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HPLC determination of ketoprofen enantiomers in human serum using a nonporous octadecylsilane 1.5 μm column with hydroxypropyl β-cyclodextrin as mobile phase additive

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Abstract

A sensitive and stereospecific high-performance liquid chromatography (HPLC) method for the quantitation of ketoprofen enantiomers in human serum was developed. The assay involves the use of an octadecylsilane solid-phase extraction for serum sample clean-up prior to HPLC analysis. Chromatographic resolution of the ketoprofen enantiomers was performed on a nonporous octyldecylsilane column with hydroxypropyl β-cyclodextrin as the mobile phase additive. The composition of the mobile phase was 98:2 v/v aqueous 0.1% trifluoroacetic acid (TFA), pH 4.00 (adjusted with triethylamine (TEA))/acetonitrile containing 10 mM hydroxypropyl β-cyclodextrin (β-CD) at a flow rate of 0.8 ml min^{-1}. Recoveries of $R(-)$-ketoprofen was $95.4 \pm 2.16\%$ and for $S(+)$-ketoprofen $96.2 \pm 1.31\%$. Linear calibration curves were obtained in the range 0.025–15 μg ml^{-1} range for each enantiomer in serum. The detection limit based on a $S/N = 3$ ratio was 10 ng ml^{-1} for each enantiomer in serum with ultraviolet detection at 220 nm. The limit of quantitation for each enantiomer was 25 ng ml^{-1}. Precision calculated as % relative standard deviation (%R.S.D.) and accuracy calculated as % error were in the range 0.2–5.2% and 0.3–2.2%, respectively, for the *R* enantiomer and 0.3–6.2 and 0.2–3.2%, respectively, for the *S* enantiomer.

Keywords: Human serum; Hydroxypropyl β-cyclodextrin; Mobile phase additive; Nonporous octadecylsilane column; Solid-phase extraction

1. Introduction

Ketoprofen, 2-(3-benzoylphenyl)propionic acid is a nonsteroidal anti-inflammatory drug (NSAID) which has potent inhibitory effects on prostaglandin synthesis. It is commonly used in the treatment of rheumatoid arthritis and osteoarthritis. Therapeutic doses of ketoprofen have proven to be as effective as those of the other commonly used NSAIDs [1,2]. Ketoprofen is marketed as the racemate although, like the other members of the 2-arylpropanoic acid class, it exhibits enantioselectivity in its action and disposition [3]. Specifically, in vitro experiments proved that the $S(+)$ enantiomer exhibited pharmaco-

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logical effects, but the $R(-)$ enantiomer was inactive. Furthermore, an in vivo experiment showed that the chiral inversion takes place from $R(-)$ to $S(+)$ with the degree of inversion varying from one animal species to another [4]. Therefore, the stereoselective determination of ketoprofen is important in relation to pharmacokinetic studies.

Native cyclodextrins such as α-, β- and γ-cyclodextrins contain 6, 7 and 8 D(+) glucopyranose units linked together by 1–4 glycosidic bonds. They are inherently chiral and undergo chiral interactions with analytes. Cyclodextrins (CDs) separate enantiomers utilizing the phenomenon of host–guest complexation, where a transient diastereomeric complex is formed between the CD and the analyte. The affinity of the analyte for the CD is due to hydrophobic interactions between the analyte and the CD cavity, or hydrogen bonding of the analyte to the hydrophilic hydroxyl groups or introduced functional groups on the CD ring [5]. Derivatization of the hydroxyl groups increases solubility and selectivity compared to the native β-CD and the hydroxyl groups also undergo additional interactions with the analytes, thereby enhancing chiral recognition [6].

Earlier reports of high-performance liquid chromatography (HPLC) methods for chiral separation of ketoprofen utilized protein-bonded silica columns [7,8], including an α_1-acid glycoprotein column which is relatively unstable [9], derivatization methods [10–12], and normal-phase HPLC with a Chiralpak AD column and liquid–liquid extraction [13,14]. The possibility of racemization during the derivatization procedure, the requirement of optically pure derivatizing reagents and the additional sample preparation steps involved often affect the sensitivity of the derivatization technique and ultimately the outcome of the separation.

This paper reports the use of hydroxypropyl β-cyclodextrin as a chiral mobile phase additive for the enantiospecific HPLC assay of ketoprofen enantiomers in human serum on a nonporous octyldecylsilane column with solid-phase extraction.

