*Journal of Chromatography*, 415 (1987) 214–220
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3478

## Note

# Determination of flunoxaprofen enantiomers in biological fluids by high-performance liquid chromatography

S. PEDRAZZINI, W. ZANOBONI-MUCIACCIA, C. SACCHI and A. FORGIONE*

*Research and Development Department, Ravizza SpA, Via Europa 35, 20053 Muggiò, Milan (Italy)*

(First received April 18th, 1986; revised manuscript received October 21st, 1986)

Flunoxaprofen, *S*(+)-2-(4-fluorophenyl)-α-methyl-5-benzoxazoleacetic acid [(*S*)-FLX], is a member of a new series of α-methyl-5-benzoxazoleacetic acids possessing anti-inflammatory activity. This compoud has demonstrated notable anti-inflammatory, analgesic and antipyretic activity in animal tests [1].

Recently, detailed studies have indicated the stereoselective inversion of (−)-α-methylarylacetic acids [2] to the (+)-enantiomers in animals and man. Stereoselective metabolism of optical isomer [3–11] involving preferential biotransformation of one isomer or differences in rates of excretion of the isomers appears to be a widespread phenomenon in the metabolism of drugs; moreover, the high degree of stereospecificity of the anti-inflammatory action of several pairs of α-methylarylacetic acid enantiomers is known [12,13]. Therefore, a method for the simultaneous determination of each optical isomer of (*R,S*)-2-(4-fluorophenyl)-α-methyl-5-benzoxazoleacetic acid in biological fluids was a prerequisite for the stereoselective metabolic and activity studies we are performing on animals and man.

Stereospecific assays have been reported for several other non-steroidal anti-inflammatory drugs (NSAIDs) of the 2-arylpropionic acid group. An example is the determination in body fluids of the enantiomers of the flunoxaprofen analogue benoxaprofen, 2-(4-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid, by high-performance liquid chromatography (HPLC) as α-methylbenzylamide formed by reaction with 1,1′-carbonyldiimidazole [14].

This paper describes a simple method for the determination of FLX optical isomers in biological fluids using S(−)-1-phenylethylamine to form diastereo-

0378-4347/87/$03.50 

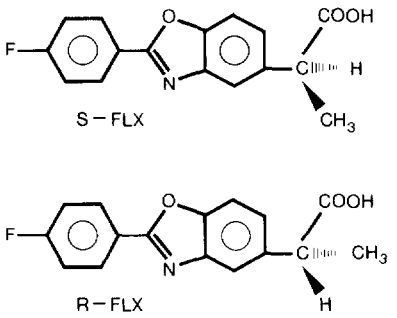


Fig. 1. Enantiomeric structures of flunoxaprofen.

isomeric amides by reaction with 1,1′-carbonyldiimidazole; the amides can be separated by HPLC using a reversed-phase $C_{18}$ stationary phase.

## EXPERIMENTAL

### *Apparatus*

Chromatography was performed with a Perkin-Elmer (Norwalk, CT, U.S.A.) Series 3B pump fitted with a Rheodyne 7125 injection valve and a 50-$\mu$l sample loop. A 125 mm $\times$ 4.6 mm I.D. reversed-phase octadecyl, 5 $\mu$m particle size, high-speed column (Perkin-Elmer) was used. A Perkin-Elmer Model LC 95 variable-wavelength UV detector was operated at 300 nm. A Perkin-Elmer LCI-100 laboratory computing integrator was used to record, store and analyse the chromatograms.

### *Reagents and chemicals*

Analytical- or reagent-grade ethanol, acetonitrile, hydrocarbon-stabilized chloroform, phosphoric acid, sodium hydroxide, sodium chloride, 1,1′-carbonyldiimidazole (CDD), acetic acid and benzyl cinnamate (BC) were obtained from Merck Schuchardt (Darmstadt, F.R.G.) and *S*(−)-1-phenylethylamine from Fluka (Buchs, Switzerland). (*S*)-FLX, racemic (*R,S*)-FLX and the *R*(−) enantiomer [(*R*)-FLX] were synthetized in our laboratories. The enantiomeric structures of FLX are shown in Fig. 1.

