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Simple and Sensitive HPLC Method with Fluorescence Detection for the Measurement of Ibuprofen in Rat Plasma: Application to a Long-Lasting Dosage Form

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A simple and sensitive high-performance liquid chromatography (HPLC) assay applied to the measurement of ibuprofen in rat plasma has been developed. Two parameters have been investigated to improve ibuprofen detectability using fluorescence detection: variation of mobile phase pH and the use of β-cyclodextrin (β-CD). Increasing the pH value from 2.5 to 6.5 and adding 5 mM β-CD enhanced the fluorescence signal (λ_{exc} = 224 nm; λ_{em} = 290 nm) by 2.5 and 1.3-fold, respectively, when using standards. In the case of plasma samples, only pH variation significantly lowered detection and quantification limits, down to 10 and 35 ng/mL, respectively. Full selectivity was obtained with a single step for plasma treatment, that is, protein precipitation with acidified acetonitrile. The validated method was applied to a pharmacokinetic study of ibuprofen encapsulated in microspheres and subcutaneously administered to rats.

Keywords ibuprofen; microspheres; HPLC; fluorescence; β-cyclodextrin; pH; plasma

INTRODUCTION

Ibuprofen, (±)-2-(*p*-isobutylphenyl)propionic acid, is a nonsteroidal anti-inflammatory (NSAID) drug, which is available in a wide variety of pharmaceutical preparations commonly used in the treatment of acute and chronic pain and inflammation, in rheumatoid arthritis, and other musculoskeletal disorders. It offers a good tolerability but a relative short plasma half-life (2–3 h), resulting in short pharmacological activity duration. To overcome this problem, various ibuprofen prodrugs (Wang, Qi, Liu, & Fang, 2005; Zhao, Chen, Li, & Wang, 2005) and formulations (Borovac et al., 2006; Fernandez-Carballido, Herrero-Vanrell, Molina-Martinez, & Pastoriza, 2004; Lamprecht, Saumet, Roux, & Benoit, 2004) have been proposed.

Numerous assays of ibuprofen in biological fluids have already been reported. Most of them rely upon separative methods, especially high-performance liquid chromatography (HPLC) techniques coupled with UV (Espinosa-Mansilla, Muñoz de la Peña, González Gómez, & Cañada-Cañada, 2006; Kot-Wasik, Debska, Wasik, & Namiesnik, 2006; Sochor, Klimes, Sedlacek, & Zahradnicek, 1995; Teng, Wang, & Davies, 2003; Zhao et al., 2005), fluorescence detection (Canaparo et al., 2000; Palmgren, Monkkonen, Jukkola, Niva, & Auriola, 2004; Quintana, Miro, Estela, & Cerdà, 2006; Santos, Aparicio, Alonso, & Callejon, 2005), and mass spectrometry (Kot-Wasik et al., 2006). Capillary electrophoresis has also been recently introduced for ibuprofen measurement but it offers a limited sensitivity for bioanalysis; it has been applied in the quality control of pharmaceutical preparations (Hamoudová & Pospíšilová, 2006) and more recently to enantiomeric pharmacokinetic studies (Główka & Karaźniewicz, 2005). Generally, HPLC methods devoted to pharmacokinetic studies of ibuprofen offer relatively high detection limits, probably due to the high doses administered in therapeutics. However, determination of very low drug levels in environmental waters (Kot-Wasik et al., 2006; Quintana et al., 2006; Santos et al., 2005) and newly developed pharmaceutical preparations with slow drug release (Lamprecht et al., 2004) imply the enhancement of ibuprofen detectability in dedicated HPLC systems. Furthermore, the determination of low concentrations of ibuprofen might still be of interest to evaluate the

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pharmacokinetics of the relatively higher doses administered in human in the late phase of elimination, that is, to get a more precise value of the elimination half-life.

Two ways have previously been investigated to improve the limit of detection (LD) of ibuprofen assays in biological and environmental matrices: (i) sample treatment including a concentration step (Kot-Wasik et al., 2006) and (ii) detection mode, especially fluorescence (Canaparo et al., 2000; Palmgren et al., 2004; Quintana et al., 2006; Santos et al., 2005). The first approach relies on off-line solid-phase extraction with an evaporation step of the cartridge effluent (a high concentration factor up to 2,000-fold [Santos et al., 2005] can be realized), and hyphenated and automated on-line devices, which also allow important concentration factors of the analyte, thus authorizing low LD values, for example, 0.36 ng/mL of ibuprofen in wastewater (Quintana et al., 2006).