2. Experimental

2.1. Reagents and chemicals

Racemic ketoprofen and the internal standard tolmetin were purchased from Sigma (St. Louis, MO, USA). $R(-)$- and $S(+)$-ketoprofen were a gift from Rhone-Poulenc Rorer Centre de Recherches (Vitry-Alforville, France). Acetonitrile was purchased from J.T. Baker (Phillipburg, NJ, USA). Triethylamine (TEA) was obtained from Fisher Scientific (Orangeburg, NY, USA) and trifluoroacetic acid (TFA) was purchased from Aldrich (Milwaukee, WI, USA). Hydroxypropyl β-cyclodextrin (HP-β-CD, degree of substitution 4.8) was supplied by American Maize Products Company (Hammond, IN, USA). All solvents were HPLC grade. Drug-free human serum was obtained from Biological Specialty (Colman, PA, USA). C18 solid-phase extraction column (100 mg cm^{-3}) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA).

2.2. Instrumentation

The HPLC system consisted of a Beckman Model 110A pump (Beckman, San Ramon, CA, USA) and a Model 728 autosampler (Micromeritics Instruments Corporation, Norcross, GA, USA) equipped with a 10 ul loop. The detector was a Waters Millipore Model 481 LC Spectrophotometer (Milford, MA USA) and a Spectra-Physics Model 4270 integrator (Spectra-Physics, San Jose, CA, USA) was used to record each chromatogram and peak height responses. The separation of the analytes was achieved on a 33×4.6 mm i.d. Micra Nonporous octyldecylsilane 1.5 μm column (Micra Scientific, Northbrook, IL, USA) equipped with a 0.2 μm Opti-solv precolumn minifilter (Optimize Technologies, Portland, OR, USA).

The mobile phase consisted of 98:2 v/v aqueous 0.1% TFA, pH 4.0 (adjusted with TEA)/acetonitrile, containing 10 mM hydroxypropyl β-CD and delivered at a flow rate of 0.8 ml min^{-1}. The mobile phase was filtered through a 0.45 μm filter (Alltech Associates, Deerfield, IL, USA) and sonicated prior to use. The column was operated at ambient temperature (23 ± 1°C).

2.3. Preparation of stock and standard solutions

Individual stock solutions of 100 μg ml^{-1} of $R(-)$- and $S(+)$-ketoprofen and 100 μg ml^{-1} of internal standard tolmetin were prepared in 10-ml volumetric flasks by dissolving the drugs in 2 ml acetonitrile followed by the addition of deionized water to volume. The solutions were stored under refrigeration at 4°C and were stable for at least 1 week. Appropriate dilutions of the $R(-)$- and $S(+)$-ketoprofen stock solutions with deionized water gave 10 and 50 μg ml^{-1} solutions which were used for spiking human serum.

2.4. Preparation of spiked human serum samples

Accurately measured aliquots of the 10, 50 and 100 μg ml^{-1} standard solutions of $R(-)$- and $S(+)$-ketoprofen were each added into 1-ml volumetric tubes and evaporated. Then 15 μl of the internal standard solution were added to the tubes and drug-free human serum was added to volume and mixed well to give final concentrations of 50, 150, 350, 750, 1000, 5000 and 10 000 ng ml^{-1} of each ketoprofen enantiomer.

2.5. Assay method

A total of 1 ml of the spiked human serum samples was diluted with 100 μl of 0.1 N HCl and vortexed for 2 min. The samples were passed through a C18 Bond-Elut solid-phase extraction (SPE) column, which had previously been conditioned with 2×1 ml of methanol followed by 2×1 ml of deionized water. The cartridge was not allowed to dry between the washing and sample application steps. The column was washed with 2×1 ml deionized water after the application of the serum sample. The analytes were eluted with 4×125 μl of 2% 0.1 N HCl in methanol and evaporated to dryness under a slow stream of nitrogen. The residue was reconstituted in 1 ml of mobile phase, filtered through a 0.2 μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI, USA) and 10 μl were injected into the liquid chromatograph.

For absolute recovery experiments, spiked samples were compared to unextracted stock solutions. Drug peak-height ratios were used to calculate the recoveries. Linear regression analysis of the peak-height ratios of each enantiomer to internal standard versus concentration of each enantiomer produced slope and intercept data which were used to calculate concentrations of $R(-)$- and $S(+)$-ketoprofen in each serum sample.

