Journal of Chromatography, 276 (1983) 230—236
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1716

Note

Rapid and simple determination of carprofen in plasma by high-performance liquid chromatography with fluorescence detection

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(First received December 16th, 1982; revised manuscript received March 11th, 1983)

Carprofen, *rac*-6-chloro-α-methylcarbazole-2-acetic acid (I) (Fig. 1) is a new non steroidal anti-inflammatory agent marketed in Europe as Imadyl [1, 2].

High-performance liquid chromatographic (HPLC) methods have been used for the determination of drugs in blood and plasma based on normal-phase [3] and reversed-phase ion-pair partition [4]; both methods involve extraction of the drug into diethyl ether, evaporation of the extracts to dryness and HPLC with fluorescence detection. HPLC has also been used to determine the stereoselective disposition of carprofen in humans [5].

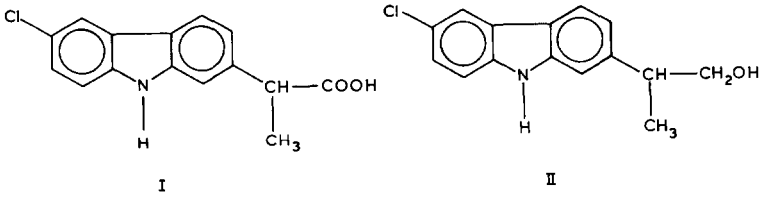

Fig. 1. Structures of compounds I and II.

In order to avoid time-consuming operations (transfer of extracts, evaporation, etc.), we have developed an HPLC method for the determination of I with the use of an internal standard, *rac*-2-(6-chloro-2-carbazolyl)-1-propanol (II) (Fig. 1), which allows the directs injection of the extracts without the evaporation step, the eluent mixture is similar to that described by Puglisi et al. [3].

0378-4347/83/$03.00

EXPERIMENTAL

Materials and reagents

The water used for the preparation of solutions was of HPLC grade, produced by a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Methylene chloride and methanol (HPLC grade), acetic acid and sodium acetate (analytical-reagent grade) were obtained from E. Merck (Darmstadt, G.F.R.).

To prepare an acetate buffer (1 *M*), a solution of sodium acetate (1 *M*) was added to 1 *M* acetic acid solution to achieve a final pH of 2.8.

Carprofen (Ro 20-5720) (I) and the internal standard (Ro 21-0134) (II) were of pharmaceutical grade (Hoffmann-La Roche, Basle, Switzerland).

Standard solutions

Stock standard solutions of compounds I and II were prepared by dissolving 10 mg of each compound in 10 ml of methanol. Working standard solutions were prepared from the stock solutions by suitable dilutions with methanol (Table I), and 50 μl were added to plasma as described below. The stock solutions and standard solutions were stored at 0—5°C in amber-coloured volumetric flasks and prepared freshly each week or each day, respectively.

TABLE I

STANDARD SOLUTIONS USED FOR THE ANALYSIS

Standard solution No.*	Compound I (ng per 50 μl)	Compound II (ng per 50 μl)	Expected concentration range of I in unkown (ng/ml)
1	3000	3000	500—7000
2	500	500	100—2800
3	150	150	20—200
4	0	3000	500—7000
5	0	500	100—2800
6	0	150	20—200

*Solutions 1, 2 and 3 are mixed standard solutions to be added to control plasma for instrument calibration (working standard solutions). Solutions 4, 5 and 6 are internal standard solutions to be added to unkown samples.

HPLC equipment and operating conditions

The chromatographic system consisted of a Model 110 constant-flow pump (Altex, Berkeley, CA, U.S.A.), a Model SFM 23 spectrofluorimetric LC detector (Kontron, Zürich, Switzerland) with a flow cell of volume 20 μl, operating at 305 nm for excitation and 375 nm for emission, a Model 7120 sample injector (Rheodyne, Berkeley, CA, U.S.A.) with a loop capacity of 20 μl, an analytical column (25 × 0.4 cm I.D.) of the Hibar type filled with LiChrosorb Si 60 (5 μm) and a pre-column (3 × 0.4 cm I.D.) filled with LiChrosorb Si 60 (7 μm) (E. Merck).

The mobile phase, consisting of methylene chloride—methanol—acetic acid

(98:1:1), was filtered through a 0.5-μm filter before use. The procedure was carried out at a constant flow-rate of 1.5 ml/min.

