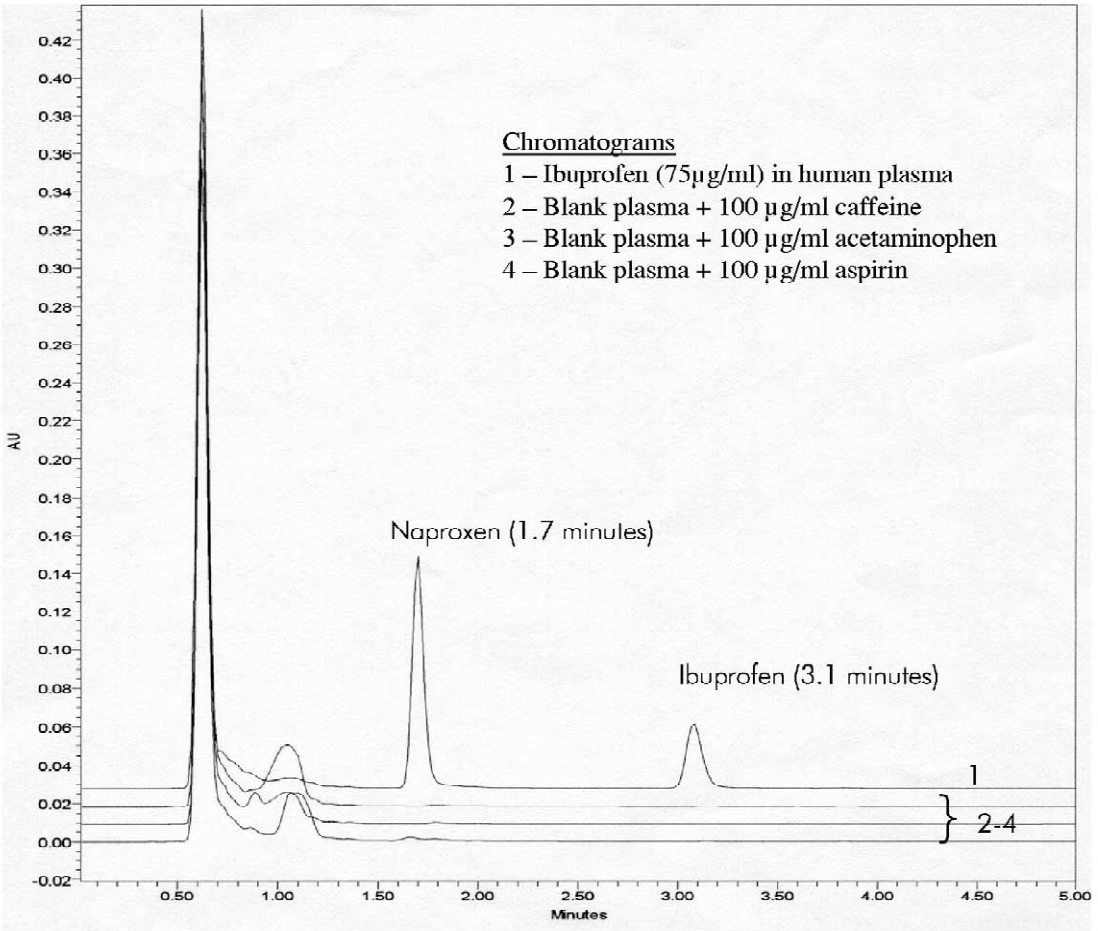

Fig. 1. Sample chromatogram of ibuprofen and naproxen in human plasma. A sample HPLC chromatogram of ibuprofen (75 µg/ml) and naproxen (internal standard) in human plasma (≠1). Also overlaid on this chromatogram are the chromatograms (≠ 2–4) of blank plasma spiked with caffeine, acetaminophen and aspirin (100 µg/ml each), demonstrating no interfering peaks.

The range of concentrations validated in these methods covered the range of concentrations observed in these bioequivalence and limited food effects studies, allowing clear description of important pharmacokinetic parameters (Table 3). There was no detectable ibuprofen in any of the pre-dose samples. The highest ibuprofen concentration in the studies using doses of 200 mg was 31.8 µg/ml, and, for doses of 800 mg, the highest concentration was 79 µg/ml. For the 200-mg tablet bioequivalence and limited food effects studies, ibuprofen was detectable in 85% of subject samples at 6 h post-dose and 55% of samples at 8 h, respectively, but only rarely detectable at 12 h. For the 800-mg studies, ibuprofen was detectable in 100% of 6-h samples, 99% of 8-h samples and 57% of 12-h samples. Thus, this assay has sufficient sensitivity to define the concentration versus time curve for usual doses of ibuprofen used in clinical settings, namely, 400- to 800-mg doses in adults every 6–8 h [1].

