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Note

High-performance liquid chromatographic assay of ketoprofen enantiomers in human plasma and urine

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Ketoprofen (KT), a 2-arylpropionic acid (2-APA) derivative non-steroidal anti-inflammatory drug (NSAID), contains a chiral center, and is marketed as a 50:50 racemic mixture of the $S(+)$- and the $R(-)$-enantiomers. The $S(+)$-isomer is usually responsible for the pharmacologic activity of the 2-APAs, whereas the R-$(-)$-isomer is less active or inactive [1]. It is therefore important to measure levels of the active enantiomer when seeking a correlation between drug levels in blood and clinical efficacy. The resultant information using stereoselective techniques likely forms the basis of a more rational approach to therapy with KT.

Singh and co-workers [2–4] previously described a stereoselective gas chromatographic method for the separation of several NSAID enantiomers, including KT. However, the method is time-consuming, as derivatization includes a lengthy incubation period. Sallustio et al. [5] reported an enantiospecific gas chromatographic assay for ketoprofen and fenoprofen. This method was also time-consuming and lacked the desired sensitivity for KT. Bjorkman [6] reported a high-performance liquid chromatographic (HPLC) assay for the enantiomers of indoprofen. The method involved extraction of unchanged drug, conversion to a mixed anhydride with ethylchloroformate, derivatization with L-leucinamide, extraction of the formed diastereoisomers with an organic solvent, evaporation and injection into the HPLC system. Using this sample preparation method, Bjorkman noticed interfering peaks in his chromatograms. We adopted the above method to assay KT enantiomers in human plasma and urine and noticed three small interfering peaks. Hence, the assay was substantially modified. After submission of this paper, it was brought to our attention by the Editor of this journal that a paper by Bjorkman [7] describing an HPLC assay of KT enantiomers in

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plasma, based on the method used in indoprofen determination, had been recently accepted for publication. To avoid the interfering peaks, Bjorkman [7] used an ion-paired system to extract KT enantiomers from plasma. His assay, however, as compared to the one reported here involved a lengthier sample preparation process and was less sensitive. This method also has the advantage of being applicable for analysis of urinary excretion of conjugated KT.

EXPERIMENTAL

Sample preparation

To 0.5-ml plasma samples containing KT were added 100 μl of an aqueous solution of 100 μg/ml racemic fenoprofen calcium (Eli Lilly, Indianapolis, IN, U.S.A.) as internal standard (I.S.) and 100 μl of 0.6 *M* sulfuric acid as acidifier. The resultant was extracted with a mixture of 5% isopropanol in isooctane after vortex-mixing for 30 s and centrifuging at 1800 relative centrifugal force for 5 min. The organic phase was transferred to a clean glass tube and 3 ml water (HPLC grade, Fisher) were added. Samples were again vortex-mixed for 30 s and centrifuged for 3 min. The organic layer was discarded and 200 μl of 0.6 *M* sulfuric acid were added. Chloroform (3 ml) was added to this, and the samples were vortex-mixed (30 s) and centrifuged (3 min). The aqueous phase was then discarded. The remaining organic phase was evaporated to dryness (Savant Speed Vac 100H concentrator–evaporator, Emerston Instruments, Scarborough, Canada). The residue was dissolved in 100 μl of 50 m*M* triethylamine in acetonitrile. To this mixture were added, at 30-s intervals, 50 μl of 60 m*M* ethyl chloroformate in acetonitrile and 50 μl of a mixture of 1 *M* L-leucinamide hydrochloride (Sigma, St. Louis, MO, U.S.A.) and 1 *M* triethylamine in methanol. After 2 min, 50 μl of water were added. Aliquots of 10–40 μl of the solution were injected into the HPLC system.

Conjugated KT was analyzed in 100–500 μl urine after alkaline hydrolysis with 25–125 μl, respectively, of 1 *M* sodium hydroxide. The samples were then acidified with 0.6 *M* sulfuric acid using 100 μl in excess of the 1 *M* sodium hydroxide volume used for hydrolysis. The sample preparation was then carried out as described for plasma.

Other drugs tested

Fenoprofen (Eli Lilly, Toronto, Canada), flurbiprofen (Boots, U.K.), ibuprofen (Upjohn, Don Mills, Canada), naproxen (Syntex, Palo Alto, CA, U.S.A.), etodolac (Ayerst, Montreal, Canada) and tiaprofenic acid (Roussel, Montreal, Canada) were also subjected to derivatization with L-leucinamide using the above sample preparation method.

Dosing

A 19-year-old healthy subject (77.8 kg) received 50 mg KT (Orudis, Rhone-Poulenc, Montreal, Canada) and donated 4 ml venous blood. The blood was collected 2 and 12 h after the dose into a heparanized Vacutainer, centrifuged, the

plasma portion separated and stored at −30°C until analysis. Urine collected from 12 to 24 h was also analyzed.

The study was approved by the Medical Ethics Committee of the University of Alberta Hospital and a written consent form was signed by the subject.