The fluorescence detector was coupled to a chromatographic computer (Sigma 10; Perkin-Elmer, Norwalk, CT, U.S.A.) for the integration of peak areas and subsequent calculations, using the internal standard method. The chromatograms were recorded on a W+W 600 recorder (Kontron).

Under these conditions, the retention times of compounds I and II were about 4 and 5.5 min, respectively. The entire system was maintained overnight at a flow-rate of 0.2 ml/min of the mobile phase. Before a period of non-use (for example, a weekend) the column had to be treated first with 50 ml of methylene chloride—methanol (98:2), then 50 ml of methylene chloride and finally 50 ml of *n*-hexane, which was the solvent used for column storage.

Procedure for plasma samples

A 50-μl aliquot of internal standard solution (Table I; depending on the expected concentration of the unknown sample) was transferred into a screw-topped test-tube (PTFE-lined caps, Sovirel 13) for each unknown sample; 1 ml of plasma was added and mixed well. For concentrations greater than 5 μg/ml, 0.5 ml or less of plasma was taken, followed by dilution to 1 ml with water.

A separate set of standards was prepared by transferring 50-μl aliquots of mixed standard solutions (Table I) into separate screw-topped test-tubes; control plasma was then added and mixed well.

To all samples, 0.5 ml of acetate buffer was added, then homogenized by slow rotation; 2 ml of *n*-butyl acetate were added and the sample was shaken on a tumble extractor (5 min at 25 rpm).

The samples were centrifuged at 2000 *g* for 5 min at 5°C on a refrigerated centrifuge (Hermle Z 365-K; Kontron); 20 μl of the upper organic phase were then injected into the HPLC system.

Calculation

The Sigma 10 computed the peak areas and the corresponding concentrations of I were obtained according to the internal standard method. The response factor was checked daily from one or more working standard solutions prepared freshly each day according to the expected concentration range of I (Tables I and III).

RESULTS

Linearity

A linear correlation between the peak-area ratio of I:II versus concentration of I was observed in the range 0.02–6 μg/ml of plasma (n = 10). The linear regression analysis performed on theoretical data (amount of I added to control plasma) versus amount found gave the equation $y = 1.0557x - 0.0213$ with a correlation coefficient of 0.9998.

Accuracy and precision

Before performing accuracy experiments using the internal standard method, the absolute recoveries of I and II were obtained from plasma spiked with the

TABLE II

ACCURACY AND PRECISION RESULTS FOR PLASMA SAMPLES SPIKED WITH I

Amount added (ng/ml)	Accuracy (%)	*n*	Intra-assay precision (%)*	Instrument reproducibility (%)**
3000	95.4	5	0.75	0.65
500	96.2	5	2.20	1.78
250	97.0	5	2.10	2.60
150	97.0	5	2.25	2.50
50	95.7	5	4.80	5.20
20	89.0	5	4.55	4.00

*Refers to the standard deviation obtained after analysing five plasma samples having the same nominal concentration during one day.
**Refers to the standard deviation obtained on chromatographing one of the five plasma extracts six times over a one-day period.

TABLE III

INTER-ASSAY PRECISION EVALUATED FROM THE WORKING STANDARD SOLUTIONS ON DIFFERENT DAYS

Working standard solution No.*	Date**	RF***	mean RF (± S.D.)	C.V. (%)
1	8.23.82	1.020	1.0432 ± 0.01948	1.9
	8.24.82	1.011		
	8.26.82	1.019		
	8.31.82	1.062		
	9. 1.82	1.071		
	9. 3.82	1.054		
	9. 6.82	1.060		
	9. 8.82	1.045		
	9. 9.82	1.038		
	9.10.82	1.052		
3	8.23.82	0.993	1.041 ± 0.0378	3.6
	8.24.82	1.002		
	8.26.82	1.000		
	8.31.82	1.040		
	9. 1.82	1.001		
	9. 3.82	1.082		
	9. 6.82	1.049		
	9. 8.82	1.100		
	9. 9.82	1.071		
	9.10.82	1.074		

*No. 1 contains I and II, both at 3000 ng/ml in plasma. No. 3 contains I and II, both at 150 ng/ml in plasma. The control plasma used for preparing the working standard solutions is the pre-dose sample of the subject involved in the pharmacokinetic study. Therefore ten different pre-dose plasma samples were tested.
**Date of the assay (month, day, year).
***Response factor, $RF = \frac{\text{Peak area (II)} \cdot C\,(\text{I})}{\text{Peak area (I)} \cdot C\,(\text{II})}$; mean of two determinations.

