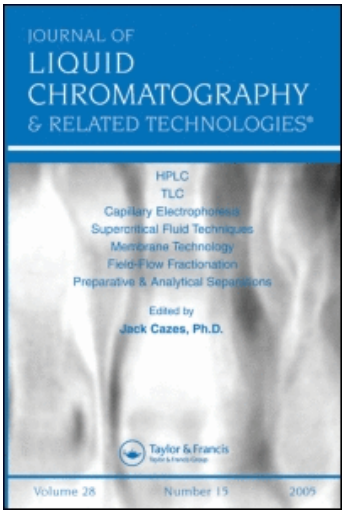

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SIMULTANEOUS MEASUREMENT OF FLURBIPROFEN, IBUPROFEN, AND KETOPROFEN ENANTIOMER CONCENTRATIONS IN PLASMA USING L-LEUCINAMIDE AS THE CHIRAL COUPLING COMPONENT

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ABSTRACT

A high performance liquid chromatographic method was developed for the quantitation of the R- and S- enantiomers of 2-arylpropionic acid namely flurbiprofen, ibuprofen and ketoprofen, in plasma.
The procedure involved extraction of drugs and internal standard from acidified plasma into dichloromethane. After evaporation of the organic layer, the compounds were derivatized with L-leucinamide after addition of ethyl chloroformate as the coupling reagent. The former diastereoisomeric amides were chromatographied at ambient temperature on a reversed phase column using 0.06M KH_2PO_4 - acetonitrile - triethylamine (51:49:0.1) as the mobile phase pumped at a flow rate of 1.8 ml/min. This assay allowed determination of 0.1µg/ml of both R- and S- enantiomers with an acceptable precision (maximum coefficient of variation of 7.5%) using a 0.5-ml plasma sample.

INTRODUCTION

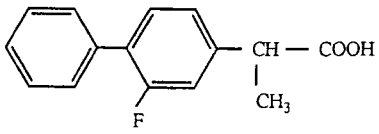
Flurbiprofen (FLU), ibuprofen (IBU) and ketoprofen (KT), potent non-steroidal anti-inflammatory drugs (NSAID) of the 2-arylpropionic acid class (Fig. 1) are currently available in their racemic form. It is well-known that the enantiomers may exert different pharmacodynamic effects. Pharmacokinetic differences have been reported as well (1). The prostaglandin synthetase inhibiting effect of these drugs is attributable to the S(+) enantiomer (2). Therefore, the stereoselective determination of drug enantiomers in plasma is of potential clinical importance (3,4).

Today, three methods are currently used to achieve chromatography enantioselective resolution of racemic compounds, especially 2-arylpropionic acids (5) :

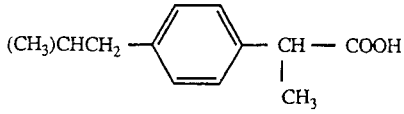
- using chiral HPLC columns
- using achiral HPLC columns with chiral mobile phase
- by derivatization with optical reagents and separation on achiral columns.

Few diastereoisomer derivatization methods were described to achieve the chromatographic separation of FLU, IBU and KETO enantiomers (Table 1). Some of these stereoselective assays entailed extensive sample preparation or lengthy chromatography times associated with a lack of sensitivity. Three of them (7, 8, 13) consist of extraction of the racemate from acidified plasma followed by conversion to a mixed anhydride with ethylchloroformate and derivatization with L-leucinamide. This procedure was first described by Björkman (15). These three methods suffer from a time-consuming extraction step (7, 13) or necessitate a cleanup extraction before injection (8).

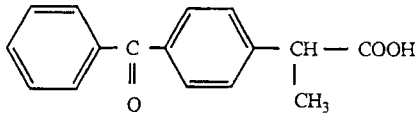
We report a simultaneous analysis of the enantiomers of FLU, IBU and KETO after formation of diastereoisomers of chiral drugs with L-leucinamide hydrochloride. The major modifications of the previously reported assays include simple extraction of unresolved drugs from acidified plasma and rapid reversed-phase HPLC separation of the diastereoisomers within 13 minutes. There is no detectable racemization of either NSAID or leucinamide during the reactions, which are complete within 3 minutes.



Flurbiprofen



Ibuprofen



Ketoprofen

FIGURE 1 : Chemical structures of the studied 2-arylpropionic acid derivatives.

