Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 796 (2003) 413–420
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Enantioselective determination of ibuprofen in plasma by high-performance liquid chromatography–electrospray mass spectrometry

Pierina Sueli Bonato*, Maria Perpetua F.M. Del Lama, Roberto de Carvalho

Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café S/N, 14040-903 Ribeirão Preto-SP, Brazil

Received 5 May 2003; received in revised form 5 August 2003; accepted 20 August 2003

Abstract

The enantioselective analysis of ibuprofen (IBU), a chiral nonsteroidal anti-inflammatory drug, in human plasma was carried out by high-performance liquid chromatography (HPLC)–mass spectrometry (LC–MS–MS). The plasma samples were prepared by liquid–liquid extraction using hexane:ethyl acetate (8:2, v/v). The HPLC chiral resolution of IBU was obtained using a chiral stationary phase based on a tris-(3,5-dimethylphenylcarbamate) amylose derivative, under reversed phase conditions (CHIRALPAK AD-RH column), using a mobile phase consisting of methanol:water (8:2, v/v), containing 0.1% of an aqueous solution of phosphoric acid at pH 2, at a flow rate of 0.6 ml/min. A make-up liquid of 4.5% (w/v) NH_4OH aqueous solution was used to assure optimum electrospray ionization in the negative mode. The coefficients of variation and deviation from nominal values were lower than 15% for both within- and between-day assays. The quantitation limit was 0.12 μg/ml and the linear range was 0.12–90.0 μg/ml for both enantiomers. The method proved to be suitable for single dose pharmacokinetic studies.

Keywords: Enantiomer separation; Ibuprofen

1. Introduction

Ibuprofen [(±)-(*R*,*S*)-2-(4-isobutylphenyl)propionic acid] (IBU), is a chiral nonsteroidal anti-inflammatory drug widely used for the treatment of several rheumatic and musculoskeletal diseases. Although ibuprofen is used as a racemic mixture, its anti-inflammatory action is mainly associated with the (+)-(*S*)-enantiomer [1–3]. Ibuprofen is extensively metabolized via the oxidation and glucuronidation routes showing selectivity for (+)-(*S*)-ibuprofen. In addition, unidirectional inversion of the (−)-(*R*)- to (+)-(*S*)-enantiomer also occurs in vivo [2,4–6]. As a consequence, plasma concentrations of (−)-(*R*)- and (+)-(*S*)-ibuprofen differ significantly, resulting in stereoselective pharmacokinetic parameters [5].

To evaluate the stereoselective kinetic disposition of ibuprofen, selective, sensitive and reproducible enantioselective analytical methods are required for the quantification of the isolated enantiomers in

* Corresponding author. Tel.: +55-166024261; fax: +55-166332960.
E-mail address: psbonato@fcfrp.usp.br (P.S. Bonato).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.08.031

plasma samples. High-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have been used for the development of these methods. HPLC methods for the enantioselective analysis of ibuprofen are based on the chiral derivatization of the drug [7–13] and on the use of chiral stationary phases [7,14–19]. The chiral analysis of ibuprofen or its metabolites in biological samples by CE has been done using maltooligosaccharides/trimethyl β-cyclodextrin [20,21], vancomycin [22] and sulphated β-cyclodextrin [23] as chiral selectors.

Among the chiral stationary phases used for the enantioselective analysis of drugs, amylose and cellulose derivatives have shown great resolving power, both under normal phase and reversed phase conditions [24–26]. Based on this, we selected a new amylose based chiral column, CHIRALPAK AD-RH, that has the advantage of being used under reversed phase conditions, to develop a sensitive and selective method for the analysis of ibuprofen in plasma.