Water was passed through a 0.2-$\mu$m SM 11607 membrane filter (Sartorius, Göttingen, F.R.G.) before use.

A stock solution of CDD (64 mg/ml) in hydrocarbon-stabilized chloroform was prepared fresh daily. Stock standard solutions of (*S*)-, (*R,S*)- and (*R*)-FLX (100 $\mu$g/ml) were prepared in 10 m*M* sodium hydroxide solution and that of BC (internal standard) (100 $\mu$g/ml) was prepared in ethanol.

### *Procedure*

The mobile phase was acetonitrile–water (52.5:47.5, v/v) at a flow-rate of 1.5 ml/min. Plasma and urine standards for calibration were prepared by spiking 1-ml aliquots of plasma and urine with stock solution of (*S*) and (*R*)-FLX to give

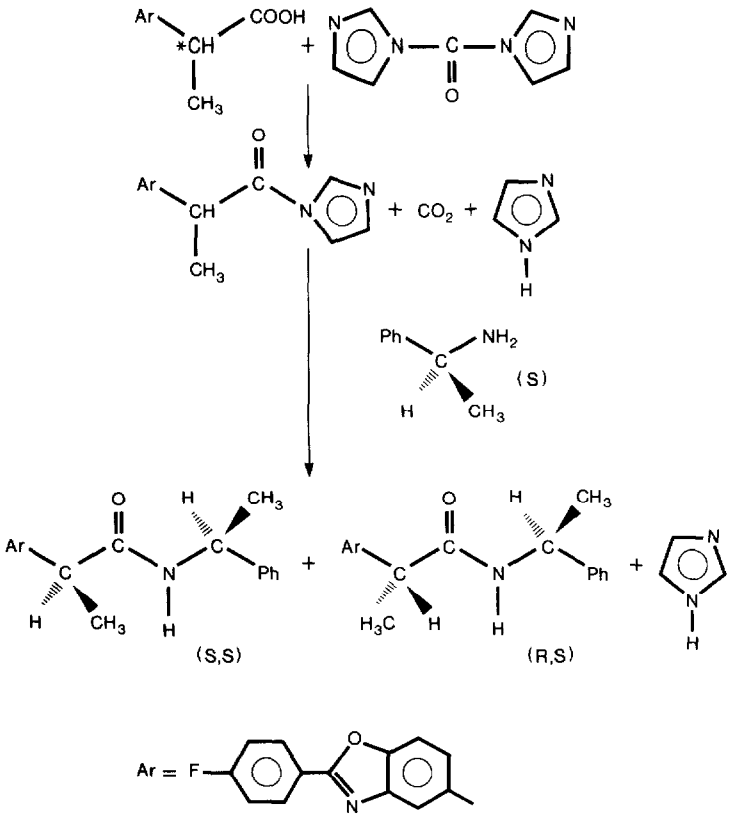


Fig. 2. Reaction mechanism and structure of flunoxaprofen enantiomer derivatives.

concentrations ranging from 1 to 15 μg/ml. The concentration of the internal standard (BC) was 10 μg/ml in each serum and urine sample.

The procedure for extraction from serum was as follows. A 1-ml volume of serum was pipetted into a glass tube and exactly 100 μl (10 μg) of internal standard (BC), 1.5 ml of 2 *M* phosphoric acid and 8 ml of chloroform were added. The tubes were then gently vortexed for 10 min. After centrifuging, the aqueous layer was separated and 5 ml of chloroform were added, again following the above-mentioned procedure. The organic layers were evaporated to dryness under a stream of nitrogen. The residue was dissolved in 500 μl of chloroform, 500 μl of CDD stock solution were added and the solution was kept at room temperature for 20 min. Then 40 μl of 10% chloroform solution in acetic acid were added and mixed; 150 μl of 10% chloroform solution of *S*(−)-1-phenylethylamine were pipetted, mixed and the solution was allowed to react for 2 h. Thereafter the solution was evaporated to dryness with nitrogen and the residue was dissolved in 500 μl of mobile phase. Aliquots of this solution were injected into the HPLC column.

