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Note

Determination of the enantiomers of ketoprofen in blood plasma by ion-pair extraction and high-performance liquid chromatography of leucinamide derivatives

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Ketoprofen, 2-(3-benzoylphenyl)propionic acid, is a non-steroidal anti-inflammatory drug (NSAID). It is used as a racemate, but of this type of propionic acid derivative, only the *S* enantiomers (generally dextrorotatory) have anti-inflammatory activity [1]. Determination of the enantiomers of ketoprofen in plasma by gas chromatography (GC) [2] and by high-performance liquid chromatography (HPLC) [3] has been described. In the GC method, ketoprofen is converted into diastereoisomeric 1-phenylethylamides by a carbodiimide-mediated reaction that takes 2 h. No accounts of precision and sensitivity were given [2]. The HPLC method is based on chromatographic isolation and quantitation of the mixture of enantiomers, followed by chiral derivatization and re-chromatography of the derivatives to give the enantiomeric ratio [3]. It also uses a time-consuming thionyl chloride-mediated reaction of ketoprofen with 1-phenylethylamine, and consequently it seems to be far from ideal for multi-sample analyses.

For the purpose of pharmacokinetic work, a previously described, facile HPLC determination [4] of the enantiomers of indoprofen, 2-[4-(1,3-dihydro-1-oxo-2*H*-isoindol-2-yl)phenyl]propionic acid, was modified for work with ketoprofen. This determination consists of extraction of indoprofen from acidified plasma, followed by conversion of it into a mixed anhydride with ethyl chloroformate. The mixed anhydride is then allowed to react with an excess of L-leucinamide. The diastereoisomeric N-"indoprofenyl"-leucinamides are then separated and quantitated by reversed-phase HPLC. There is no detectable racemization of either indoprofen or leucinamide during the reactions, which, moreover, are complete within 2.5 min.

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EXPERIMENTAL

Reagents and chemicals

Racemic ketoprofen and internal standard, 2-(4-benzoylphenyl)butyric acid, were gifts from Bayer (Leverkusen, F.R.G.) and Rhone-Poulenc (Paris, France). (*S*)-(+)-Naproxen was a gift from Astra Syntex Scandinavia (Södertälje, Sweden). The compounds were dissolved in 10 m*M* phosphate buffer (pH 8.6) and the appropriate stock solutions were prepared by dilution with distilled water. Tetrabutylammonium (TBA) hydrogen sulphate was purchased from Labkemi (Gothenburg, Sweden). A 1 *M* solution in water was prepared, neutralized with 1 *M* sodium hydroxide solution and washed with dichloromethane (five times) and heptane (twice). A 0.1 *M* sodium carbonate buffer (pH 9.6) containing 0.5 *M* TBA was then prepared. Ethyl chloroformate, triethylamine and L-leucinamide hydrochloride were purchased from Fluka (Buchs, Switzerland).

Diethyl ether (BDH, Poole, U.K.), 1-propanol (Nordic Pharmacopoea quality), dichloromethane (6044; E. Merck, Darmstadt, F.R.G.), cyclohexane (9666; Merck), toluene (8325; Merck), ethyl acetate (9623; Merck), acetonitrile (30; Merck) and methanol (6009; Merck) were used without further purification. The water was freshly distilled and collected in a stainless-steel vessel.

Instrumentation and chromatographic conditions

The liquid chromatograph consisted of an LDC/Milton Roy Constametric III pump (Riviera Beach, FL, U.S.A.), a PROMIS autosampler (Spark Holland, Emmen, The Netherlands) and an LDC Spectro Monitor III variable-wavelength UV detector. A LiChroCart RP-18 7-μm column (250×4 mm I.D.) was used in conjunction with a Hibar precolumn (30×4 mm I.D.) filled with Perisorb RP-18 (all from Merck). The mobile phase was, unless otherwise stated, acetonitrile–10 m*M* phosphate buffer, pH 6.5 (38:62). The flow-rate was 2.0 ml/min and the detection wavelength was 260 nm.