TABLE 1
Previous HPLC Enantiomeric Assays on Human Plasma

Authors	Derivatizing reagent	MQC[a] (µg/ml)	Sample volume [b](ml)
Ketoprofen			
Sallustio *(1986)* (6)	R-2-phenylethylamine	0.2	0.2
Björkman *(1987)* (7)	L-leucinamide	0.25	0.5 - 1
Foster *(1987)* (8)	L-leucinamide	0.1	0.5
Hayball *(1991)* (9)	S-1-phenylethylamine	0.15	1
Ibuprofen			
Mehvar *(1988)* (10)	S-(-)-1-(1-naphthyl)ethylamine	0.1	0.5
Wright *(1992)* (11)	R-(+)-α-phenylethylamine	0.25	0.5
Lemko *(1993)* (12)	S-(-)-1-(1-naphthyl)ethylamine	0.1	0.5
Flurbiprofen			
Berry *(1988)* (13)	L-leucinamide	0.1	0.5
Knadler *(1989)* (14)	S-(α)-methylbenzylamine	0.025	0.5

[a] MQC : minimum quantifiable concentration for each enantiomer
[b] sample volume : volume plasma required for assay

MATERIALS AND METHODS

Reagents and Chemicals

Racemic FLU, IBU, KETO and the two internal standards indomethacin and S (+) naproxen were purchased from Sigma (St Quentin Fallavier, France). Pure R- and S- enantiomers of each drug were kindly supplied by The Boots Company Ltd. (Nottingham, England) for FLU and IBU and by Rhône-Poulenc Rorer (Vitry-Alforville, France) for KETO, respectively. Acetonitrile, dichloromethane and triethylamine were supplied by Info-Labo (Sainte-Foy-la-Grande, France) and were of HPLC reagent grade. Methanol (HPLC) grade was supplied by Prolabo (Paris, France). Ethyl chloroformate and L-leucinamide were obtained from Sigma. Water was deionized and doubly-glass distilled. All others reagents were of analytical grade.

Apparatus and Chromatographic Conditions

The analysis were performed on a Waters Assoc. (Milford, MA, USA) chromatographic system, including a Model 45 constant-flow pump, a Wisp Model 710 B automatic injector and a Lambda Max Model 480 ultraviolet detector operated at 225 nm (IBU analysis) or 275 nm (FLU and KETO analysis), respectively. Samples were chromatographied on an Ultrabase C_{18} 5µ (250 mm x 4.6 mm i.d.) stainless steel column (Shandon HPLC, Cheshire, United Kingdom). Chromatograms were recorded on a 10-mV recorder Omniscribe (Houston Instruments) at a chart-speed of 0.5 cm/min. The mobile phase consisted of 0.06 M KH_2PO_4 - acetonitrile - triethylamine (51 : 49 : 0.1, v/v) pumped at a flow rate of 1.8 ml/min. Before use, the mobile phase was filtered through a 0.45 µm filter (Sartorius, Göttingen, FRG).

Standard Solutions

Stock solutions of (±) FLU, (±) IBU, (±) KETO and internal standars S(+)

naproxen and indomethacin (1 mg/ml) were prepared in methanol and stored at -20°C until use. Appropriate dilutions of each racemate were made in drug-free human plasma to give final enantiomer concentrations of 1, 2, 4, 6, 8 and 10 µg/ml. A solution of S(+) naproxen at 2.5 µg/ml was used for enantiomeric assay of IBU and solutions of indomethacin at 25 and 50 µg/ml were prepared for stereospecific analysis of FLU and KETO, respectively. Ethyl chloroformate (60mM) was prepared in acetonitrile. The derivatizing reagent, L-leucinamide (0.1 M) was prepared in triethylamine - methanol (0.14 : 1).

Work-up and Derivatization of Plasma Samples

To an aliquot of plasma (500 µl of standard or sample) were added 100µl of the appropriate internal standard solution, 500 µl of 0.6M sulfuric acid and 15 ml of dichloromethane. The constituents were then mixed for 20 minutes. After centrifugation at 2000g for 5 minutes, the organic layer was transferred to clean tubes and evaporated to dryness under a gentle stream of nitrogen at ambient temperature. The residue was reconstituted in 100 µl of triethylamine in acetonitrile (50mM), followed by the addition of 50µl of ethyl chloroformate solution (60mM in acetonitrile) and vortexed for 30s. A 50-µl aliquot of L-leucinamide solution (0.1M) was added, vortex-mixed briefly and allowed to stand. After 2 minutes, the reaction was terminated by the addition of 50 µl of bi-distilled water. Aliquots of 10-50 µl of the resulting solutions were injected into the HPLC system.

Extraction Efficiency

To assess the efficiency of the extraction step, standard solutions of each racemic compound (FLU , IBU, KETO) were extracted in the absence of internal standard using the above extraction procedure. The chromatographic conditions were identical to that described for the enantiospecific determinations. The peak heights after injection of racemic NSAID extracted from plasma were compared to those generated from direct injections of aqueous solutions of these compounds.

