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Development and validation of a liquid chromatographic method for the quantitation of ibuprofen enantiomers in human plasma

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Abstract: A method for the quantitation of ibuprofen enantiomers in human plasma has been developed and validated. Separation of *R*- and *S*-ibuprofen was achieved on a silica-bonded β-cyclodextrin column with a mobile phase of acetonitrile–0.02% (v/v) triethylamine in water adjusted to pH 4.0 with glacial acetic acid in water (60:40, v/v). The UV detection was performed at 220 nm. The established linearity range was 1–25 μg ml^{-1} ($r > 0.99$). The limit of quantitation was designed as 1 μg ml^{-1} for each enantiomer. Interday precision and accuracy for the standards were 2.2–5.9% relative standard deviation (RSD) and −2.9–+3.5% relative error for *R*-ibuprofen, and 1.9–6.3% RSD and −7.1–+4.4% relative error for *S*-ibuprofen. Interday precision and accuracy for quality controls at 2.5, 7.5 and 17.5 μg ml^{-1} were 6.1–6.4% RSD and −1.4–+0.8% relative error for *R*-ibuprofen, and 5.7–5.9% RSD and −1.2–+2.8% relative error for *S*-ibuprofen. *p*-Isopropylbenzoic acid was used as an internal standard. The run time was 26 min. Interference from various lots of human plasma were not observed. Stability results of on-system, re-injection, bench-top, freeze–thaw cycles and sample storage were established.

Keywords: *R- and S-ibuprofen; enantiomers; LC chiral separation; β-cyclodextrin CSP.*

Introduction

Ibuprofen is a non-steroidal anti-inflammatory analgesic and antipyretic drug. It has one asymmetric centre (Fig. 1). *S*-ibuprofen exhibits pharmacological effects but the *R*-form is inactive. A unidirectional inversion from the *R*- *to S*-form occurs during metabolism [1–4]. This inversion was shown to occur both presystemically in the gastrointestinal tract [4] and systemically in circulation [5]. The *R*-isomer was shown to have higher protein binding than the *S*-isomer [6]. These recent results and other stereochemical issues raised further interest in question to the stereoselectivity of the bioavailability and disposition of *S*-ibuprofen, with aspects on dose-dependent pharmacokinetics and drug-interaction. The measurement of the ibuprofen enantiomers in body fluids is necessary for these studies.

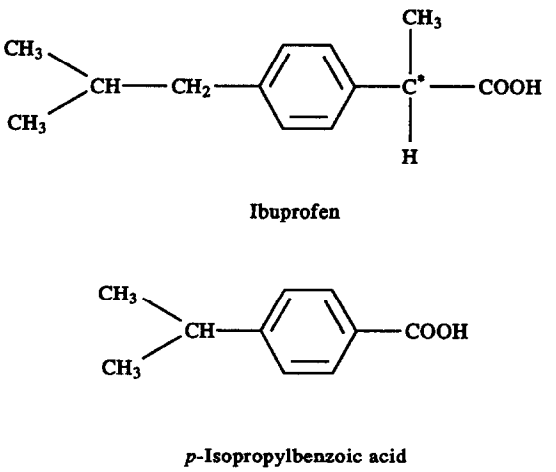

Figure 1
Chemical structure of ibuprofen and *p*-isopropylbenzoic acid (IS). The asterisk denotes the chiral centre.

A number of LC methods for the determination of ibuprofen enantiomers have appeared in the literature. Pre-column derivatizations on the carboxylic acid moiety to form diastereomers allowed subsequent separations by HPLC using achiral columns [7, 8]. These derivatization methods involve multiple derivatization and extraction steps and are time-consuming. Enantiomers of ibuprofen derivatized with 1-naphthalene methylamide or 3,5-dinitrobenzoylamide were separated on Pirkle-type covalent chiral columns [9, 10]. These methods have not been reported to be used for analysis of ibuprofen enantiomers in body fluids. Direct separation of ibuprofen enantiomers on an α_1-acid glycoprotein (AGP) column was described [11]. However, this

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method suffered from poor sensitivity due to the broad peak shape of ibuprofen enantiomers and to the limited load capacity of chiral AGP columns. Determination of the ratio of ibuprofen enantiomers on a β-cyclodextrin column has been reported using an additional conventional reversed-phase system to determine the racemic ibuprofen [12]. This required higher costs of setting up and maintaining two HPLC systems as well as more time on pharmacokinetic data treatment. The objective of this study was to develop a chiral separation method that was simple, rugged and applicable for the analysis of large numbers of samples in pharmacokinetic studies of ibuprofen.