Work-up and derivatization of plasma samples

To 0.50- or 1.00-ml samples of plasma were added 0.50 ml of internal standard solution (4.0 or 8.0 μg/ml) and 0.50 ml of TBA in sodium carbonate buffer (see *Reagents and chemicals*). The samples were extracted with 4 ml of 1-propanol–dichloromethane (1:99) on a Hook and Tucker rotamixer, and the solvent layers were separated by centrifugation at 1200 *g*. The organic layer was transferred to another tube and the solvents were evaporated under a stream of dry air. Five drops of toluene were then added and evaporated in the same way to remove traces of water.

The residue was taken up in 200 μl of 50 m*M* triethylamine–acetonitrile solution. To this mixture were added in sequence, at 30-s intervals, 100 μl each of (1) a 60 m*M* solution of ethyl chloroformate in acetonitrile and (2) a solution of L-leucinamide hydrochloride (1 *M*) and triethylamine (1 *M*) in methanol. After 2 min, 0.5 ml of 0.25 *M* hydrochloric acid were added and the derivatives were extracted with 4 ml of ethyl acetate. The solvents were evaporated as above, and the residue was taken up in 100 μl of acetonitrile. This solution was diluted with

400 μl of 10 mM phosphate buffer (pH 6.5) and 60 μl of this mixture were injected by means of the autosampler.

Extraction yield

To 0.50-ml samples of aqueous ketoprofen solution (10 μg/ml) were added 0.50 ml of TBA in sodium carbonate buffer. The samples were extracted with 4.00 ml of 1-propanol–dichloromethane (1:99) or 1-propanol–dichloromethane–cyclohexane (1:50:50). The concentrations of ketoprofen were determined in both phases. To 0.50 ml of the aqueous phase, 0.50 ml of internal standard solution (10 μg/ml in methanol) and 100 μl of 1 M hydrochloric acid were added. To 2.00 ml of the organic phase, the same amount of internal standard solution was added, the solvents were evaporated and the residue was taken up in acetonitrile and phosphate buffer as described above. Aliquots (60 μl) of these solutions were injected into the chromatograph. The mobile phase was in this case a mixture of methanol–10 mM phosphate buffer (pH 3.5) (60:40). The k' values of ketoprofen and internal standard in this system are 3.5 and 6.0, respectively.

In order to find out whether the presence of plasma proteins influences the extraction yields, the extraction with 1-propanol–dichloromethane (1:99) was performed as above, but from spiked plasma samples, 0.50 or 1.0 ml, instead of from pure ketoprofen solutions.

Extraction of ketoprofen, in its acid form, from plasma acidified with 0.10 ml of 0.6 M sulphuric acid, was also tried. The solvents used were mixtures of propanol and cyclohexane (1:99, 2:98 or 4:96) or cyclohexane and diethyl ether (1:1 or 2:1).

Standard curves and precision

Plasma samples (0.50 ml) spiked with (racemic) ketoprofen to concentrations of 1.0, 2.0, 4.0, 8.0 and 16 μg/ml (3.9–62.9 μM) were analysed in duplicate on addition of internal standard to a concentration of 8.0 μg/ml, and standard curves for the enantiomers were drawn, using peak heights for quantitation. Another standard curve was made on samples spiked with 0.25, 0.50, 1.0 and 2.0 μg/ml (0.98–7.87 μM) and 4 μg/ml of internal standard. The accuracy and precision of the method were assessed by the analysis of eight plasma samples spiked with 8.0 μg/ml and of another eight spiked with 0.50 μg/ml of racemic ketoprofen.

Derivatization reaction

The rate of formation of the derivatives was investigated as described for indoprofen [4]. Samples of pure ketoprofen (1.0 μg) were derivatized by the ordinary procedure, with one modification. The 0.25 M hydrochloric acid, which stops the reaction between the mixed anhydride and the leucinamide, was added 15, 30, 60, 120 or 240 s after the leucinamide solution (duplicate samples for each reaction time). Before the extraction with ethyl acetate, a solution of leucinamide derivative of internal standard was added. The amount of formed ketoprofen derivative was quantitated relative to the added standard.

The experiment was repeated with samples of pure internal standard (1 μg).