The recent technological advances in coupling mass spectrometry to liquid chromatography (LC–MS and LC–MS–MS) brought new insight into quantitative bioanalysis, including enantioselective analysis. The use of conventional columns such as C8, C18, CN involves no serious problem with mobile phase selection because the atmospheric pressure ionization interfaces were developed to be used with this kind of column [27,28]. However, chiral columns require specific mobile phases for the resolution of the sample that are not compatible with the MS system. Protein-based stationary phases require inorganic buffers (phosphate buffer) and/or high aqueous mobile phases, which can lead to significant ion-suppression during MS detection [29]. To overcome this problem, a post-column addition of organic solvents could be used. The most used chiral columns, i.e. polysaccharide-based stationary phases, are frequently used with mobile phases based on hexane, a flammable solvent that requires safety measures when used in conjunction with electrospray or atmospheric pressure chemical ionization interfaces. The use of a make up liquid such as acetate/isopropanol mixtures before the column effluent enters the MS interface avoids the risk of explosion and improves analyte ionization [29].

As a result, there are still relatively few methods described in the literature for the enantioselective analysis of drugs and metabolites in biological samples using this powerful technique. Ikegawa et al. [30] reported the use of LC–MS for the resolution of ibuprofen glucorunides (diastereoisomeric isomers) using a non chiral reversed phase column. CE–MS chiral analysis of ibuprofen has been reported in the literature by Tanaka et al. [31] and Fanali et al. [22], but not with applications to biological samples. In addition, this is the first report on the use of chiral LC–MS–MS for the analysis of ibuprofen in plasma samples.

2. Experimental

2.1. Chemicals and reagents

rac-IBU (99.9%) was kindly supplied by Knoll Pharmaceuticals (Nottingham, England). Stock standard solutions were prepared with methanol (HPLC grade) in the concentration range of 4.8–3600 μg/ml and were stable for at least 3 months when stored at −20 °C. Spiked plasma samples were obtained by the addition of 25 μl of these standard solutions to 0.5 ml plasma prior to extraction. The internal standard solution, naproxen, was prepared with methanol at the concentration of 50 μg/ml.

All chemicals were of analytical grade or HPLC grade and were purchased from Merck (Darmstadt, Germany) or EM Science (Gibbstown, NJ, USA). The water used to prepare the solutions or mobile phase was purified in a Milli-Q-plus system (Millipore, Bedford, MA, USA).

2.2. Equipment and methods

A Shimadzu (Kyoto, Japan) HPLC system consisting of two LC10AD solvent pumps, an SLC 10A system controller, a CTO-10AS column oven and a 7125 Rheodyne injector with a 20 μl loop was used. Separations were carried out at 22 °C on a CHIRALPAK AD-RH column (150 mm × 4.6 mm i.d., 5 μm particle size, Chiral Technologies, Exton, PA, USA). A CN guard column (4 mm × 4 mm i.d., Merck, Darmstadt, Germany) was used to protect the analytical column. The mobile phase for the analysis of IBU and the internal standard consisted of methanol:water (8:2, v/v), containing 0.1% of an aqueous solution of phosphoric acid at pH 2, at a flow rate of 0.6 ml/min.