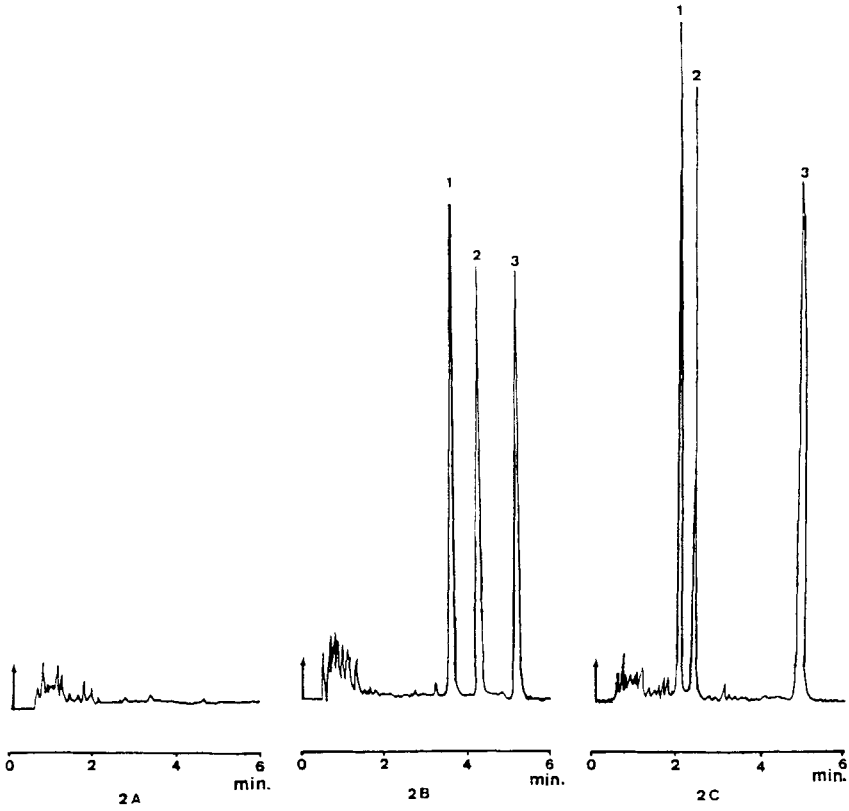


FIGURE 2 : Chromatograms of blank plasma (figure 2A) and drug-spiked human plasma with 6 µg/ml of FLU (figure 2B), KETO (figure 2C) as racemates.

Peak 1 : R(-) enantiomer ; peak 2 : S(+) enantiomer ; peak 3 : internal standard

λ : 275 nm ; AUFS : 0.05

RESULTS AND DISCUSSION

The diastereoisomers of chiral FLU, IBU and KETO were formed at ambient temperature in less than 3 minutes by utilizing L-leucinamide and ethylchloroformate. The amide derivatives formed by reaction of the carboxylic group of the NSAID with the chiral amine, L-leucinamide are separated on an achiral column in a reversed-phase system.

Representative chromatograms of blank and drug-spiked samples are shown in Figures 2 and 3. We did not observe additional peaks formed under the conditions used for derivatization. The elution order of the

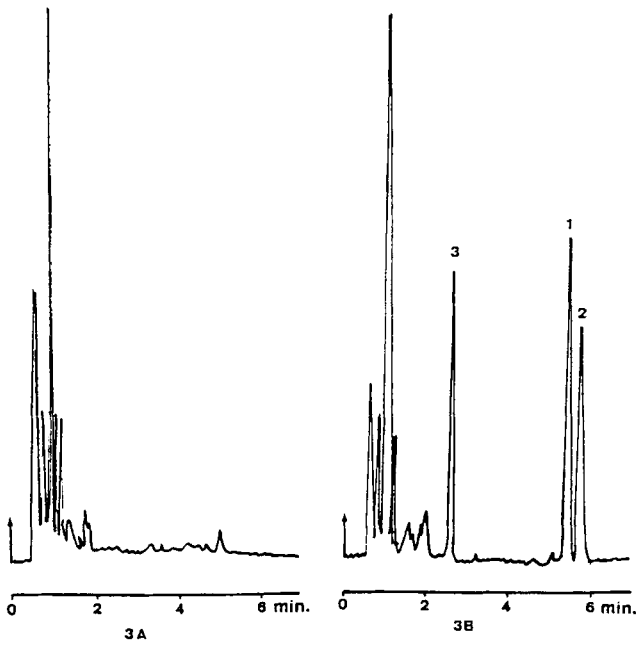


FIGURE 3 : Chromatograms of blank plasma (figure 3A) and drug-spiked human plasma with 6 µg/ml of IBU (figure 3B) as racemates.
Peak 1 : R(-) enantiomer ; peak 2 : S(+) enantiomer ; peak 3 : internal standard
λ : 225 nm ; AUFS : 0.05