Many authors have used the native fluorescence properties of ibuprofen for its detection in a HPLC system, with a 2.5 times improvement in LD value; however, UV spectrophotometric detection, using no sample purification step, gave a LD value within 20 ng/mL (Palmgren et al., 2004). On the contrary, cyclodextrins (CDs), the cyclic oligosaccharides consisting of six or more D-(+)-glucopyranose units, are well known to have inclusion complexing properties with guest molecules that possess suitable polarity and dimension. They have been extensively used in pharmaceutical formulations to improve water solubility, to prevent degradation, and to modify bioavailability of drugs, especially in the field of NSAID drugs (Ravelet et al., 2002; Szejtli, 1998). Moreover, CDs can act as chiral selectors of racemic drugs in separative systems (Główka & Karaźniewicz, 2005), and increase the fluorescence intensity of numerous analytes, as the movement of their fluorophore (generally a planar aromatic ring) is restricted by its inclusion inside the hydrophobic cavity of CDs, as demonstrated for ibuprofen (Hergert & Escandar, 2003; Oh, Lee, Lee, Shin, & Park, 1998).

In the case of ibuprofen, the binding constant corresponding to its inclusion complex with β-cyclodextrin (β-CD) has been reported to be 2,600 M^{-1}, which is considerably higher than with α-CD and γ-CD (55 and 59 M^{-1}, respectively; Szejtli, 1998). Thus, the cavity size of β-CD seems convenient for the inclusion process of the aromatic part of ibuprofen. Association constants between β-CD and either protonated (IBH) or deprotonated (IB^-) form of ibuprofen have been calculated to be 1,900 and 8,700 M^{-1} for [IBH–β-CD] and [IB^-–β-CD], respectively (Manzoori & Amjadi, 2003). These values clearly show the prevailing role of the carboxylic group and its ionization degree in the stability of the complex. Both complexes have 1:1 stoichiometries.

Lower constant values (in the range 400–700 M^{-1}) have been reported for the complex between ibuprofen and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), which has the main advantages to exhibit higher water solubility and lower toxicity than β-CD (Oh et al., 1998). Upon complexation with CDs, ibuprofen exhibits enhanced fluorescence efficiency without any significant shift of excitation and emission wavelengths (224 and 290 nm, respectively). The fluorescence emission signal of IB^- and IBH is increased by about two- and fourfold, respectively, on β-CD addition and the LD of the corresponding fluorescence method is 30 ng/mL (Manzoori & Amjadi, 2003). The previously reported spectroscopic studies describing the influence of pH and CD inclusion complexation on fluorescence properties of ibuprofen have only been carried out in batch solutions (Hergert & Escandar, 2003; Oh et al., 1998). This work is focused on the optimization of the eluting conditions in a reversed-phase (RP)-HPLC system, especially with regard to (i) pH values of the mobile phase and (ii) the use of β-CD, to enhance the fluorescence signal and to give rise to a lower LD value of the ibuprofen HPLC assay in rat plasma.

EXPERIMENTAL PROCEDURE

Chemicals and Standards

All chemicals and solvents were of analytical or HPLC reagent grade and were used without further purification. Ibuprofen (batch number 450025) was a gift from Knoll Pharmaceuticals (Nottingham, UK). The ibuprofen stock solution was prepared in methanol at a concentration of 0.1 mg/mL and stored at 4°C for a maximum period of 3 months. Carprofen, diclofenac, ketoprofen, indomethacin, and acetylsalicylic acid were obtained from Sigma (Saint-Quentin Fallavier, France); β-CD hydrate was purchased from Acros Chemicals (Geel, Belgium) and HP-β-CD from Sigma; DL-poly(lactic-co-glycolic) acid (PLGA) 50:50 (m/m) Resomer RG 504 S (MW 48,000; viscosity: 0.47 dL/g) was purchased from Boehringer Ingelheim Inc. (Ingelheim, Germany). Polyvinylalcohol (PVA, MW 30,000, 88% hydrolyzed) was supplied by Sigma and sorbitan monostearate Span® 60 by Seppic (Paris, France).