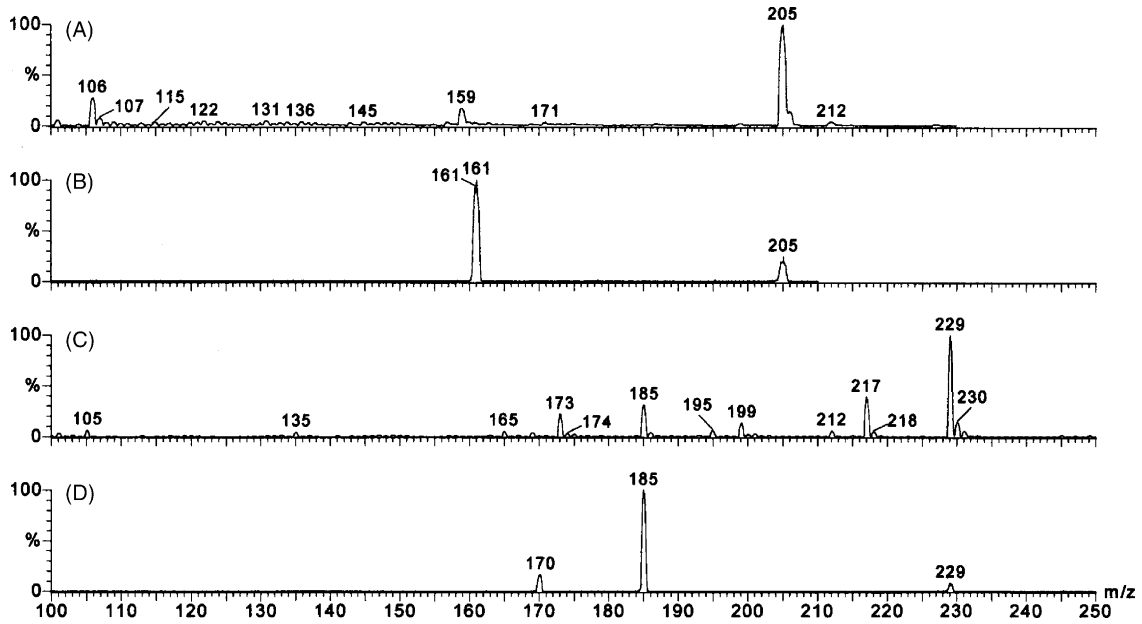

Fig. 1. Negative electrospray scan spectrum of ibuprofen (A) and naproxen (C) and product ion scan spectrum of ibuprofen (B) and naproxen (D).

The MS system was a Quatro LC triple-stage quadrupole (Micromass, United Kingdom), fitted with a Z-electrospray interface operating in the negative ion mode and calibrated with sodium iodide/cesium iodide in the 50–2000 Da range. The source block and desolvation temperatures were 100 and 250 C, respectively. Nitrogen was used as both drying and nebulizing gas and argon was used as collision gas. Cone (15 and 12 V for ibuprofen and internal standard, respectively) and collision cell (12 and 10 eV for ibuprofen and internal standard, respectively) voltages as well as other MS parameters were optimized by direct infusion of ibuprofen and internal standard solutions prepared in the mobile phase:4.5% NH_4OH solution (5:1) at a flow rate of 20 μl/min. The HPLC eluent was split by a Valco valve and a flow rate of approximately 0.1 ml/min was further mixed with 4.5% (w/v) NH_4OH solution (delivered by a syringe pump at a flow rate of 20 μl/min) and introduced into the stainless steel capillary probe.

Quantitation was performed by MRM (dwell time of 0.4 s) of the deprotonated molecules ($[MH]^-$) and their corresponding product ion (Fig. 1) using an internal standard calibration method with peak area ratios and $1/x$ weighting. The peak area ratios for calibration curves and quantitation were obtained using a Micromass Masslynx 3.0 software.

2.3. Extraction procedure

Plasma samples (0.5 ml) were spiked with 25 μl of the internal standard solution (naproxen, 50 μg/ml), acidified with 300 μl 1 mol/l HCl solution and extracted with 3 ml hexane:ethyl acetate (8:2, v/v). The tubes were capped and submitted to mechanical shaking at 200 rpm for 20 min and then centrifuged at 1800 × *g* for 5 min. The upper organic phases were then transferred to conical tubes and evaporated dry under an air flow at room temperature. The residues were dissolved in 100 μl of the mobile phase and 20 μl were chromatographed.

2.4. Method validation

To determine absolute recovery, plasma samples spiked with 0.12, 0.60, 9.0 and 45 μg/ml of each IBU enantiomer were extracted in triplicate by the procedure proposed. The organic phases were transferred to clean tubes and after the addition of 25 μl of the internal standard solution, the solvent was evaporated

under an air flow. The concentrations of these samples were determined on the basis of a calibration curve obtained with the data for the analyte and internal standard not submitted to extraction.