TABLE 2
Precision of the FLU, IBU and KETO assays

Compound	Theorical concentration (µg/ml)	Experimental concentration mean (SD) (µg/ml)	CV (%)[a]
S(+) FLU	0.5	0.51 (0.037)	7.2
	2.5	2.69 (0.16)	5.9
	5	5.11 (0.28)	5.5
	7.5	7.67 (0.44)	5.7
R(-) FLU	0.5	0.53 (0.04)	7.5
	2.5	2.74 (0.2)	7.3
	5	5.2 (0.32)	6.1
	7.5	7.74 (0.5)	6.4
S(+) IBU	0.5	0.48 (0.046)	9.6
	2.5	2.57 (0.19)	7.3
	5	5.27 (0.41)	7.8
	7.5	7.65 (0.61)	7.9
R(-) IBU	0.5	0.55 (0.04)	7.3
	2.5	2.52 (0.21)	8.4
	5	5.37 (0.37)	6.5
	7.5	7.44 (0.52)	7.0
S(+) KETO	0.5	0.45 (0.037)	8.2
	2.5	2.10 (0.097)	4.6
	5	4.21 (0.23)	5.4
	7.5	6.33 (0.4)	6.3
R(-) KETO	0.5	0.45 (0.03)	6.7
	2.5	2.13 (0.12)	5.4
	5	4.2 (0.22)	5.3
	7.5	6.39 (0.39)	6.0

[a] : coefficient of variation

diastereoisomers was confirmed by derivatization and chromatography of optically pure R- and S- enantiomers. Using these samples, it was shown that L-leucinamide derivatives of R-FLU, R-IBU and R-KETO always eluted prior to their respective S-isomers.

The precision and repeatability of the method were assessed by triplicate analysis of four control plasma samples containing 1, 5, 10, 15 µ/ml of FLU, IBU and KETO, respectively. These analyses were repeated during three consecutive days (n=9). Thus the corresponding concentrations of each enantiomer S(+) and R(-), were 0.5, 2.5, 5 and 7.5 µg/ml, respectively. The results are presented in Table 2.

TABLE 3
Linear Least-Square Regression Analysis for Each Drug Enantiomer

Compound	Regression equation [a]	Regression coefficient	p-value
S (+) FLU	y = 0.201 (0.005) x - 0.027	0.988	p = 0.0001
R (-) FLU	y = 0.227 (0.0004) x - 0.019	0.992	p = 0.0001
S (+) IBU	y = 0.156 (0.005) x - 0.009	0.982	p = 0.0001
R (-) IBU	y = 0.182 (0.004) x + 0.009	0.988	p = 0.0001
S (+) KETO	y = 0.197 (0.003) x + 0.019	0.993	p = 0.0001
R (-) KETO	y = 0.226 (0.005) x + 0.005	0.989	p = 0.0001

[a] x = enantiomer concentration ;
y = peak-height ratio of compound / internal standard

The precision of the assay for FLU, IBU and KETO enantiomers at low and high concentrations were within acceptable limits for clinical studies. Over the entire concentration range studied, the coefficients of variation for determination of S- and R- FLU enantiomers were less than 7.5%. Similar coefficients of variation were obtained for IBU and KETO enantiomers, being less than 9.6% and ≤8.2%, respectively.

The calibration curves generated over the enantiomeric concentration range from 1 to 10 µg/ml (supplied as racemic drug) were linear for both S- and R- enantiomers of each drug. Linear least-square regression analysis for nine calibration curves of each enantiomeric drug is depicted in Table 3. The slopes of the lines describing both FLU, IBU and KETO enantiomers are not statistically different (Wilcoxon - test : NS, $p<0.01$) indicating non-stereoselectivity in the extraction and/or derivatization methods.

The extraction yields for the racemic mixture were 88.6 ± 8.4 % (FLU), 81.9 ± 9.5 %(IBU) and 80.8 ± 7.1 % (KETO), respectively.

The limit of detection (LOD, signal to noise ratio = 3) was found to be 0.1 µg/ml for each enantiomer.

CONCLUSION

The present method is common to the determinations of R- and S-enantiomers of FLU, IBU and KETO. It is simpler than most of the methods reported in the literature. This HPLC procedure only requires one extraction step of racemates from plasma and a rapid derivatization of less than 3 minutes. This analytical procedure employing relatively simple HPLC technology is sensitive enough for use in single-dose pharmacokinetic studies.

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