HPLC System and Operating Conditions

The HPLC system (model HPLC 10A VP, Shimadzu, Champs-sur-Marne, France) consisted of a low-pressure gradient solvent delivery pump, an autosampler, a column oven, a spectrofluorimetric detector (model RF-10A XL), and a data processing software. Both guard (8 mm × 3 mm i.d.) and analytical (150 mm × 3 mm i.d.) columns were packed with Uptisphere ODB (porosity: 12 nm; particle size: 5 μm) (Interchim, Montluçon, France). The different mobile phases tested consisted of various mixtures of methanol or acetonitrile and 0.05 M phosphate buffers. They were prepared from a 0.05 M sodium dihydrogenphosphate solution adjusted to pH = 2.5, 4.5, or 6.5 with either concentrated hydrochloric acid (HCl) solution or 40% sodium hydroxide solution. β-CD was added at a final concentration of 5 mM (Table 1). They were filtered through a 0.45-μm filter, degassed before use, and run at a flow rate of 0.6 mL/min and at a column temperature of 30°C. Spectrofluorimetric detection was operated at an excitation

TABLE 1
Influence of Mobile Phase pH and β-CD Addition on the Ibuprofen Peak Parameters (Calculated According to European Pharmacopoeia Rules)

Mobile Phase Composition	k[a]	N (m^{-1})[b]	S/N[c]
Influence of pH buffer			
Acetonitrile–0.05 M phosphate buffer pH 2.5 (45:55, vol/vol)	8.5	29,500	9.3
Acetonitrile–0.05 M phosphate buffer pH 4.5 (45:55, vol/vol)	7.3	39,950	9.3
Acetonitrile–0.05 M phosphate buffer pH 6.5 (20:80, vol/vol)	27.3	36,200	25
Methanol–0.05 M phosphate buffer pH 6.5 (60:40, vol/vol)	4.1	8,900	23.5
Influence of β-CD addition			
Acetonitrile–0.05 M phosphate buffer pH 6.5 (20:80, vol/vol)	27.3	36,200	25
Acetonitrile–0.05 M phosphate buffer pH 6.5 + 5 mM β-CD (20:80, vol/vol)	6.6	8,550	32

[a] t_0 was measured according to baseline disturbance marking void volume (presently, 1.5 min).
[b] $5.54\ (tr/w_{h/2})^2$.
[c] Calculated at an ibuprofen concentration of 100 ng/mL.
β-CD, β-cyclodextrin.

wavelength of 224 nm and emission wavelength of 290 nm (gain: × 4; sensitivity: medium).

Microsphere Preparation and Characterization

Microspheres were prepared by the O/W solvent extraction method (Soppimath & Aminabhavi, 2002). Briefly, 50 mg of ibuprofen, 400 mg of PLGA, and 200 mg of Span® 60 were dissolved in 20 mL of ethylacetate. The organic solution was poured into 50 mL of 0.1% (wt/vol) PVA aqueous solution and stirred mechanically (1,000 rpm) using a three-bladed propeller for 30 s at 25 ± 2°C to obtain a pre-emulsion (O/W). This pre-emulsion was added to 2 L of purified water and magnetically stirred at 600 rpm for 10 min, to obtain the final O/W emulsion. Upon solvent extraction during 15 min, the polymer precipitates (due to the good miscibility and fast diffusion of the organic polymer solvent in water) and the microsphere cores solidify. Microspheres were then collected by filtration through a 0.45-μm HA membrane (Millipore, Molsheim, France) and dried at 25 ± 2°C for 48 h.

Mean diameter and size distribution of microspheres were analyzed by laser diffraction in a particle size analyzer (Mastersizer S, Malvern Instruments, Orsay, France). Each sample was measured in triplicate. The amount of ibuprofen entrapped within polymeric particles was determined spectrophotometrically at 222 nm (UV–visible spectrophotometer model UV-160 1PC, Shimadzu, Kyoto, Japan) by measuring the amount of nonentrapped ibuprofen in the external aqueous solution (indirect method), which was recovered after filtration and washing of microparticles. A standard calibration curve was performed with the ibuprofen solution (aqueous solution of 0.1% PVA with 1% acetone). The established linearity range was 2–10 μg/mL ($r > .998$).