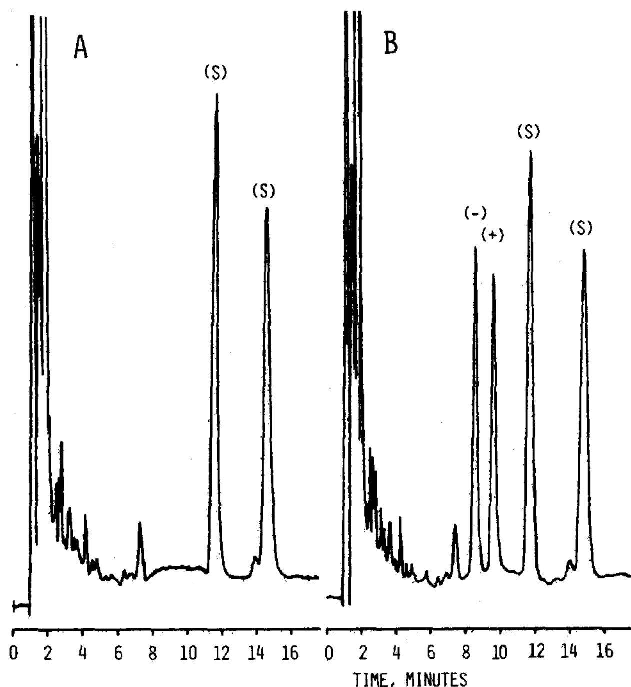

Fig. 1. Chromatograms of derivatized samples. (A) Extract of blank pooled plasma, 0.50 ml, with internal standard (8.0 μg/ml, as racemate). (B) A similar sample spiked with ketoprofen (4.0 μg/ml, as racemate). Detector, 0.02 a.u.f.s.; recorder, 10 mV, 5 mm/min. Peaks: (−) = the leucinamide derivative of (−)-ketoprofen; (+) = the derivative of (+)-ketoprofen; (*S*) = the derivative of any enantiomer of the internal standard.

Quantitation of formed derivative was then relative to added ketoprofen leucinamide.

Identification of the enantiomers

Ketoprofen was partially resolved as an (*S*)-1-phenylethylammonium salt, as described for ibuprofen [5], and the optical rotation of the isolated free acid was measured on a Perkin-Elmer Model 241 polarimeter.

Pharmacokinetic trial of the method

A healthy, male volunteer took 100 mg of ketoprofen as an oral solution 1 h after breakfast. Samples of venous blood were drawn in heparinized Venoject® tubes. The plasma was separated and frozen at −20°C until analysis.

RESULTS

Chromatograms

Typical chromatograms are shown in Fig. 1. The k' values for the ketoprofen derivatives are 8.1 and 9.3, which gives a separation factor, $\alpha = k'_{(+)}/k'_{(-)}$, of

1.14. The derivatives of the internal standard elute with $k' = 11.6$ and 15.0, which gives $\alpha = 1.29$. The leucinamide derivative of (*S*)-(+)-naproxen gave a single peak, $k' = 9.4$, which overlapped with the (+)-ketoprofen leucinamide peak. Naproxen could therefore not be used as internal standard.

Extraction yield

The ion-pair extraction of ketoprofen with 1-propanol–dichloromethane (1:99) from a pure, aqueous solution was apparently quantitative. No ketoprofen could be detected in the aqueous phase. Extraction with 1-propanol–dichloromethane–cyclohexane (1:50:50) gave a 93% yield. When spiked plasma samples were extracted, the aqueous phases could not be analysed because of endogenous interfering peaks. Judging from the analysis of the organic phases, however, the extraction yields relative to that from pure water was 104% with 0.5 ml of plasma and 103% with 1.0 ml of plasma present.

When ketoprofen was extracted from acidified plasma, derivatized and analysed by the ordinary procedure, endogenous interfering peaks corresponding to ca. 0.1 μg/ml of ketoprofen were found in the chromatograms. With ion-pair extraction, no such interfering peaks were found.

Standard curves and precision

The standard curves of the ketoprofen enantiomers were calculated as the height ratios of the first ketoprofen peak to the first internal standard peak and the second ketoprofen peak to the second internal standard peak. The correlation coefficients were greater than 0.999 for the high-concentration (1–16 μg/ml) standard curves and greater than 0.998 for the low-concentration (0.25–2.0 μg/ml) standard curves. The eight samples spiked with 8.0 μg/ml ketoprofen showed the following concentrations (mean $\pm$S.D.) and coefficients of variation: (−)-ketoprofen, 4.06 ± 0.089 μg/ml (2.2%); (+)-ketoprofen 3.99 ± 0.076 μg/ml (1.7%). Those spiked with 0.50 μg/ml showed: (−)-ketoprofen, 0.247 ± 0.012 μg/ml (4.8%); (+)-ketoprofen, 0.249 ± 0.013 μg/ml (5.1%).