Experimental

Materials and reagents

Racemic ibuprofen was obtained from the USP, the internal standard (IS) *p*-isopropylbenzoic acid was purchased from Sigma (St Louis, MO, USA). *R*- and *S*-ibuprofen were purchased from Research Biochem, Inc., (Natick, MA, USA). All other reagents were of analytical grade and purchased from Sigma or Fisher (Fair Lawn, NJ, USA). Deionized water was purified by a NANOpure® system from Barnstead. Control heparinized human plasma was purchased from Worldwide Biological (Cincinnati, OH, USA). Two primary stock solutions of ibuprofen were prepared from separate weighings for standards and quality control samples (QCs). Ibuprofen primary stock and substock were prepared in a solution of methanol–water (50:50, v/v). Working standards were prepared fresh daily by spiking 50 μl of 10-fold concentrated solutions into 0.5 ml of blank control heparinized plasma. The final concentrations of *R*- or *S*-ibuprofen in plasma standards were 1, 2, 3.5, 5, 10, 15, 20 and 25 μg ml^{-1}. Three levels of QCs, 2.5, 7.5 and 17.5 μg ml^{-1}, were prepared, aliquoted and stored frozen at −20°C.

Instrumentation

The LC system consisted of a Beckman 110B solvent delivery module, a Perkin–Elmer ISS-100 autosampler, a Waters 481 LC UV detector set at 220 nm, and VG® Multichrom data system from VAX®/VMS. The flow rate was 1.0 ml min^{-1} and the run time was set at 26 min. The analytical column, a β-cyclodextrin column (25 × 0.46 cm i.d.) and the guard β-cyclodextrin column (5 × 0.46 cm i.d.) were purchased from Astec. The temperature for the analytical column was maintained at 20°C by a Brinkmann RM6 cooling system. The mobile phase was acetonitrile–0.02% (v/v) of triethylamine (TEA) in water adjusted to pH 4.0 with glacial acetic acid (HAc) in water (60:40, v/v).

Data treatment

Chromatographic data were reduced using a VG® Multichrom data system. The raw data output was acquired on a VG® Chromserver and transferred to the VAX®/VMS. A weighted $1/y$ linear regression was used to determine slopes, intercepts and correlation coefficients, where y = the ratio of the compound peak height to the internal standard peak height. The resulting parameters were used to calculate concentrations: concentrations = [ratio − (y-intercept)]/slope.

Extraction procedures

To 0.5 ml of plasma sample, 150 μl of IS solution (100 μg ml^{-1}) and 100 μl of 2.4 M hydrochloric acid were added. After mixing, 5 ml of hexane–ethyl ether (4:1, v/v) was added to extract the compounds. Three millilitres of the organic phase was evaporated to dryness under nitrogen and reconstituted in 300 μl of mobile phase. A 30 μl volume was injected into the chromatograph.

Results and Discussion

Resolution

Figure 2(a–c) shows chromatograms of extracted blank control plasma, standard at 1 μg ml^{-1} and QC at 17.5 μg ml^{-1}. The peaks of *R*- and *S*-ibuprofen were sufficiently separated, enabling the accurate quantitation of *R*-ibuprofen at low concentration in the presence of *S*-ibuprofen at a disproportionately higher concentration as shown in Fig. 2(d). Selectivity towards naproxen, hydrocodone, ketoproten, acetylsalicylic acid and warfarin was investigated; none of them interfered with the quantitation of *R*- and *S*-ibuprofen. Separation of *R*- and *S*-ibuprofen was inferior with a mobile phase of acetonitrile–0.1% triethylaminonium acetate buffer, pH 7.5 (30:70, v/v) previously reported [12]. No interference was observed in any of the eight different lots of control plasma tested. The interaction of compounds and β-cyclodextrin is highly structure-dependent. Most of the interferences that were