The linearity of the analytical method was assessed in the plasma concentration range of 0.12–90 μg/ml for each ibuprofen enantiomer.

Sensitivity was evaluated by determining the quantitation limit. Plasma samples ($n = 5$) were spiked with ibuprofen at the concentrations of 0.12 μg/ml, and analyzed under the conditions previously established. Criteria for the quantitation limit was established as the lowest concentration of the calibration curve, provided the deviation from nominal value were lower than 20%.

The precision and accuracy of the method were evaluated by within-day ($n = 10$) and between-day ($n = 5$) assays using plasma samples spiked with ibuprofen at the concentrations of 0.4, 4.0 and 40.0 μg/ml of each enantiomer and the results obtained were expressed as relative standard deviations (coefficient of variation, CV) and relative error.

2.5. Preliminary human experiment

In order to evaluate the applicability of the method, several plasma samples collected from a healthy volunteer after administration of a single dose of 600 mg of (*R,S*)-ibuprofen (Advil®, Wyeth-Whitehall Ltd.) were analyzed under the conditions established in the present study. Venous blood was drawn into heparinized tubes immediately before and 0.5, 1, 1.5, 2, 5, 7, 9 and 12 h after drug administration. After centrifugation for 10 min at 1800 × *g*, plasma samples were transferred to clean tubes and stored at −20 °C until analysis. The volunteer gave written informed consent to participate in the study, which was approved by the Ethics Committee of Escola de Enfermagem de Ribeirão Preto, USP (0081/2000).

3. Results and discussion

3.1. Optimization of the LC–MS–MS method

The chiral resolution of ibuprofen in biological samples has been carried out using several chiral columns, particularly those based on proteins [14–16,19]. The major problem in using these columns when MS detection is performed is the incompatibility of the mobile phase with the most frequently used ion sources, electrospray and atmospheric pressure chemical ionization, due to the use of non volatile buffers and high aqueous mobile phases. On the other hand, polysaccharide-based stationary phases, also used for the resolution of ibuprofen [7], are frequently employed with mobile phases consisting of hexane–alcohol that also require a post-column addition of additives (i.e. acetate buffer/isopropanol) to avoid the risk of explosion inside the ion source and to improve analyte ionization.

Based on this, we selected a new amylose tris-(3,5-dimethylphenylcarbamate) derived stationary phase that could be used under reversed phase conditions but with relatively mild conditions. Furthermore, the CHIRALPAK AD-RH column used in the present study has not been described for the chiral resolution of ibuprofen. The use of neutral mobile phases (methanol:water or acetonitrile:water) or mobile phases acidified with formic or acetic acid resulted in non symmetric peaks or unacceptable resolution. The chiral resolution of ibuprofen (Rs = 1.25, $\alpha = 1.20$) was only possible when a trace amount of phosphoric acid was used in the mobile phase (0.1% of an aqueous solution of phosphoric acid at pH 2). This mobile phase is compatible with the chiral column but it could be dangerous for the MS system; to avoid this effect the concentration of the phosphoric acid solution was maintained as low as possible, although this resulted in a detrimental effect on column efficiency and chiral resolution. Although the use of this slightly acidic solution as the mobile phase allowed the detection of the drugs, the calibration curves observed were not linear in the range established (0.12–90 μg/ml) as a result of non reproducible analyte ionization. Attempts for optimization of cone, collision cell, and multiplier voltage, etc. did not resulted in linear calibration curves. This problem was solved when the column effluent was mixed with a 4.5% NH_4OH solution before entering the electrospray ion source. Although only a sixth part of the column effluent was introduced into the ion source and the additional dilution by post-column addition of the NH_4OH solution, the method was sensitive enough to obtain detection limits of 1 ng/ml (S/N ratio = 3). Fig. 2 shows the total ion and selected reaction monitoring chromatograms for ibuprofen and internal standard (naproxen) under