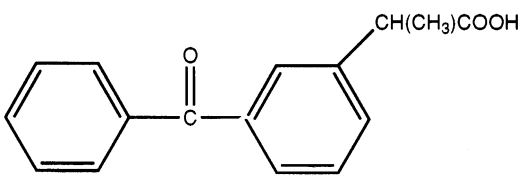
3. Results and discussion

Chemical modification of cyclodextrins has been shown to 'stretch' the cavity mouth and therefore change the hydrophobicity of the molecule and the stereoselectivity of the inclusion process. The mouth of the cyclodextrin hydrophobic cavity is surrounded by secondary hydroxyl groups which are considered to be important in chiral recognition [15]. In a derivatized HP-β-CD, some hydroxyl groups are substituted with hydroxypropyl functional groups. This modification allows for a more stereospecific and stronger interaction between the hydroxyl groups and hydrogen-bonding moiety present in the ketoprofen structure. In addition to the two aromatic rings which form inclusion complexes with the CD, ketoprofen has a carboxylic acid group at the chiral center that could participate in additional interactions with the rim hydroxypropyl groups of the HP-β-CD [15].

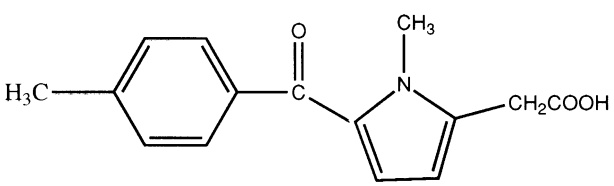
The chemical structures of ketoprofen and the internal standard tolmetin are shown in Fig. 1. In the course of investigating the chiral chromatographic behavior of various drugs using cyclodextrin and cyclodextrin derivatives as mobile phase additives, the enantiomers of ketoprofen were successfully resolved on a nonporous octadecylsilane 1.5 μm column [16]. The separation method developed was applied to the determination of the enantiomers of ketoprofen in human serum. Two unique features of the new nonporous column are the use of small amounts of organic modifier and the short retention times of the analytes. The use of small amounts of organic modifier is especially important in chiral mobile phase additive separation because the addition of organic modifiers to the mobile phase is known to greatly decrease the

solubility of CDs [17]. Fig. 2 shows chromatograms of blank human serum and serum spiked with the ketoprofen enantiomers and internal standard tolmetin. The ketoprofen enantiomers were baseline resolved (R values of 1.5–1.7) at concentrations used in this work.

Recoveries of ketoprofen enantiomers from human serum were assessed using spiked samples at concentrations ranging from 25 to 15 000 ng ml^{-1}. The mean absolute recoveries of the ketoprofen enantiomers at 25 and 15 000 ng ml^{-1} using the C18 solid-phase extraction column were 95.4 ± 2.16% ($n = 8$), for the $R(-)$ enantiomer, and 96.2 ± 1.31% ($n = 8$), for the $S(+)$ enantiomer, eluting with a solution of 2% 0.1 N HCl in methanol. Absolute methanol used alone gave recoveries of 70% for each enantiomer. Selection of tolmetin as the internal standard was based on its structural similarity to ketoprofen, its satisfactory retention time and similar extraction behavior to ketoprofen through the sample preparation steps.



KETOPROFEN



TOLMETIN

Fig. 1. Chemical structures of ketoprofen and tolmetin (internal standard).

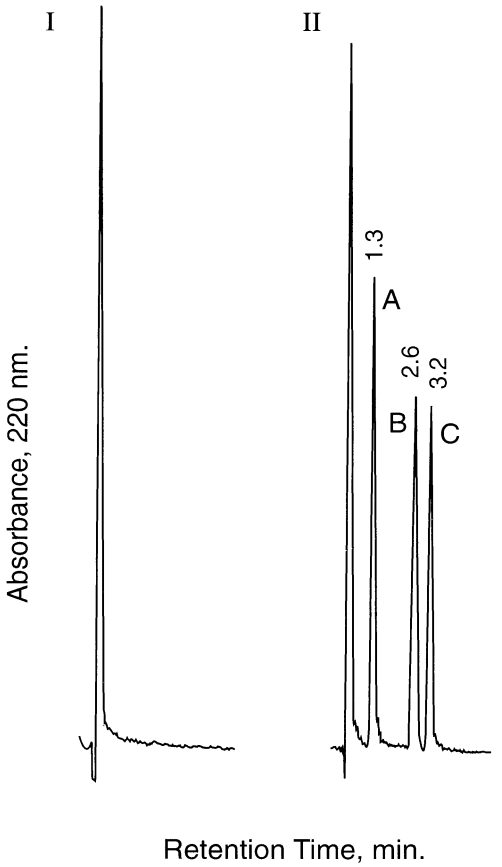


Fig. 2. Representative chromatograms of (I) blank human serum and (II) human serum spiked with (A) internal standard tolmetin (1.3 min, 1.5 μg ml^{-1}), (B) $S(+)$-ketoprofen (2.6 min, 150 ng ml^{-1}) and (C) $R(-)$-ketoprofen (3.2 min, 150 ng ml^{-1}). (See Section 2 for chromatographic parameters).