The urine extraction procedure for the free compound was as follows. A 1 g amount of sodium chloride and exactly 100 μl (10 μg) of internal standard (BC)

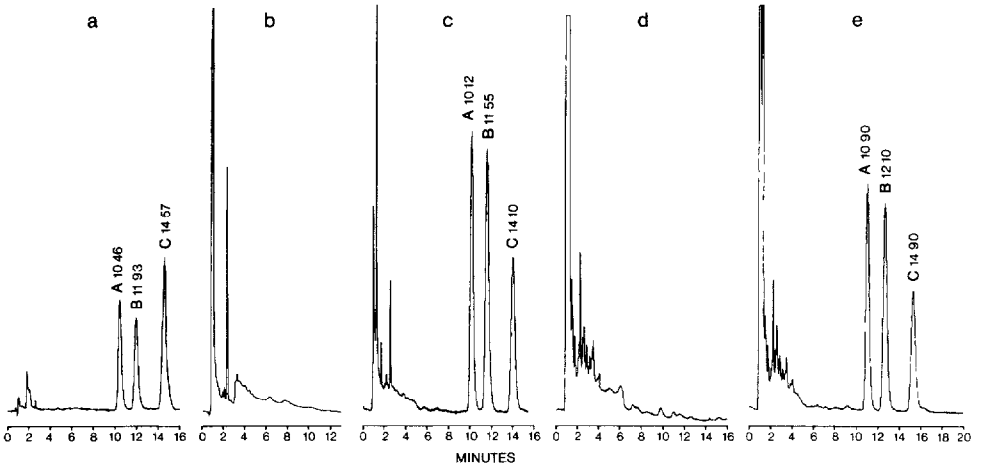


Fig. 3. Chromatograms of: (a) standard solutions of (*R,S*)-FLX (5 μg/ml) and internal standard (BC) (10 μg/ml); (b) extracts from 1 ml of blank serum; (c) serum obtained from a man 2 h after the administration of 100 mg of (*R,S*)-FLX orally; the concentrations were found to be 6.40 μg/ml for (*S*)-FLX and 6.0 μg/ml for (*R*)-FLX; (d) extracts from 1 ml blank urine; (e) 24- urine samples obtained from the same subject; the concentrations found were 6.6 μg/ml for (*S*)-FLX and 5.8 μg/ml for (*R*)-FLX. Peaks: A = (*S*)-FLX (retention time 10.4 min); B = (*R*)-FLX (retention time 12.0 min); C = internal standard (BC) (retention time 14.5 min).

were added to 1 ml of urine and then acidified to pH 2 with 2 *M* phosphoric acid. The subsequent procedure was as described for serum.

For the free and bound compound (glucuronide derivative), 1 g of sodium chloride, 1 ml of posphate buffer (pH 6.8) and 100 μl of β-glucuronidase–arylsulphatase (12 and 60 U/ml, respectively; from *Helix pomatia*, Merck-Schuchardt) were added separately to 1 ml of urine. Each sample was mixed briefly following each addition using a vortex mixer. The tubes were allowed to react for 40 h at 37 °C, then the described procedure was followed.