Standard curves

The enantiomers were quantified against sets of standard solutions prepared by spiking aliquots of 0.5 ml blank plasma with racemic KT (Rhone Poulenc). Final concentrations of the enantiomers were 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 μg/ml. Aliquots of 0.5 ml urine were similarly spiked with KT to give final concentrations of 0.5, 2.5 and 5.0 μg/ml. The solutions were subjected to analysis according to the method described for samples.

Extraction efficiency

To assess the efficiency of the extraction method, spiked solutions of racemic KT (1.0 and 5.0 μg/ml in plasma and 1.0 and 10.0 μg/ml in urine) were extracted ($n=3$) in the absence of I.S. using the above method. After evaporation of the chloroformic layer, the extracted racemic KT was dissolved in 200 μl methanol, and 100 μl I.S. solution were added. Aliquots of 10–40 μl of these solutions were chromatographed according to a non-stereospecific HPLC assay [8]. The area ratios (racemic KT/I.S.) of the eluting peaks were compared with those of solutions of similar concentrations which were injected directly into the HPLC system without extraction. The order of elution of the enantiomers was determined by testing the retention time of a peak eluted from a pure $S(+)$-KT (Rhone-Poulenc) solution.

Instrumentation

The HPLC system consisted of a Waters (Mississauga, Canada) Model 590 pump, Model 712 WISP automatic sample processor, Model 481 UV spectrophotometer, and a Model 3390A Hewlett-Packard (Avondale, PA, U.S.A.) integrator. At ambient temperature, a 10-cm reversed-phase column (Partisil 5 ODS-3, Whatman, Clifton, NJ, U.S.A.) attached to a 5-cm guard column packed with 37–53 μm C_{18} material was utilized throughout the experiment. The mobile phase, 0.06 *M* potassium dihydrogen phosphate–acetonitrile–triethylamine (64:36:0.02) was pumped at a flow-rate of 1.0 ml/min and the detector wavelength was 275 nm. The peak-area method (enantiomer/I.S.) was used to calculate response.

RESULTS AND DISCUSSION

Fig. 1 depicts chromatograms of blank plasma, blank urine and a plasma sample spiked with 0.25 μg/ml of each enantiomer. Diastereoisomers of $R(-)$- and $S(+)$-KT and $R(-)$- and $S(+)$-I.S. eluted at 9.8, 11.3, 17.7 and 19.9 min, respectively.

No interfering peaks were observed. An excellent linear correlation between peak-area ratios (enantiomers/I.S.) and enantiomer concentrations was found

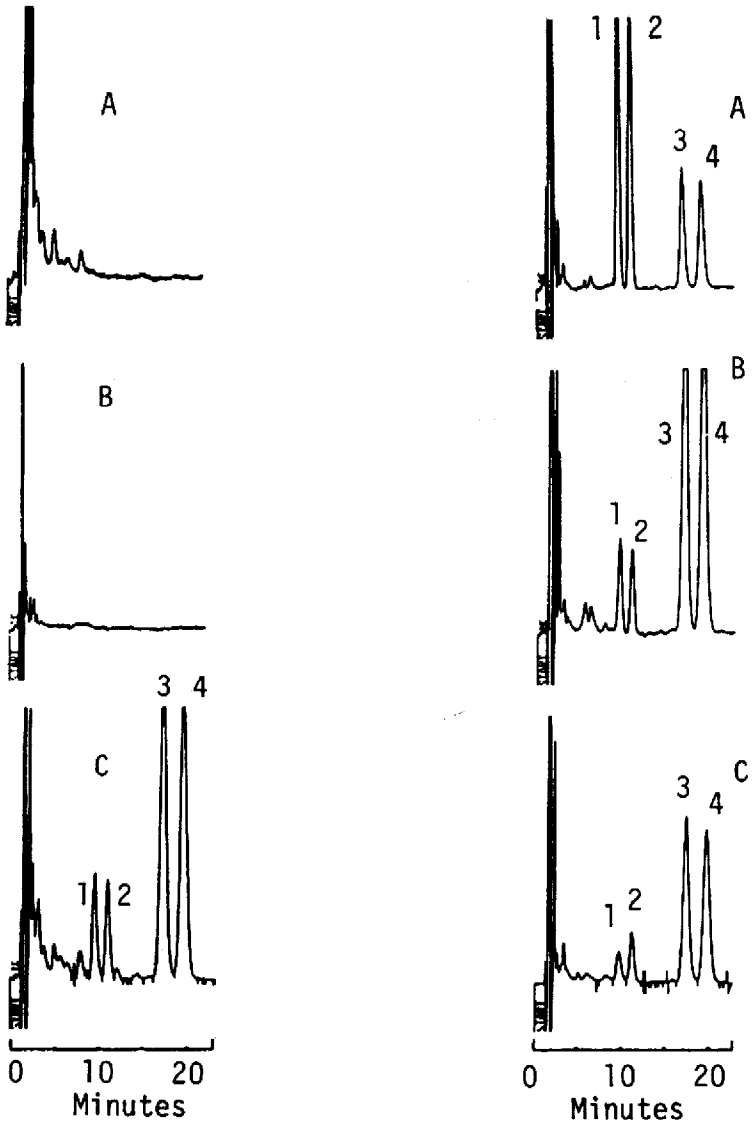

Fig. 1. HPLC profiles of blank plasma (A), blank urine (B) and plasma spiked with 0.25 μg/ml of each enantiomer of KT (C). Injection volumes: 40 μl of plasma and 20 μl of urine solutions. Attenuation: plasma, changed from 1 to 2 at 14.5 min; urine, 2. Peaks: 1 = *R*(−)-KT; 2 = *S*(+)-KT; 3 = *R*(−)-I.S.; 4 = *S*(+)-I.S.