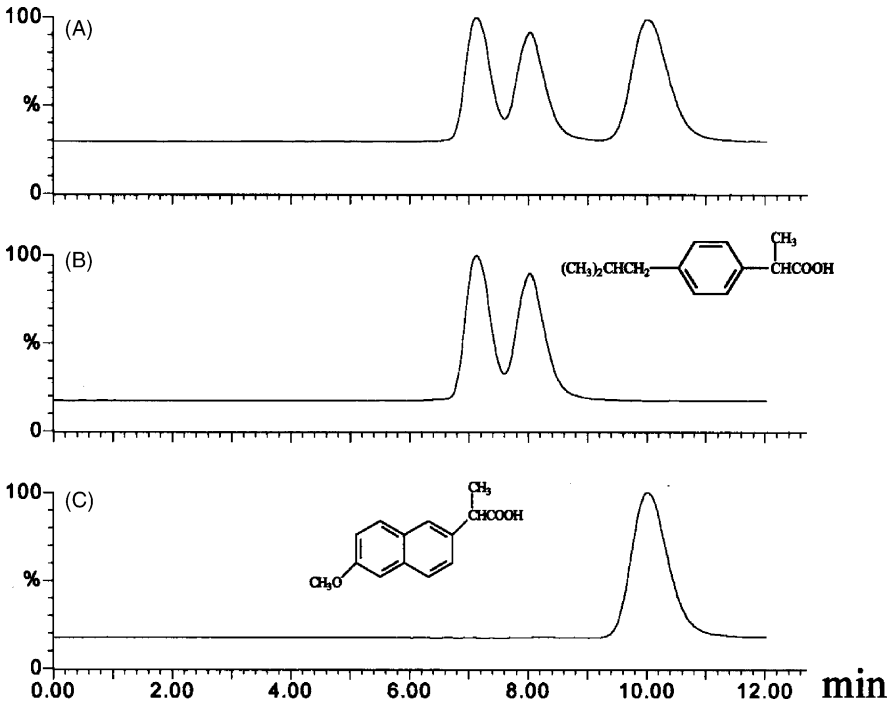

Fig. 2. Total ion (A) and multi reaction monitoring chromatograms for ibuprofen (B) and naproxen (C). Conditions: CHIRALPAK AD-RH column; mobile phase consisting of methanol:water (8:2, v/v), containing 0.1% of an aqueous solution of phosphoric acid at pH 2; flow rate of 0.6 ml/min; make-up liquid: 4.5% NH_4OH aqueous solution. Elution order: (−)-(*R*)-ibuprofen followed by (+)-(*S*)-ibuprofen.

the optimized conditions. The elution order was established on the CHIRALPAK AD-RH column using individual enantiomers previously isolated and characterized by semi-preparative chromatography on a CHIRALCEL OJ column [23].

3.2. Validation of the methods

The developed method was validated by evaluating recovery, linearity, selectivity, precision, accuracy and quantitation limit. Coefficients of variation and relative errors of less than 15% were considered acceptable, except for the quantitation limit, for which these values were established at 20%, as recommended in the literature [32,33].

Sample preparation was performed by liquid–liquid extraction using hexane:ethyl acetate (8:2, v/v) as extracting solvent after acidification of plasma samples. Table 1 shows mean recoveries of 73.9 and 73.1% for (−)-(*R*)- and (+)-(*S*)-ibuprofen, respectively. The co-

Table 1
Mean relative recovery percentages of ibuprofen enantiomers from plasma

Concentration spiked (μg/ml)	(−)-(*R*)-Ibuprofen		(+)-(*S*)-Ibuprofen	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
0.12	79.3	9.7	73.7	13.2
0.60	69.9	10.9	69.3	4.3
9.0	69.4	11.2	59.8	11.9
45.0	84.1	9.1	81.6	3.5
Mean	73.9		73.1	

$n = 3$ for each concentration; CV: coefficient of variation.