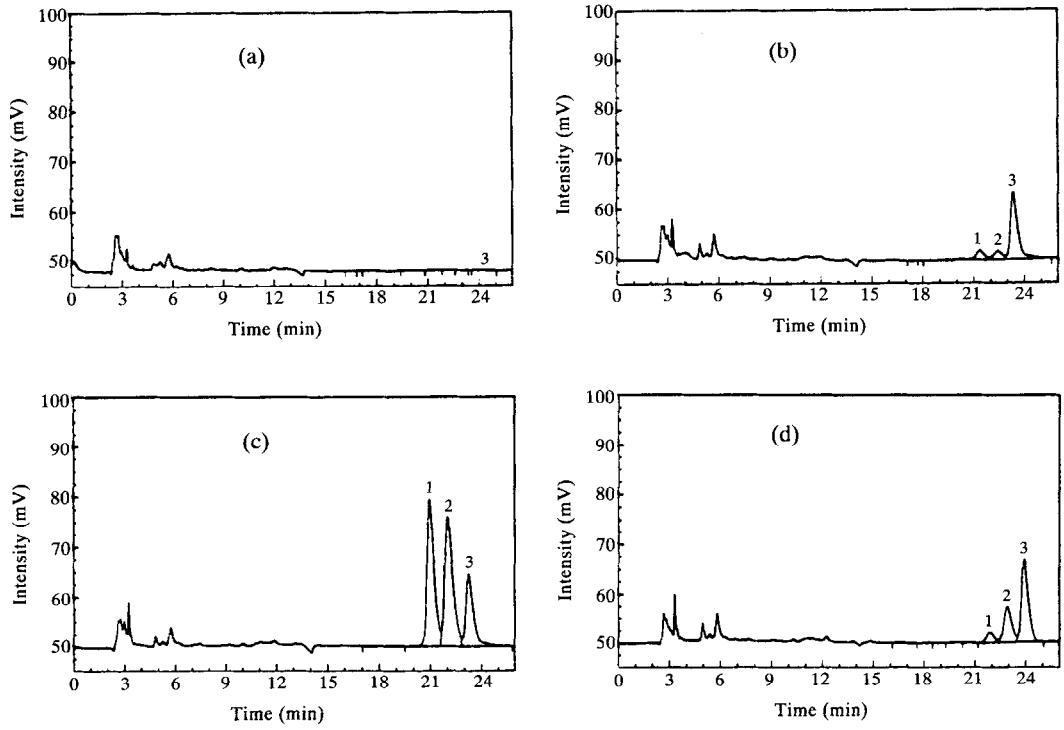

Figure 2
HPLC separation of extracted blank control plasma, standard, QC and test. (a) Blank control plasma; (b) standard at 1 μg ml^{-1} for each enantiomer; (c) QC at 17.5 μg ml^{-1} for each enantiomer; (d) test at 1 μg ml^{-1} for *R*-ibuprofen and 4 μg ml^{-1} for *S*-ibuprofen. Peak identification: 1, *R*-ibuprofen; 2, *S*-ibuprofen; 3, *p*-isopropylbenzoic acid (IS). HPLC conditions: Column, β-cyclodextrin (5 μm, 250 × 4.6 mm) fitted with a guard column β-cyclodextrin (5 μm, 50 × 4.6 mm) maintained at 20°C; mobile phase, acetonitrile–0.02% (v/v) triethylamine in water adjusted to pH 4.0 with glacial acetic acid (60:40, v/v); flow rate 1.0 ml min^{-1}; detection, 220 nm.

seen in many of the literature methods had weaker interaction with β-cyclodextrin than the ibuprofen enantiomers and were eluted out much earlier. The typical retention times were 21.0, 22.1 and 23.3 min for *R*-ibuprofen, *S*-ibuprofen and IS, respectively. The run time was 26 min. The analytical column was stable for up to at least 300 injections and no significant column-to-column variability on performance was observed.

Extraction recovery

A simple single step of liquid–liquid extraction was used for sample clean-up. Table 1 shows that the overall extraction recoveries were 93 and 94% for *R*-ibuprofen and *S*-ibuprofen, respectively. This simple extraction step allowed a large number of samples to be processed in a short time.

Validation

Seven validation curves were run on 7 separate days over a 3-week period. Table 2 shows the interday and intraday accuracy and precision of standard curves. The results showed excellent correlation coefficients for both *R*- and *S*-ibuprofen. The standards show a linear range of 1–25 μg ml^{-1}, with a limit of quantitation at 1 μg ml^{-1} (RSD% of 5.1 for *R*-ibuprofen and 6.3 for *S*-ibuprofen). The standard curve range and limit of quantitation are sufficient for most pharmacokinetic studies. Only one-tenth of the reconstituted sample was injected onto the analytical column. At a concentration of 1 μg ml^{-1} for each enantiomer the ratio of peak signal to the background noise is 50, indicating that a lower concentration of ibuprofen enantiomers can be quantitated. Since the baseline noise is very small, the limit of quantitation can be further significantly improved by injecting more sample. Table 3 presents the interday and intraday accuracy and precision of QCs. The accuracy and precision data show that this method is consistent and reliable with low values in relative error and relative standard deviation for standards and QCs at the entire concentration range.