4. Discussion and conclusions

Reports are available in the medical literature which describe methods for the chromatographic analysis of ibuprofen which have utility in different settings [6–10]. However, there are also some limita-

Table 1
Assay validation: results of intra-day and inter-day validation in human plasma

QC sample	LOQ	Low	Medium	High
Nominal concentration (μg/ml)	1.56	4.5	40	85
Intra-day validation				
Number of samples	6	6	6	6
Mean concentration (μg/ml)	1.78	4.56	39.1	84.7
Standard deviation (μg/ml)	0.10	0.09	0.59	2.25
Coefficient of variation (%)	5.70	1.86	1.50	2.66
Accuracy (%)	114.3	101.3	97.9	99.6
Inter-day validation				
Number of samples	18	18	18	18
Mean concentration (μg/ml)	1.57	4.45	39.9	84.9
Standard deviation (μg/ml)	0.17	0.12	2.45	3.57
Coefficient of variation (%)	10.57	2.77	6.13	4.21
Accuracy (%)	100.6	98.8	99.8	99.9
Recovery (%)	101	92	87	90

tions to these methods, including solvent evaporation in the extraction process [7–9], larger plasma volumes required than used in the method described in this report [6–9] or no validation in human plasma [10]. While there is increasing interest in the measurement of the specific *R* and *S* enantiomers of ibuprofen, because of differences in their pharmacologic activity, the ability of the assay reported herein to differentiate between these enantiomers was not evaluated. As discussed by Bhushan and Martens [11], approaches to differentiate between these enantiomers require specific chiral techniques, including the use of chiral HPLC columns, derivatization with optically pure chiral reagents, chiral mobile phases and thin-layer chromatography with chiral selectors. Thus, this assay would not be expected to differentially measure the concentrations of specific ibuprofen enantiomers.

Table 2
Proof of applicability: results of analysis of ibuprofen quality control samples

QC sample	Low	Medium	High
Studies of 200-mg tablets			
Nominal concentration (μg/ml)	4.5	30	65
Number of QC samples	52	51	51
Mean concentration (μg/ml)	4.3	29.0	64.0
Standard deviation (μg/ml)	0.2	3.8	5.5
Coefficient of variation (%)	5.2	2.6	3.4
Accuracy (%)	95.6	98.2	99.5
Studies of 800-mg tablets			
Nominal concentration (μg/ml)	4.5	40	85
Number of QC samples	58	58	58
Mean concentration (μg/ml)	4.2	40.3	89.3
Standard deviation (μg/ml)	0.1	0.3	1.4
Coefficient of variation (%)	2.5	0.7	1.6
Accuracy (%)	93	101	105

This report describes a new method for the analysis of samples of human plasma for ibuprofen which will be useful in clinical and regulatory research. The volume of plasma required for extraction and analysis is only 50 μl, a useful characteristic allowing re-analysis of specimens, analysis of specimens in duplicate and for studies in which blood volumes will be limited (e.g. pediatric studies). The sample extraction methods are simple requiring few tube transfers and steps, especially if the 96-well extraction plate is used. There is also the added advantage of this extraction method that evaporation steps are eliminated and, using the extraction plate, the samples can be eluted directly into injection vials. Thus, the simplified extraction process would be expected to reduce the likelihood of laboratory error. The volume of sample available after extraction is 500 μl, of which only 25 μl is required

Table 3
Proof of applicability: pharmacokinetics assessing bioequivalence and limited food effects

	Mean pharmacokinetic parameters		
	C_{max} (μg/ml)	AUC_{0-t} (μg h/ml)	AUC_{0-inf} (μg h/ml)
Fasting studies of 200-mg tablets			
Test product[a]	18.4	58.6	65.8
Reference product[a]	18.9	57.9	66.3
Ratio of test to reference (%)[b]	97.7	101.1	99.0
Fasting studies of 800-mg tablets			
Test product[a]	58.1	208.0	218.4
Reference product[a]	62.8	213.4	220.8
Ratio of test to reference (%)[b]	91.5	96.5	98.0
Limited food effects studies			
Variation for 200-mg tablets (%)[c]	−13	+6.0	+17
Variation for 800-mg tablets (%)[c]	−6.8	+2.4	+8.8

[a] The test products were IBU®200 and IBU®800 by BASF Corporation. The reference listed products were Motrin®IB 200 mg tablets and Motrin® 800 mg tablets by McNeil Consumer Products Company.

[b] For bioequivalence studies, the ratio is defined as the antilog of the averages of the log of the ratio of the test to reference values for the individual subjects. Bioequivalence is defined as having the ratio between 80 and 125% for these pharmacokinetic parameters.

[c] The percent variation is the variation for the mean values of the test compared to the reference product in subjects taking the study drug after a meal. A comparable food effect is assumed by a percent variation of ±20% for these pharmacokinetic parameters.

for injection onto the HPLC. Thus, there is adequate sample available for multiple injections or for re-injection if necessary (e.g. in the event of HPLC failure). The extracted samples are stable for at least 48 h at room temperature, assuring sample stability while in an autosampler. Finally, this method uses a short run time of 5 min per HPLC sample run allowing faster laboratory throughput.

This method has been used to analyze plasma samples with unknown ibuprofen concentrations in bioequivalence and limited food effects studies supporting abbreviated new drug applications for these formulations of ibuprofen. These applications have received full approval from the US FDA (approval letters on file with BASF Corporation and the US FDA). The different doses used, indicate that this method has sufficient sensitivity within the range of ibuprofen concentrations expected with the clinical use of this drug.

Therefore, the report describes a fully validated method for the analysis of ibuprofen in human plasma and it meets the requirements for applicability for clinical utility and for clinical and regulatory research [2,3].

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