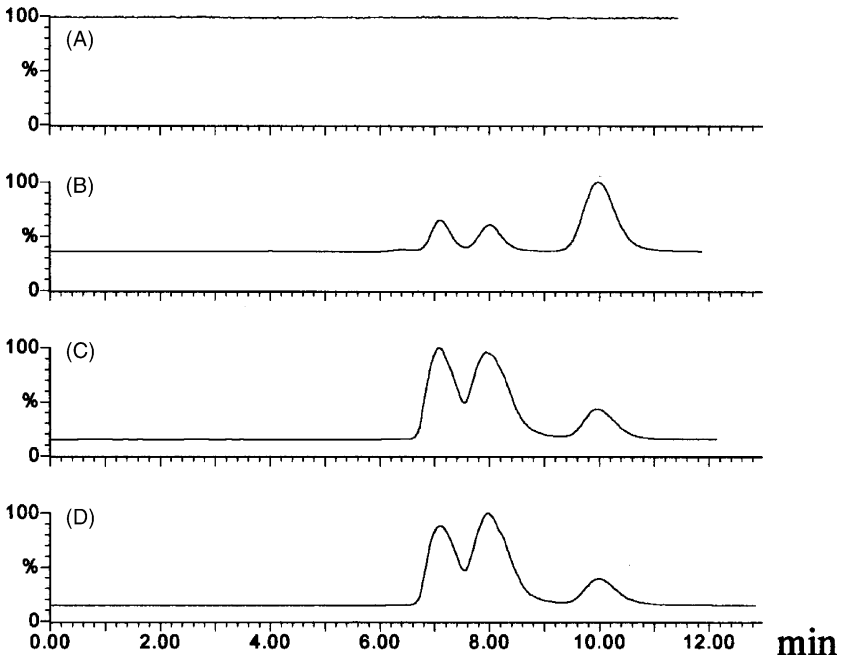

Fig. 3. Representative LC–MS–MS chromatograms from blank human plasma (A), extracted human plasma spiked with 10 μg/ml ibuprofen enantiomers (B) and extracted human plasma samples collected 1.5 h (C) and 2 h (D) after dosing with 600 mg *rac*-ibuprofen from a healthy volunteer. Conditions were as specified in Fig. 2.

efficients of variation lower than 15% confirm the repeatability of the extraction procedures.

The high selectivity of the MS–MS detection system can be seen in the analysis of a blank human plasma sample, illustrated in Fig. 3A, showing no endogenous interferents. The simultaneous analysis of a drug free plasma sample using UV detection (220 nm) showed peaks eluting at the same retention times observed for ibuprofen enantiomers that were not detected by the MS.

The linearity of the method was evaluated in the range of 0.12–90.0 μg/ml. The data were subjected

Table 2
Precision and accuracy of the method for the enantioselective analysis of ibuprofen

	Nominal concentration (μg/ml)					
	(−)-(*R*)-Ibuprofen			(+)-(*S*)-Ibuprofen		
	0.4	4.0	40.0	0.4	4.0	40.0
Within-day						
Measured concentration (ng/ml)	0.40	3.87	43.81	0.43	3.76	39.50
Precision (CV, %)	9.7	4.3	3.0	6.6	8.5	3.3
Accuracy (%)	0.0	−3.2	9.5	7.5	−6.0	−1.2
Between-day						
Measured concentration (ng/ml)	0.36	3.80	41.65	0.39	3.70	41.35
Precision (CV, %)	9.9	9.2	6.7	11.1	8.9	6.9
Accuracy (%)	−10.0	−4.7	4.1	−2.5	−7.5	3.4

Number of determinations: 10 for within-day assay and 5 for between-day assay; CV: coefficient of variation.