Table 1
Recovery of *R*- and *S*-ibuprofen in human plasma

	R-ibuprofen (μg ml^{-1})			*S*-ibuprofen (μg ml^{-1})		
	1	10	25	1	10	25
Extracted peak heights	2738	24956	69939	2595	23107	63773
RSD (%)	6.2	6.4	8.6	5.2	6.6	8.6
Unextracted peak heights	2836	28877	72614	2645	26486	65779
RSD (%)	5.2	8.1	7.4	4.2	7.8	7.1
n	6	6	6	6	6	6
Recovery (%)	97	86	96	98	87	97
Mean recovery (%)		93			94	

RSD = relative standard deviation.
n = Number of determinations.

Table 2
Precision and accuracy of *R*- and *S*-ibuprofen standards

	Standard conc. (μg ml^{-1})								
Standard curve No.	1.00	2.00	3.50	5.00	10.0	15.0	20.0	25.0	*r*
R-ibuprofen									
A	0.893	1.99	3.67	4.98	10.6	15.9	19.6	24.0	0.9985
B	0.995	1.95	3.57	5.10	9.87	15.5	19.0	25.6	0.9991
C	1.04	1.92	3.40	4.97	9.87	16.0	21.2	23.3	0.9975
D	0.955	1.99	3.59	5.12	10.1	15.2	20.1	24.6	0.9998
E	0.924	1.97	3.64	4.82	10.6	17.0	20.1	22.9	0.9954
F	0.999	1.88	3.63	5.14	10.7	14.1	18.9	26.3	0.9978
G	0.989	2.01	3.34	5.03	10.7	14.9	20.1	24.5	0.9994
Mean	0.971	1.96	3.55	5.02	10.3	15.5	19.9	24.5	0.9982
RSD (%)	5.1	2.4	3.6	2.2	3.7	5.9	4.0	4.9	0.15
RE (%)	−2.93	−2.00	+1.39	+0.457	+3.49	+3.43	−0.714	−2.17	
S-ibuprofen									
A	0.831	1.98	3.81	5.17	10.8	16.0	19.6	23.6	0.9925
B	0.944	1.95	3.62	5.23	10.0	15.7	18.7	25.4	0.9986
C	1.03	1.89	3.35	5.10	10.0	16.2	21.1	23.1	0.9969
D	0.921	1.98	3.64	5.22	10.3	15.2	20.0	24.3	0.9995
E	0.906	1.96	3.67	4.82	10.6	17.1	20.1	22.8	0.9949
F	0.937	1.92	3.66	5.24	10.9	14.3	18.8	25.9	0.9976
G	0.937	1.99	3.40	5.18	10.9	15.1	20.0	24.1	0.9989
Mean	0.929	1.95	3.59	5.14	10.3	15.7	19.8	24.2	0.9977
RSD (%)	6.3	1.9	4.5	2.9	3.7	5.7	4.2	4.7	0.15
RE (%)	−7.06	−2.36	+2.65	+2.74	+5.00	+4.38	−1.21	−3.31	

r = Correlation coefficient.
RE = relative error.
RSD = relative standard deviation.

Stability

Stabilities of the processing (freeze–thaw, bench-top), chromatography (on-system and re-injection), and sample storage were tested and established. The data are presented in Table 4. QCs were subjected to various cycles of freezing and thawing. The samples were then analysed. Table 4 shows that both enantiomers were stable after multiple cycles of freezing and thawing. After three cycles of freezing and thawing, the values of QC samples were 99–100% of that for one cycle for *R*-ibuprofen, and 100–101% for *S*-ibuprofen. Bench-top stability after 4 h at room temperature was 100–102% for *R*-ibuprofen and 96–99% for *S*-ibuprofen, compared with the corresponding values of normal time. The on-system stability after 22 h was 101–105 and 98–101% for *R*- and *S*-ibuprofen, compared with the corresponding values at the original time. Samples re-injected after 24.5 h were 101–106 and 100–102% of the original corresponding values for *R*- and *S*-ibuprofen. Sample storage stability was tested after QCs were stored in a −20°C freezer for 56 days. The values of the stored samples were 94–107 and 95–108% of

Table 3
Precision and accuracy of *R*- and *S*-ibuprofen quality controls

	R-ibuprofen (μg ml^{-1})			*S*-ibuprofen (μg ml^{-1})		
	2.50	7.50	17.5	2.50	7.50	17.5
Interday						
Mean	2.49	7.64	17.4	2.52	7.78	17.4
RSD (%)	8.2	7.8	7.4	7.8	7.3	7.0
RE (%)	−0.28	+1.88	−0.75	+0.77	+3.79	−0.54
n	42	42	41	42	42	41
Intraday						
Mean	2.44	7.56	17.2	2.45	7.69	17.2
RSD (%)	3.1	5.9	8.2	3.5	6.0	8.2
RE (%)	−2.5	+0.8	−1.8	−2.1	+2.6	−1.6
n	6	6	6	6	6	6

RSD = Relative standard deviation.
RE = Relative error.
n = Number of determinations.