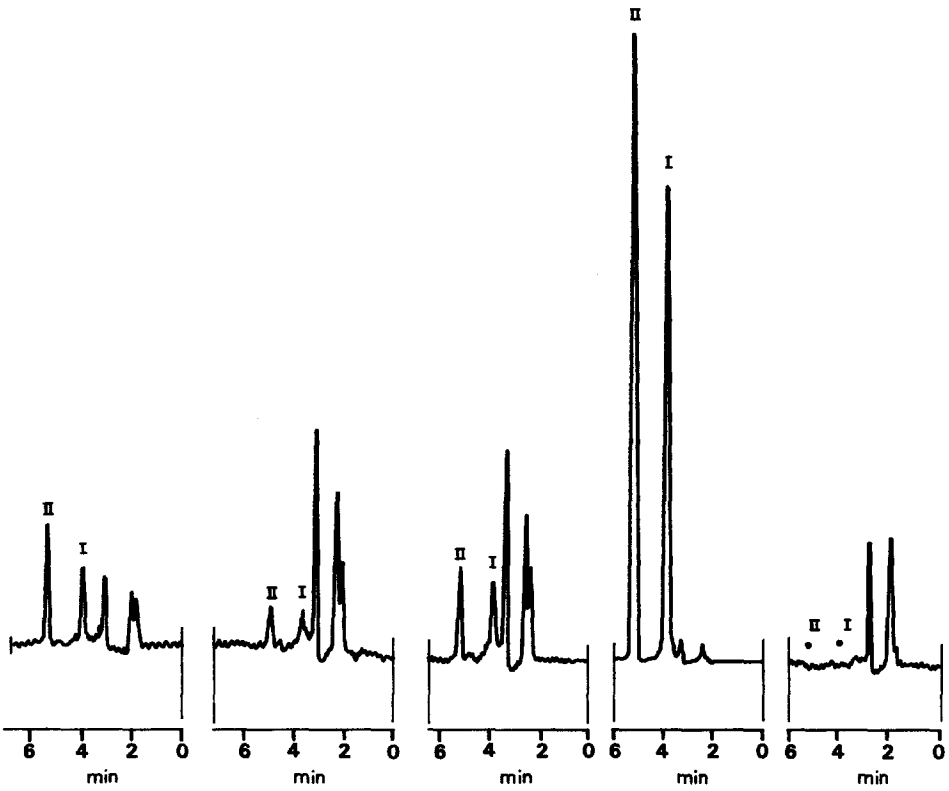

Fig. 2. Chromatogram of control plasma extract. Instrument settings: sensitivity adjustment (coarse) = High HV var; sensitivity adjustment (fine) = 10; variable photomultiplier high voltage adjustment = 4. Recorder range = 100 mV.

Fig. 3. Chromatogram of authentic standards recovered from control plasma, I and II, 3 μg/ml in plasma. Instrument settings: sensitivity adjustment (coarse) = High; sensitivity adjustment (fine) = 5.

Fig. 4. Chromatogram of authentic standards recovered from control plasma, I and II, 50 ng/ml in plasma. Instrument conditions as in Fig. 2.

Fig. 5. Chromatogram of authentic standards recovered from control plasma, I and II, 20 ng/ml in plasma. Instrument conditions as in Fig. 2.

Fig. 6. Chromatogram of plasma extract from a volunteer dosed rectally with 150 mg of carprofen. Plasma sample 36 h after the dose. Instrument settings: sensitivity adjustment (coarse) = High; sensitivity adjustment (fine) = 10. Concentration of I found: 0.21 μg/ml.

two compounds; it was about 96% for both substances over a wide range of concentrations.

Accuracy studies were performed on control plasma spiked with I. The accuracy, defined as (amount found/amount added) · 100, was found to be about 96% over a wide range of concentrations (Table II). Table II also reports intra-assay and inter-assay precision results (Figs. 2—4).

The inter-assay precision results, verifying the long-term reproducibility of the assay, are reported in Table III.