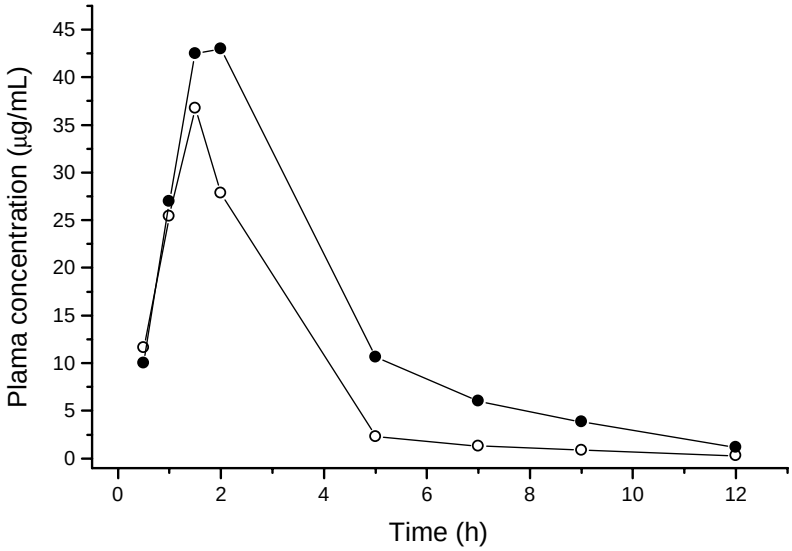

Fig. 4. Plasma concentration–time profiles of ibuprofen enantiomers after administration of 600 mg *rac*-ibuprofen to a healthy volunteer. Solid circles: (+)-(*S*)-ibuprofen; open circles: (−)-(*R*)-ibuprofen.

to linear regression analysis and the calibration graphs showed good linearity, with typical correlation coefficients higher than 0.998 for both ibuprofen enantiomers. Typical equations for the calibration curves for the (−)-(*R*)- and (+)-(*S*)-ibuprofen were $y = 0.4636(\pm 0.0274)x + 0.0182(\pm 0.0076)$ and $y = 0.5599(\pm 0.0410)x + 0.0116(\pm 0.0088)$, respectively. In addition, a 15% deviation from nominal values was considered acceptable for all concentrations except for the concentration of 0.12 μg/ml, established as the quantitation limit.

The precision and accuracy were assessed in both within-day (10 spiked plasma samples for each concentration on the same day) and between-day (two spiked plasma samples for each concentration on five consecutive days) assays. Table 2 demonstrates the results achieved with three concentrations in the evaluation of the precision and accuracy of the method; neither CVs nor systematic errors exceeded a value of 15%, in agreement with literature recommendations [32,33].

The chromatograms (Fig. 3) and plasma concentration-time profiles (Fig. 4), referring to the analysis of ibuprofen enantiomers in plasma samples collected after oral administration of 600 mg *rac*-ibuprofen to a healthy volunteer showed higher plasma concentrations for (+)-(*S*)-enantiomer, in agreement with data reported in the literature [6].

4. Conclusion

This paper reports for the first time the enantioselective analysis of ibuprofen in plasma by LC–MS–MS. The enantioselective method described here is simple, rapid, and reproducible. The quantification limit obtained using 0.5 ml plasma is similar to those reported in the literature and is small enough for the method to be used in single dose pharmacokinetic studies. The major advantage of this method when compared to the previously published methods is the high selectivity due to the use of the MS detection system. This kind of detection avoid almost any kind of interference from endogenous compounds and other co-administered drug, resulting in easy method development.

Acknowledgements

The authors are grateful to Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and CNPq (Conselho Nacional de Desenvolvimento Científico e

Tecnológico) for financial support and for granting research fellowship.