Fig. 2. HPLC profiles of 10 μl of a 2-h plasma sample (A), 40 μl of a 12-h plasma sample (B) and 40 μl of a 12–24 h urine sample (C) after a 50-mg oral dose of KT. Attenuation plasma, changed from 1 to 2 at 14.5 min; urine, 2. Peaks: 1 = *R*(−)-KT; 2 = *S*(+)-KT; 3 = *R*(−)-I.S.; 4 = *S*(+)-I.S.

($r^2 > 0.995$) over the enantiomer concentration range 0.05–5.0 μg/ml for plasma and 1.0–5.0 μg/ml for urine. The best-fit lines passing through the data points were described using the peak-area ratio (y) and the concentration in μg/ml (x), where $y = 0.4161x + 0.0067$ and $y = 0.4243x - 0.0037$ for *R*(−)- and *S*(+)-enantiomers in plasma, respectively. The standard curves describing urine samples

were $y=0.5813x+0.0059$ and $y=0.5880x+0.0422$ for $R(-)$- and $S(+)$-enantiomers, respectively. The coefficient of variation (C.V.) over the examined concentration ranges for plasma and urine was less than 10%.

The 2-h plasma sample had 2.27 μg/ml $R(-)$- and 2.03 μg/ml $S(+)$-enantiomer, while the 12-h sample had 0.13 μg/ml of each enantiomer (Fig. 2). Based on these observations, the minimum quantifiable concentration (MQC) in plasma of 0.05 μg/ml found for this assay was essential if the drug concentrations were to be followed for up to 12 h post-dosing. Bjorkman [7] set his MQC at 0.25 μg/ml while he also reported KT enantiomer concentrations of approximately 0.1 μg/ml 7 h after a single dose of KT. Our assay, therefore, offers a higher sensitivity over that reported by Bjorkman [7].

The extraction yields from plasma (peak-area ratio comparisons of extracted versus directly chromatographed solutions) for the racemic mixture were 77.58% and 78.15% for 1.0 and 5.0 μg/ml, respectively. For urine samples, the yields were 75.51% and 73.96% for 1.0 and 10.0 μg/ml racemic KT, respectively.

The maximum UV absorbance for derivatized KT enantiomers was 250 nm. However, as three small interfering peaks were consistently present at this wavelength, the assay was carried out at 275 nm. These interfering peaks, which were also observed by Bjorkman during the analysis of indoprofen [6] and ketoprofen [7], seem to be a major problem in using the method originally utilized by Bjorkman [6]. For analysis of KT enantiomers, Bjorkman [7] used ion-pair extraction to avoid these peaks. Our simple and convenient sample preparation method also removed the unwanted peaks from the chromatograms. Furthermore, preparation was rapid, as twelve samples required approximately 60 min.

It is worth mentioning that the applicability of this method was also tested for fenoprofen, flurbiprofen, ibuprofen, naproxen, etodolac and tiaprofenic acid. While all of the compounds formed diastereoisomers with L-leucinamide, satisfactory resolution of the enantiomers was noted only for fenoprofen, naproxen and flurbiprofen. With the exception of naproxen, peaks representing the enantiomers of other compounds did not interfere with those of *R*- and *S*-KT.

This assay is stereospecific, convenient and suitable for determination of plasma and urine concentrations of KT following therapeutic doses.

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REFERENCES

1 A.J. Hutt and J. Caldwell, Clin. Pharmacokinet., 9 (1984) 371.
2 N.N. Singh, F.M. Pasutto, R.T. Coutts and F. Jamali, J. Chromatogr., 378 (1986) 125.
3 N.N. Singh, F. Jamali, F.M. Pasutto, A.S. Russell, R.T. Coutts and K.S. Drader, J. Pharm. Sci., 75 (1986) 439.

4 N.N. Singh, F. Jamali, F.M. Pasutto, R.T. Coutts and A.S. Russell, J. Chromatogr., 382 (1986) 331.
5 B.C. Sallustio, A. Abas, P.J. Hayball, Y.J. Purdie and P.J. Meffin, J. Chromatogr., 374 (1986) 329.
6 S. Bjorkman, J. Chromatogr., 339 (1985) 339.
7 S. Bjorkman, J. Chromatogr., 414 (1987) 465.
8 F. Jamali, A.S. Russell, B.W.Berry and C. Lehmann, J. Chromatogr., 310 (1984) 327.