Derivatization reaction

For both enantiomers of ketoprofen, plateaus in the peak-height ratios of formed leucinamide derivative relative to added internal standard derivative were found from 30-s reaction time and onwards. No single value deviated from the mean by more than 3.7%. The results were quite similar with the enantiomers of internal standard, with no single value deviating by more than 5.9% after 30-s reaction time.

Identification of the enantiomers

A sample of ketoprofen with an enantiomer excess showing up as a ca. 2:1 ratio of the first to the second ketoprofen derivative peak was obtained. This sample of free ketoprofen was levorotatory in dichloromethane solution. Consequently, the leucinamide derivative with the lower k' value stems from (*R*)-(−)-ketoprofen.

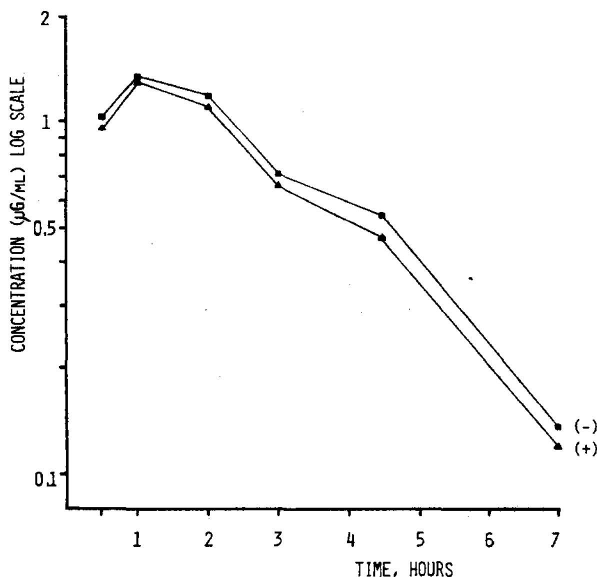

Fig. 2. Plasma concentration–time curves of the ketoprofen enantiomers after oral administration of 100 mg of racemic ketoprofen.

Pharmacokinetic trial of the method

Fig. 2 shows the plasma concentration curves obtained. At least in this person, the difference in disposition between the enantiomers is very small.

DISCUSSION

This method for the determination of the enantiomers of ketoprofen corresponds roughly to the previously reported one for indoprofen [4]. However, since the plasma concentrations of total ketoprofen found within four half-lives after a therapeutic dose is 0.2–10 µg/ml [6], compared with 1–100 µg/ml for total indoprofen [4,7,8], a somewhat higher sensitivity is necessary for the determination of the ketoprofen enantiomers than for the determination of the indoprofen enantiomers. Also, solvent extraction of ketoprofen from acidified plasma (the procedure chosen for indoprofen) gave interfering peaks from endogenous plasma constituents. This problem has been encountered previously [3]. By the use of ion-pair extraction, the peaks from endogenous compounds can be eliminated.

The reaction between a 2-arylpropionic acid mixed anhydride and leucinamide goes without racemization [4]. There is no apparent enantioselectivity in the reaction rate, neither with indoprofen and its butyric acid homologue [4], nor with ketoprofen and its homologue. It has been stated [3] that the reaction between the acyl chloride of ketoprofen and *R*-2-phenylethylamine (misprint for *R*-1-phenylethylamine?) is stereoselective for *R*-ketoprofen, but no experimental evidence for this is given in the paper. A very small stereoselectivity (2% isomer excess in the final product) was found for *S*-indoprofen in the reaction of the

acyl chloride of racemic indoprofen with S-1-phenylethylamine [9]. Since this enantioselectivity was very reproducible with different amounts of indoprofen, it was of no consequence for the analytical results. In general, stereoselectivity in the formation of diastereoisomeric 2-arylpropionic acid amide derivatives does not seem to be a problem.

In conclusion, a facile method for the enantiospecific determination of ketoprofen in blood plasma is described. The sensitivity and precision are satisfactory for pharmacokinetic work.

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