References

[1] A.M. Evans, Clin. Rheumatol. 20 (2001) 9.
[2] A.M. Evans, J. Clin. Pharmacol. 36 (1996) S7.
[3] S.C. Tan, B.K. Patel, S.H.D. Jackson, C.G. Swift, A.J. Hutt, Enantiomer 4 (1999) 195.
[4] M.F. San Martin, M.O. Tapia, A.L. Soraci, Acta Bioq. Clin. Latinoam. 32 (1998) 457.
[5] N.M. Davies, Clin. Pharmacokin. 34 (1998) 101.
[6] S.C. Tan, B.K. Patel, S.H.D. Jackson, C.G. Swift, A.J. Hutt, Xenobiotica 32 (2002) 683.
[7] R. Bhushan, J. Martens, Biomed. Chromatogr. 12 (1998) 309.
[8] Y.Y. Lau, J. Liq. Chromatogr. Relat. Technol. 19 (1996) 2143.
[9] F. Pehourcq, F. Lagrange, L. Labat, B. Bannwarth, J. Liq. Chromatogr. 18 (1995) 3969.
[10] M.R. Wright, S. Sattari, D.R. Brocks, F. Jamali, J. Chromatogr. Biomed. Appl. 583 (1992) 259.
[11] R. Canaparo, E. Muntoni, G.P. Zara, C.D. Pepa, E. Berno, M. Costa, M. Eandi, Biomed. Chromatogr. 14 (2000) 219.
[12] S.C. Tan, S.H.D. Jackson, C.G. Swift, A.J. Hutt, Chromatographia 46 (1997) 23.
[13] C.H. Lemko, G. Caille, R.T. Foster, J. Chromatogr. Biomed. Appl. 619 (1993) 330.
[14] J.X. Devries, E. Schmitzkummer, D. Siemon, J. Liq. Chromatogr. 17 (1994) 2127.
[15] T.A.G. Noctor, G. Felix, I.W. Wainer, Chromatographia 31 (1991) 55.
[16] K.J. Pettersson, A. Olsson, J. Chromatogr. Biomed. Appl. 563 (1991) 414.
[17] F.K. Glowka, Chem. Anal. 43 (1998) 79.
[18] N.D. Weng, J.W. Lee, J. Pharm. Biomed. Anal. 12 (1994) 551.
[19] B. Vermeulen, J.P. Remon, J. Chromatogr. B 749 (2000) 243.
[20] H. Soini, M. Stefansson, M.L. Riekkola, M.V. Novotny, Anal. Chem. 66 (1994) 3477.
[21] I. Bjornsdottir, D.R. Kepp, J. Tjornelund, S.H. Hansen, Electrophoresis 19 (1998) 455.
[22] S. Fanali, C. Desiderio, G. Schulte, S. Heitmeier, D. Strickmann, B. Chankvetadze, G. Blaschke, J. Chromatogr. A 800 (1998) 69.
[23] V.A.P. Jabor, V.L. Lanchote, P.S. Bonato, Electrophoresis 23 (2002) 3041.
[24] Y. Okamoto, Y. Kaida, J. Chromatogr. A 666 (1994) 403–419.
[25] K. Oguni, H. Oda, A. Ichida, J. Chromatogr. A 694 (1995) 91–100.
[26] E. Yashima, Y. Okamoto, Bull. Chem. Soc. Jpn. 68 (1995) 3289–3307.
[27] K. Vékey, J. Chromatogr. A 921 (2001) 227.
[28] S.R. Needham, P.R. Brown, K. Duff, D. Bell, J. Chromatogr. A 869 (2000) 159.
[29] R. Bakhtiar, L. Ramos, F.L.S. Tse, Chirality 13 (2001) 63.
[30] S. Ikegawa, N. Murao, J. Oohashi, J. Goto, Biomed. Chromatogr. 12 (1998) 317.
[31] Y. Tanaka, Y. Kishimoto, S. Terabe, J. Chromatogr. A 802 (1998) 83.
[32] R. Causon, J. Chromatogr. B 689 (1997) 175–180.
[33] F. Bressole, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3–10.