Rat Treatment and Plasma Collection and Preparation

An ibuprofen microsphere aqueous suspension or an ibuprofen solution (Pedea®, Orphan Europe SARL, Paris la Défense, France, 5 mg/mL) was administered subcutaneously at the dose of 1 mg of ibuprofen/kg, to overnight fasted rats (male Sprague–Dawley, 300 ± 20 g; $n \geq 4$). There were five groups of four rats making a total of 20 rats. Indeed, it was not possible to withdraw blood samples from the same rats at each time. Each rat was only sampled twice between 15 min and 10 h. Starting at 24 h, the groups were made larger and there were a minimum of six rats at a time as there was more time for rats to recover. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (15 mg/kg). A volume of 400 μL of blood was collected by cardiac puncture at 15, 30, and 45 min and 1, 2, 4, 6, 8, 10, 24, 48, and 80 h after administration, into 1.5-mL polypropylene vials containing 60 μL of 0.129 M sodium citrate solution. After centrifugation at 3,000 × g for 10 min at 18°C, the obtained plasma was immediately stored at –20°C.

The 5-point calibration curve was built by spiking blank (drug-free) plasma samples (180 μL) with ibuprofen (20 μL of ibuprofen standard solutions prepared in mobile phase) to give concentrations ranging from 35 to 200 ng/mL. Frozen plasma samples (200 μL) were thawed in a water bath at 37°C and proteins were precipitated by vortex mixing with 200 μL of a mixture of acetonitrile–1 M HCl (99:1, vol/vol) for 4 min. After centrifugation at 42,000 × g for 10 min at 4°C, the supernatant was transferred into HPLC sample vials and a 50 μL volume was injected into the HPLC system.

RESULTS AND DISCUSSION

HPLC System Development for Enhancement of Fluorescence Signal

Different eluting conditions of ibuprofen were evaluated on an RP column to precisely study the influence of two parameters, that is, pH of mobile phase and use of CD, on both chromatographic parameters and fluorescence signal of the considered analyte. The corresponding data are summarized in Table 1.

First, three pH values were tested, that is, 2.5, 4.5, and 6.5, which are below the ibuprofen pK_a (= 4.41, according to Palmgren et al., 2004), close to pK_a, and higher than pK_a, respectively. Shifting the mobile phase pH from 2.5 to 6.5 decreased the retention factor (k) and the number of theoretical

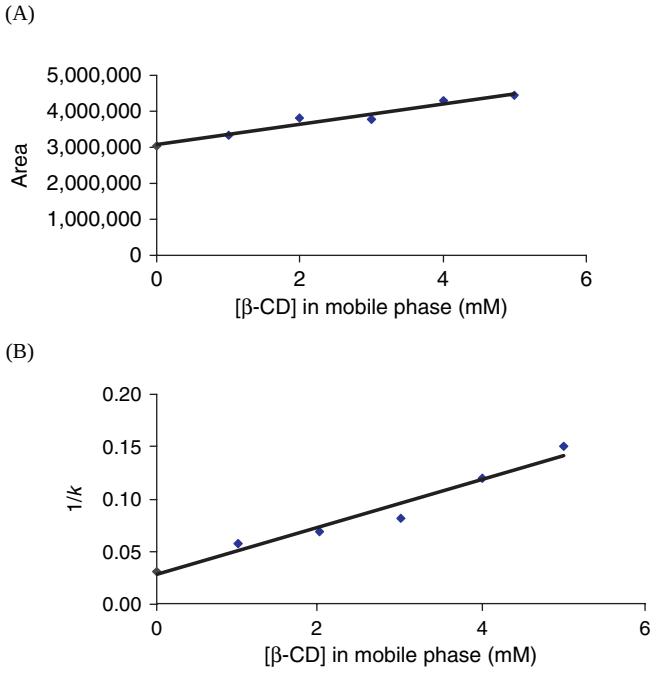

FIGURE 1. Influence of β-cyclodextrin (β-CD) concentration added to the mobile phase (CH_3CN–phosphate buffer pH 6.5; 20:80, vol/vol) on the ibuprofen peak area (A) and plot of 1/*k* versus β-CD concentration at a column temperature of 30°C (B). Values are the mean of two experimental data.

plates (*N*) of ibuprofen peak, and increased 2.5-fold the signal-to-noise (S/N) ratio of the ibuprofen peak. Thus, a pH value of 6.5 appeared favorable to improve the LD of ibuprofen in the RP-HPLC system, as its deprotonated form has a higher fluorescence signal; these data confirm the previous ones obtained in batch solutions (Manzoori & Amjadi, 2003).