TABLE I

REPRODUCIBILITY OF THE ASSAY OF OPTICAL ENANTIOMERS

| Enantiomer | Nominal concentration (μg/ml) | Concentration found (mean ± S.E., $n = 4$) (μg/ml) | Coefficient of variation (%) |
|---|---|---|---|
| (*S*)-FLX | 1.0 | 0.9 ± 0.05 | 11.5 |
| | 2.5 | 2.2 ± 0.01 | 0.6 |
| | 5.0 | 4.2 ± 0.08 | 4.0 |
| | 10.0 | 8.8 ± 0.06 | 1.3 |
| (*R*)-FLX | 1.0 | 0.9 ± 0.05 | 11.4 |
| | 2.5 | 2.3 ± 0.01 | 1.0 |
| | 5.0 | 4.2 ± 0.06 | 2.7 |
| | 10.0 | 8.8 ± 0.04 | 0.8 |

TABLE II

RECOVERIES OF $S(+)$- AND $R(-)$-FLX FROM HUMAN SERUM AND URINE

| Enantiomer | Added (μg/ml) | Serum | | Urine | |
|---|---|---|---|---|---|
| | | Found (μg/ml) | Recovery (%) | Found (μg/ml) | Recovery (%) |
| $S(+)$-FLX | 1 | 0.94 | 94.0 | 0.89 | 89.1 |
| | 2.5 | 2.23 | 89.2 | 2.31 | 92.4 |
| | 5 | 4.21 | 84.1 | 4.10 | 82.0 |
| | 7.5 | 6.84 | 91.2 | 7.00 | 93.9 |
| | 10 | 8.85 | 88.5 | 9.13 | 91.3 |
| | 15 | 14.21 | 94.7 | 16.00 | 106.7 |
| Mean ± S.E. | | | 90.3 ± 1.59 | | 92.5 ± 3.29 |
| $R(-)$-FLX | 1 | 0.93 | 93.0 | 0.91 | 91.0 |
| | 2.5 | 2.26 | 90.4 | 2.27 | 90.8 |
| | 5 | 4.18 | 83.6 | 4.31 | 86.2 |
| | 7.5 | 6.76 | 90.1 | 6.36 | 84.9 |
| | 10 | 8.85 | 88.5 | 8.83 | 88.3 |
| | 15 | 13.27 | 88.5 | 15.56 | 103.7 |
| Mean ± S.E. | | | 89.0 ± 1.27 | | 90.8 ± 2.76 |

## RESULTS AND DISCUSSION

Earlier investigations [15] showed that CDD was a useful reagent for the facile esterification of arylacetic acids in human plasma extracts. The imidazolide intermediates of $(S)$- and $(R)$-FLX formed very rapidly ($\leq 1$ min) and were highly reactive. A series of aqueous samples containing known amounts of $(S)$- and $(R)$-FLX was prepared to determine the optimal reaction times for imida-

TABLE III

AMOUNT OF FREE AND BOUND FLX ENANTIOMERS EXCRETED IN URINE SAMPLES AT DIFFERENT TIME INTERVALS BY A PATIENT TREATED WITH 100 mg OF RACEMIC FLX ORALLY

| Time (h) | Amount excreted (mg) | | | |
|---|---|---|---|---|
| | $S(+)$ free | $S(+)$ bound | $R(-)$ free | $R(-)$ bound |
| 0–6 | 3 5 | 11.76 | 3.11 | 8.37 |
| 6–12 | 1.9 | 4.45 | 1.25 | 1.80 |
| 12–24 | 1.08 | 4.21 | 0.91 | 2.40 |
| 24–48 | 0.61 | 4.78 | 0.53 | 3.70 |
| 48–72 | 0.09 | 0.77 | 0.09 | 0.44 |
| Total | 7.18 | 25.97 | 5.89 | 16.71 |

zolide formation and for amide formation from the imidazolides and $S(-)$-1-phenylethylamine. The results indicated that imidazolide formation from $(S)$- and $(R)$-FLX was complete within 1 min. Amide formation from the imidazolides derivatives was complete within 1 h. The reaction mechanism and structures are shown in Fig. 2.