Linear calibration curves were obtained in the 25–15 000 ng ml^{-1} range for each ketoprofen enantiomer which includes normal serum levels (0.5–5 mg l^{-1}) after a 50 mg single oral dose of ketoprofen [1]. Standard curves were fitted to the linear regression equation $y = ax + b$, where y represents the ratio of drug/internal standard peak heights, a and b are constants, and x is the concentration of ketoprofen enantiomer. Typical regression parameters of a (slope), b (y-intercept) and the correlation coefficient were calculated to be 0.000975, 0.223078 and 0.999, respectively, for $R(-)$-ketoprofen and 0.000997, 0.209505 and 0.999, respectively, for $S(+)$-ketoprofen. The

Table 1
Accuracy and precision data for ketoprofen enantiomers in spiked human serum

Analyte	Conc. added (ng ml^{-1})	Conc. found[a] (ng ml^{-1})	Error (%)	R.S.D. (%)
Intra-day				
R(−)-ketoprofen	50	48.90 ± 1.85	2.2	3.7
	150	150.82 ± 1.10	0.6	0.7
	350	351.37 ± 0.93	0.4	0.3
	750	753.67 ± 3.91	0.5	0.5
	1000	1015.85 ± 17.25	1.6	1.7
	5000	5090.72 ± 66.43	1.8	1.3
	10000	10186.34 ± 146.82	1.8	1.5
S(+)-ketoprofen	50	48.40 ± 2.02	3.2	4.0
	150	148.80 ± 1.30	0.8	0.9
	350	349.20 ± 1.10	0.2	0.3
	750	756.14 ± 2.63	0.4	0.4
	1000	1017.23 ± 18.86	1.7	1.9
	5000	5102.66 ± 71.75	2.1	1.4
	10000	10174.45 ± 133.24	1.7	1.3
Inter-day				
R(−)-ketoprofen	50	48.10 ± 2.60	1.8	5.2
	150	149.20 ± 1.13	0.5	0.8
	350	348.70 ± 2.20	0.4	0.6
	750	752.16 ± 1.17	0.4	0.2
	1000	1014.35 ± 14.42	1.4	1.4
	5000	5087.81 ± 67.26	1.7	1.3
	10000	10154.46 ± 131.42	1.5	1.3
S(+)-ketoprofen	50	48.40 ± 3.12	3.2	6.2
	150	148.65 ± 2.41	0.9	1.6
	350	351.10 ± 1.34	0.3	0.4
	750	753.32 ± 2.96	0.4	0.4
	1000	1015.75 ± 13.82	1.6	1.4
	5000	5089.96 ± 66.23	1.8	1.3
	10000	10168.85 ± 144.16	1.7	1.4

[a] Based on $n = 3$ for intra-day assay and $n = 9$ for inter-day assay.

precision and accuracy of the method were ascertained using spiked human serum samples at concentrations ranging from 50 to 10 000 ng ml^{-1} levels (see Table 1).

The intra-day precision and accuracy for *R*(−)-ketoprofen as expressed by %R.S.D. and % error were in the range 0.3–3.7 and 0.4–2.2% ($n = 3$), respectively. For *S*(+)-ketoprofen, intra-day precision and accuracy were in the range 0.3–4.0 and 0.2–3.2% ($n = 3$), respectively. The inter-day precision and accuracy for *R*(−)-ketoprofen were in the range 0.2–5.2 and 0.3–1.8% ($n = 9$), respectively. For *S*(+)-ketoprofen, the inter-day precision and accuracy were 0.4–6.2 and 0.3–3.2% ($n = 9$), respectively. The minimum detectable concentration of each enantiomer was determined to be 10 ng ml^{-1} ($S/N = 3$). The lowest quantifiable level was found to be 25 ng ml^{-1} for each enantiomer: *R*(−), 4.3% R.S.D., 3.1% error; *S*(+), 4.6% R.S.D., 4.2% error.

In conclusion, a HPLC method has been developed and validated for the assay of *R*(−)- and *S*(+)-ketoprofen in human serum using HP-β-CD as the mobile phase additive on a nonporous octadecylsilane 1.5 μm column. The method utilizes solid-phase extraction for sample clean-up and is applicable to the separation and quantitation of each enantiomer in the 25–10 000 ng ml^{-1}

range. The main advantages of the novel non-porous column are the use of small amounts of organic modifier and the fast chromatographic run times.

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