Second, the addition of β-CD at a concentration of 5 mM to different mobile phases buffered at pH 6.5 was tested. When they contained a high proportion of organic solvent, either acetonitrile (45%, vol/vol) or methanol (60%, vol/vol), the ibuprofen retention time and fluorescence signal did not change, which demonstrated that the inclusion process of ibuprofen into CD cavity did not occur. When a low content of acetonitrile (20%) was used, the retention time of ibuprofen decreased from 50 to 11.5 min with a peak broadening under addition of β-CD, proving a complex formation between ibuprofen and β-CD. A linear increase of fluorescence, as observed through the variations of the ibuprofen peak area, was also noted (Figure 1A). The addition of HP-β-CD in the same eluting conditions (20% acetonitrile; [HP-β-CD] = 5 mM) changed ibuprofen retention time from 50 to approximately 13 min but did not increase ibuprofen peak area. Only β-CD was used in further experiments. The apparent formation constant (K_f) of the complex between ibuprofen and β-CD was calculated to verify that the inclusion process really takes place in the present chromatographic conditions. For that purpose, the following equation was used (Flood et al., 2000; Ravelet et al., 2002):

$$1/k = [\beta - CD]K_f/k_0 + 1/k_0,$$

where *k* is the retention factor of ibuprofen at a particular concentration of β-CD and k_0 is its retention factor in the absence of β-CD. For a compound with a 1:1 stoichiometry with β-CD, a plot of 1/*k* versus [β-CD] yields a straight line which has a slope equal to K_f/k_0. The determination coefficient r^2 of the regression line was .96 (Figure 1B), and K_f = 875 M^{-1} at 30°C, which is a lower value than those already reported: 2,600 (temperature not indicated) and 7,100 M^{-1} (32°C) according to Szejtli (1998) and Manzoori and Amjadi (2003), respectively. In this study, K_f was calculated in the presence of an organic solvent (acetonitrile) in the mobile phase, which modified the low-energy bonds between ibuprofen and β-CD, thus explaining the lower K_f value obtained.

The enhancement of the S/N ratio observed when adding β-CD to the mobile phase and using ibuprofen standards was about 30% (Table 1) but this gain was not confirmed when evaluating the LD with real biological samples. As a matter of fact, the slopes of the calibration curves built with plasma samples fortified with ibuprofen standards were 16,139 ± 687 (*n* = 3) in the absence of β-CD and 19,204 ± 1,090 in the presence of 5 mM β-CD. The resulting LD values were 9 and 10 ng/mL with and without addition of β-CD to the mobile phase, respectively, which can be considered as very similar values.

All these data demonstrate the usefulness of varying pH but no influence of using β-CD to enhance the florescence signal of ibuprofen in the present HPLC system. Thus further experiments in this work (including validation process and pharmacokinetic studies) were carried out using the mobile phase without β-CD, that is, methanol–0.05 M phosphate buffer (pH 6.5; 60:40, vol/vol).

Validation of the Ibuprofen HPLC Assay in Rat Plasma

First, the selectivity of the overall analytical method was tested versus other NSAIDs, that is, carprofen, ketoprofen, acetyl salicylic acid, indomethacin, and diclofenac, by injecting a 100 ng/mL standard solution. No compound was detected within a 1-h elution period. The use of a single step for plasma preparation before injecting into the HPLC system, that is, protein precipitation with acidified acetonitrile, afforded full selectivity versus endogenous compounds (three different batches of blank plasma were tested) (Figure 2).

The stability of ibuprofen plasma extracts was tested over a 15-h period, by injecting every hour a standard and an extract of plasma sample fortified at a concentration of 100 ng/mL: no significant variation of the ibuprofen peak area was observed (the signal variation observed in the 15-h period was not higher than the relative standard deviation (RSD) value calculated at the same ibuprofen concentration, that is, 5.6% (Table 2)); thus, the HPLC measurements were further run overnight.