Table 4
Stability of quality-control samples in human plasma

	R-ibuprofen (μg ml^{-1})			*S*-ibuprofen (μg ml^{-1})		
	2.50	7.50	17.5	2.50	7.50	17.5
Freeze–thaw stability						
(*n* = 6)						
1st Cycle	2.35 (3.6)	7.31 (4.1)	16.6 (2.3)	2.44 (2.3)	7.56 (3.9)	16.7 (2.4)
2nd Cycle	2.33 (2.7)	7.28 (2.9)	16.6 (1.9)	2.42 (5.0)	7.50 (2.9)	16.7 (1.9)
As % of 1st Cycle	99	100	100	99	99	100
3rd Cycle	2.33 (3.0)	7.32 (1.7)	16.5 (1.8)	2.47 (3.4)	7.60 (1.1)	16.7 (1.9)
As % of 1st Cycle	99	100	99	101	101	100
Bench-top stability						
(*n* = 6) 0 h	2.49 (6.2)	7.56 (6.1)	17.3 (6.4)	2.51 (5.7)	7.71 (6.1)	17.3 (5.9)
4 h	2.53 (2.6)	7.74 (2.1)	17.3 (3.6)	2.42 (2.4)	7.57 (2.2)	17.2 (3.9)
As % of 0 h	102	102	100	96	98	99
On-system stability						
(*n* = 3) 0–50 min	2.34 (1.6)	7.25 (0.9)	16.7 (0.7)	2.38 (1.3)	7.35 (1.2)	16.6 (0.3)
22 h	2.46 (1.4)	7.49 (1.6)	16.8 (0.8)	2.34 (1.1)	7.46 (1.7)	16.8 (0.3)
As % of 0–50 min	105	103	101	98	101	101
Re-injection stability						
(*n* = 3) initial	2.34 (1.6)	7.25 (0.9)	16.7 (0.7)	2.38 (1.3)	7.35 (1.2)	16.6 (0.3)
24.5 h	2.47 (1.9)	7.48 (0.7)	16.9 (1.2)	2.37 (1.5)	7.46 (0.8)	16.9 (0.7)
As % of initial	106	103	101	100	101	102
Sample storage stability						
(*n* = 6) initial	2.39 (4.4)	7.67 (3.2)	18.3 (2.1)	2.40 (4.3)	7.74 (3.1)	18.1 (2.1)
56 days	2.56 (4.5)	7.44 (4.6)	17.5 (10.4)	2.58 (4.9)	7.60 (4.7)	17.2 (8.3)
As % of initial	107	97	94	108	98	95

RSD (%) are indicated in parentheses.

the corresponding values at the original assay for *R*- and *S*-ibuprofen.

The stability of the enantiomers against interconversion was tested during the extraction step and subsequent analysis. Samples containing 1 μg ml^{-1} of *R*-ibuprofen and 4 μg ml^{-1} of *S*-ibuprofen were prepared. Table 5 shows that there was no enantiomer interconversion during extraction or analysis, since none of the enantiomer concentrations was significantly changed after sample extraction and upon re-injection after 32 h.

Conclusions

A simple and reliable HPLC method for the quantitation of underivatized ibuprofen enantiomers in human plasma was developed. The

Table 5
Extraction and on-system stability against racemization

	0–75 min mean conc.		32–34 h mean conc.	
	R-ibuprofen (1 μg ml^{-1})	*S*-ibuprofen (4 μg ml^{-1})	*R*-ibuprofen (1 μg ml^{-1})	*S*-ibuprofen (4 μg ml^{-1})
	0.89	3.80	0.98	4.23
	0.88	3.82	0.92	4.17
	0.95	4.05	0.95	4.09
Mean	0.91	3.89	0.95	4.16
RSD (%)	4.2	3.6	3.2	1.7
RE (%)	−9.0	−2.8	−5.0	+4.0
n	3	3	3	3

method was validated to meet the pharmaceutical industry guidelines [13], with additional tests on enantiomer interconversion through processing and chromatography. No degradation and transformation of enantiomers were observed during the extraction and injection process. Unlike other methods using complicated derivatizing procedures, relatively unstable protein columns, or multiple HPLC systems, this simple method reported here can be handled and the system maintained with ease.

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