Fig. 3a is a representative chromatogram of FLX enantiomers and the internal standard. The identification of $(S)$- and $(R)$-FLX was achieved by comparing the retention times of peaks from standard solutions of the two enantiomers; under our experimental conditions the retention times were 10.46 min for $(S)$-FLX, 11.93 min for $(R)$-FLX and 14.57 min for the internal standard (BC). Excellent linearity of the calibration graph was achieved for the concentration range 1–15 $\mu$g/ml for $(S)$-FLX and $(R)$-FLX [$r=0.9997$ for $(S)$-FLX and $r=0.9998$ for $(R)$-FLX].

The HPLC method described for the assay of FLX enantiomers in urine and serum was applied to samples collected from a human volunteer after an oral dose of 100 mg of the racemic drug. Fig. 3b and d show the absence of interfering peaks from extract blanks of serum and urine; Fig. 3c and e represent chromatograms of FLX enantiomers from 1 ml of serum and 1 ml of urine from a patient treated with 100 mg of $(R,S)$-FLX orally. The limit of detection is 0.5 $\mu$g/ml for the enantiomers in serum and urine. The reproducibility for $(S)$- and $(R)$-FLX is presented in Table I.

Average recoveries when the enantiomers were extracted from spiked serum and urine were $90.3 \pm 1.6$ and $92.5 \pm 3.3\%$ for $(S)$-FLX and $89.0 \pm 1.3$ and $90.8 \pm 2.8\%$ for $(R)$-FLX, respectively (Table II). The recoveries were essentially quantitative.

The method presented is precise, specific and sensitive for the assay of FLX enantiomers in serum and urine and has been used to quantitate FLX enantiomer levels in animal and clinical pharmacokinetic studies. The amounts of free and bound FLX enantiomers excreted in urine samples at different time intervals by a patient treated with 100 mg of racemic FLX orally are reported in Table III.

These preliminary results indicate that a larger amount of $(S)$-FLX than $(R)$-FLX is excreted. Further studies will be useful in ascertaining the biochemical mechanism responsible for the selective dextro-isomeric elimination.

REFERENCES


1 D.W. Dunwell, D. Evans, T.A. Hicks, C.J. Cashin and A. Kitchen, J. Med. Chem., 18 (1975) 53.
2 R.J. Bopp, J.F. Nash, A.S. Ridolfo and E.R. Shepard, Drug Metab. Dispos., 7 (1979) 356.
3 S.S. Adams, E.E. Cliffe, B. Lessel and J.S. Nicholson, J. Pharm. Sci., 56 (1967) 1686.
4 P. Jenner and B. Testa, Drug Metab. Rev., 2 (1973) 117.
5 S.F. Sisenwine, H.B. Kimmel, A.L. Liu and H.W. Ruelius, Drug Metab. Dispos., 3 (1975) 150.
6 R.F.N. Mills, S.S. Adams, E.E. Cliffe, A. Dickinson and J.S. Nicholson, Xenobiotica, 3 (1973) 589.
7 W.J. Wechter, D.G. Loughead, R.J. Reischer, G.J. van Giessen and D.G. Kaiser, Biochem. Biophys. Res. Commun., 61 (1974) 833.
8 S.S. Adams, P. Bresloff and C.G. Mason, J. Pharm. Pharmacol., 28 (1976) 256.
9 K.J. Kripalani, A. Zein El-Abdin, A.V. Dean and E.C. Schreiber, Xenobiotica, 6 (1976) 159.
10 A.V. Dean, S.J. Lan, K.J. Kripalani, L.T. Difazio and E.C. Schreiber, Xenobiotica, 7 (1977) 549.

11 A.J. Hutt and J. Caldwell, J. Pharm. Parmacol., 35 (1983) 693.
12 T.Y. Shen, Angew. Chem., Int. Ed., Engl., 11 (1972) 460.
13 B. Testa, Trends Pharmacol. Sci., 7 (1986) 60.
14 S.W. McKay, D.N.B. Mallen, P.R. Shrubsall, B.P.Swann and W.R.N. Williamson, J. Chromatogr., 170 (1979) 482.
15 D.G. Kaiser and G.J. van Giessen, J. Pharm. Sci., 63 (1974) 219.