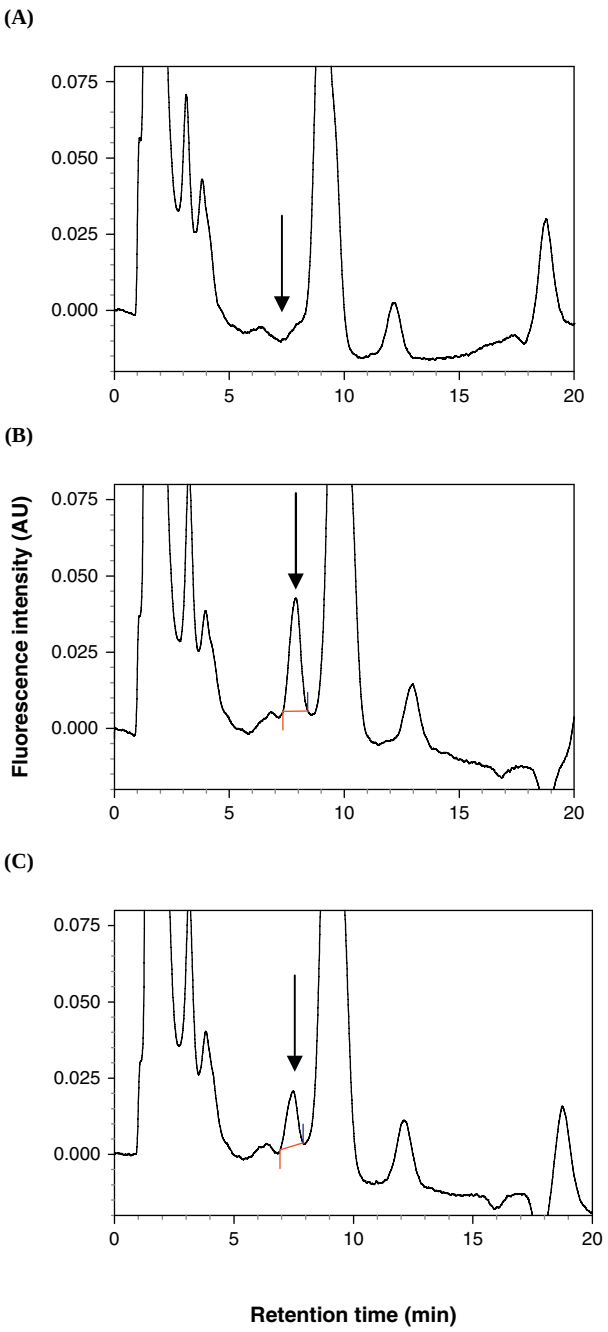

FIGURE 2. Typical chromatograms corresponding to samples of rat blank plasma (A), rat plasma fortified with ibuprofen at a concentration of 75 ng/mL (B), and rat plasma collected after 48 h of ibuprofen-loaded microparticles administration by subcutaneous route (dose: 1 mg/kg) (C). Eluting conditions: methanol–0.05 M phosphate buffer pH 6.5 (60:40, vol/vol) at a flow rate of 0.6 mL/min and at 30°C.

The main validation parameters are summarized in Table 2. The resulting LQ of 35 ng/mL was lower than the values previously obtained in HPLC assays with UV detection (in the range 0.5–1 µg/mL) (Wang et al., 2005; Zhao et al., 2005) and with fluorescence detection in urine (100 ng/mL) (Fan et al., 2005)

TABLE 2
Main Validation Parameters for the HPLC Assay of Ibuprofen Using Mobile Phase: Methanol–0.05 M Phosphate Buffer pH 6.5 (60:40, vol/vol) and Plasma Samples Fortified with Standard Solutions

Slope ± *SD*	16,139 ± 687
Intercept ± *SD*	1,254 ± 55,154
Determination coefficient r^2	.9948
LD[a] (ng/mL)	10
LQ[a] (ng/mL)	35
Repeatability: RSD (%; *n* = 3) at	
50 ng/mL	4.9
100 ng/mL	5.6
200 ng/mL	4.9
Recovery: (%; *n* = 3) at	
50 ng/mL	84 ± 3
100 ng/mL	83 ± 3
200 ng/mL	90 ± 2

[a]LD and LQ values were calculated as follows:
LD = (mean value of intercept + 3 *SD* of the intercept)/mean value of the slope.
LQ = (mean value of intercept + 10 *SD* of the intercept)/mean value of the slope.

and in wastewater samples (1.6 µg/mL) (Santos et al., 2005). The present assay appeared to be convenient for pharmacokinetic studies of a long-lasting form.