Sensitivity

The detection limit was about 20 ng/ml in plasma, with a signal-to-noise ratio of approximately 3:1 (Fig. 5).

Application of the method to biological specimens

The assay was applied to the quantitation of I in the plasma of volunteers dosed orally and rectally with carprofen (150 mg, single dose) in a cross-over study (Fig. 6).

The plasma concentration–time course curve in a volunteer is shown in Fig. 7. In this example, the high sensitivity of the method was not required, but it could prove necessary for drug determinations 48 h or more after drug administration, or in cases of lower doses (for instance, 50 mg).

The reported example refers to a bioavailability study performed during a drug product development (suppository). The relative bioavailability, in this case (the standard dosage form was oral tablets) was low (about 50%) because of critical factors of formulation; recently further biopharmaceutical studies

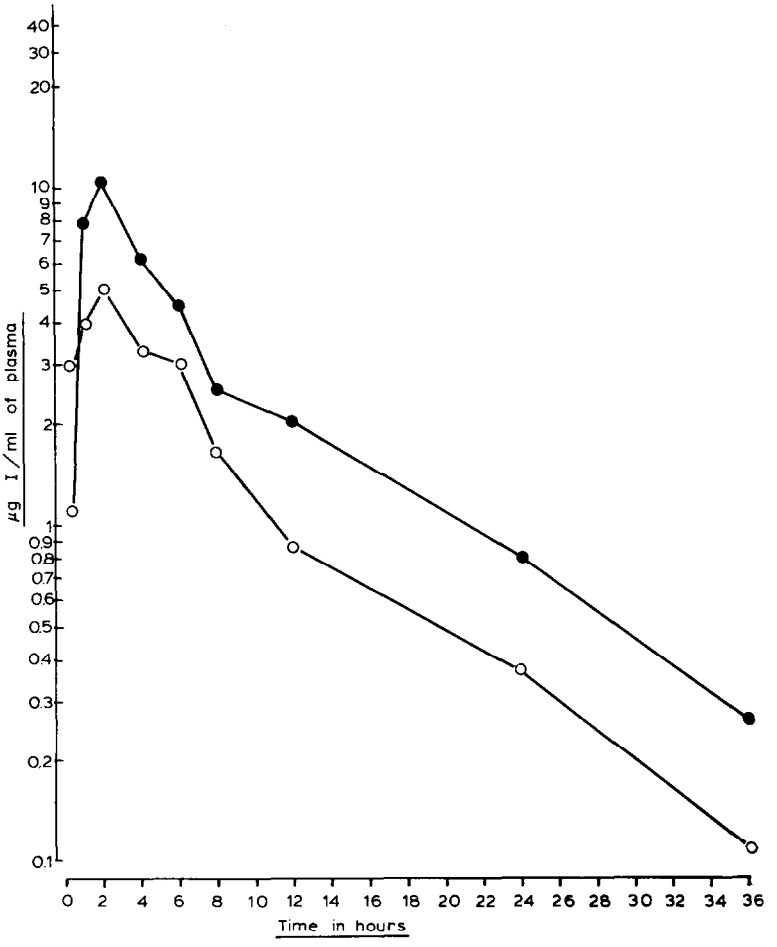

Fig. 7. Drug plasma profile of I in a volunteer dosed orally (•) and rectally (○) with 150 mg of carprofen (between the two administrations a 2-week "wash-out" period was allowed).

have led to a formulation with about 100% relative bioavailability. The pharmacokinetics of carprofen in humans was reported recently [6].

DISCUSSION

The main advantage of the procedure described is associated with the sample preparation stage. Time-consuming transfers of extracts and evaporation are avoided.

Carprofen and the internal standard could be quantitatively extracted from plasma with halogenated solvents, but this necessitated the removal of the upper aqueous layer prior to injection. The pH selected was that which allowed the minimum volume of *n*-butyl acetate to be used (2 ml).

The strong fluorescence of carprofen allowed the injection of diluted plasma extracts which, in turn, led to a relatively long column life.

The method was readily adapted to automatic injection and allowed the rapid analysis of large numbers of samples arising from clinical studies.

ACKNOWLEDGEMENT

The authors are grateful to Dr. D. Dell (Biological Pharmaceutical Research Department, Hoffmann-La Roche, Basle, Switzerland) for helpful suggestions and correcting the manuscript.

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