Pharmacokinetic Studies

Most of the analytical techniques used for determining ibuprofen in plasma have relatively high LQ (i.e., in the 200–1,000 ng/mL range). Although this is generally acceptable for the high doses of ibuprofen administered in man (200–400 mg as a single dose a few times daily), there is a need for more sensitive methods. For instance, this is the case when ibuprofen is used as an orphan aqueous solution drug (i.e., Pedea®) to close the patent ductus arteriosus in newborn humans (Aranda & Thomas, 2006). In the case, the administered dose is 5 mg/kg. An injectable and long-lasting dosage form of ibuprofen has presently been developed after encapsulation of the drug into biodegradable polymers. Such polymers are commonly used in man and some dosage forms are already on the market mainly for the treatment of prostatic cancer when the release of drugs for weeks is needed. The ibuprofen microparticles presently obtained had an average diameter of 31 ± 7 µm (*n* = 3) and the encapsulation efficiency was 70% with regard to the initial amount of ibuprofen in the preparation. Therefore, they could easily pass through a 21-G gauge needle for subcutaneous administration. The ibuprofen plasma concentration profile was followed up to 80 h; at this time, the mean ibuprofen concentration was still 49.1 ng/mL with the microparticles

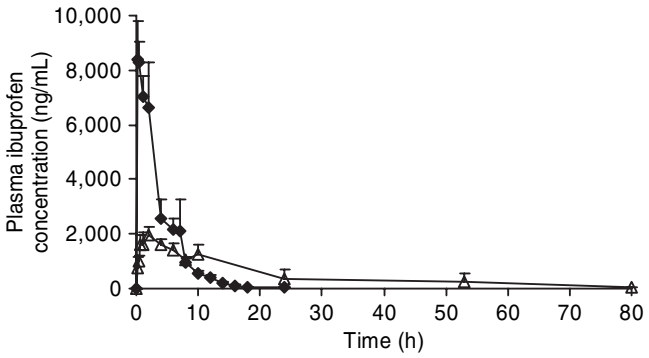

FIGURE 3. Profile of ibuprofen concentration in rat plasma after subcutaneous administration of ibuprofen solution (◆) and DL-poly(lactic-co-glycolic) acid (PLGA) microspheres (△) (dose: 1 mg/kg; n = 4 rats).

(Figure 3). By comparison, plasma concentrations were not detectable after 24 h with the aqueous solution of ibuprofen. C_{max} was dramatically reduced with the microparticles suspension (from 8,400 to 2,000 ng/mL for the solution and suspension, respectively) as expected due to the slow release from the microparticles. This property of the microparticles can also be related to the change in T_{max}, which is much earlier for the solution (15 min) than for the suspension (120 min). After C_{max}, the ibuprofen plasma concentrations followed a pseudo-plateau until 10 h then decreased slowly till 80 h, which was the final sampling point. The newly ibuprofen assay also allowed to follow significant plasma concentrations to the last sampling point with accuracy. Based on the areas under the curves obtained with both the solution and the microparticles suspension, it was possible to calculate the relative bioavailability of the microparticles with regard to the solution. The relative bioavailability is about 73% after 24 h, which demonstrated that most of the encapsulated ibuprofen was released into the systemic circulation from the subcutaneous depot. Taking into account the plasma level at 80 h (which is more than the quantification limit equal to 35 ng/mL) leads to a relative bioavailability of 99%. Such a high figure can be considered as a total bioavilability and definitely reflects a very good diffusion of the drug from the microspheres. However, it should be remembered that different animals are sampled at each time which may slightly overestimates the actual 99% bioavailability figure. Nevertheless, the goal of developing an injectable slow release dosage form of ibuprofen was demonstrated successfully, thanks to the new ibuprofen assay.

CONCLUSIONS

A long-lasting dosage form was developed using encapsulation of ibuprofen into biodegradable PLGA microspheres. Microspheres characteristics and particularly their mean diameter allowed them to be administered subcutaneously through a 21-G gauge needle. The optimized HPLC method allowed ibuprofen to be monitored during 80 h in rat plasma and demonstrated an almost total drug availability from the microspheres. With regard to the newly developed method, although β-CD addition to the mobile phase has not the expected effect on the improvement of the biological HPLC assay sensitivity, the pH increase has a real benefit. Further improvements in sensitivity should be performed through the development of a liquid/solid extraction step including a high concentration factor of the analyte.

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