Note

Determination of (d, l)-6-chloro-α-methylcarbazole-2-acetic acid in plasma by high-performance liquid chromatography

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The drug (d,l)-6-chloro-α-methylcarbazole-2-acetic acid (Ro 20-5720) is used as an antiinflammatory agent. For determination of the substance in the normal dose range, an analytical method with high specificity and a sensitivity in the nanomole range is necessary. Previously described methods for the analysis of the drug lack both specificity and sensitivity[1].

The high-performance liquid chromatographic (HPLC) method described here was based on reversed-phase ion-pair partition. This technique gives separation systems of high efficiency. The retention is determined by the properties of the mobile phase[2–4]. By the use of fluorimetric detection, high sensitivity can be achieved in the analysis of fluorescent compounds. The fluorescence of carbazole compounds was described by Bender et al.[5].

EXPERIMENTAL

Reagents

An acetate buffer solution (0.1 M, pH 5.0) was prepared. Diethyl ether (Uvasol), methanol (Uvasol), acetic acid (Merck, Darmstadt, G.F.R., p.a. quality) and tetrabutylammonium hydroxide (TBA-OH) (Eastman, Kodak, Rochester, N.Y., U.S.A. 25.9 % in methanol) were used.

Standard solutions

Compound 1 (Fig. 1), molecular weight 273.72, melting point 206.0–208.0° (with decomposition), of pharmaceutical grade supplied by Hoffmann-La Roche (Basle, Switzerland) was used as an analytical standard. A stock solution was prepared by dissolving 100 mg of the compound in 100 ml of methanol (A) and diluting 1:9 to yield a solution of 0.1 mg/ml (B). From solutions A and B working solutions in plasma were made, containing 20 and 1 μg/ml. The working solutions were diluted with plasma to give 10 batches covering the range from 0.07 to 14.6 μmoles/l (20 ng/ml to 4 μg/ml).
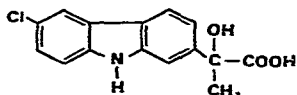
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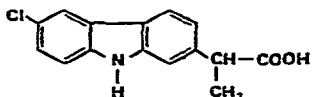
Compound 1   pKa = 4.73
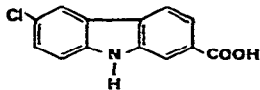
Compound 2

Compound 3   pKa = 3.70

Fig. 1. Structures of compounds. 1 = (d,l)-6-Chloro-α-methylcarbazole-2-acetic acid; 2 = 6-chloro-2-carbazolecarboxylic acid (internal standard); 3 = 6-chloro-α-hydroxy-α-methylcarbazole-2-acetic acid (metabolite).

Internal standard solution

Compound 2 (Fig. 1), 6-chloro-2-carbazolecarboxylic acid, was supplied by Hoffmann-La Roche. A stock solution was prepared by dissolving 10.0 mg of the compound in 100 ml of methanol; 1.0 ml of the stock solution was added to 1000 ml of the buffer solution (250 μl to 1000 ml of buffer for the lower standard curve, 0.07–0.7 μmole/l).

Chromatographic apparatus and preparation of the mobile phase

The liquid chromatography system consisted of a high-pressure pump (Waters Assoc., M-6000), a loop injector (Waters Assoc., U6K) and a fluorescence detector (Schoeffel, FS 970). The excitation wavelength was 300 nm and a 370-nm cut-off filter was used. A μBondapak C_{18} separation column ,30 cm × 4 mm, was used.

The mobile phase, containing the counter ion, was prepared from de-gassed, triply distilled water and methanol (30:70). A 10.0-ml volume of TBA-OH solution was added to 1000 ml of the water–methanol mixture and the pH was adjusted to 7.3 with acetic acid. In order to prevent contamination of the mobile phase with air, the solution was stored under nitrogen and the air inlet of the bottle was fitted with a charcoal filter. The flow-rate of the mobile phase was 1.5 ml/min.

Analysis of plasma

In the analytical procedure, an autopipette (Autochem Instruments, Lidingö, Sweden) was used to increase the reproducibility of the pipettings.

Into a 15-ml tube, 100 μl of plasma (1.0 ml for concentrations below 1.46 μmole/l) and 1.0 ml of buffer solution containing the internal standard (4.0 ml for concentrations below 1.46 μmole/l) were introduced. The mixture was extracted with 5 ml of diethyl ether on a reciprocating shaker for 10 min. After centrifugation at 1000 g for 10 min, the supernatant ether phase was transferred into another 15-ml tube with a conical bottom. The diethyl ether was evaporated off under a stream of nitrogen at 30°, the residue was dissolved in 100 μl of methanol and 10 μl of the solution were injected for HPLC–fluorescence analysis.

In vivo study

Six healthy volunteers (four males and two females) were used. One 75-mg capsule or one 75-mg tablet was ingested on an empty stomach (overnight fasting) together with 0.1 l of water. Venous blood samples were drawn 0.5, 1, 2, 3, 4, 6, 12, 24 and 48 h following administration, no food intake being allowed until after the 4-h samples had been taken. The trial was performed with the cross-over method. The drug was administered in a random manner in the order of capsule–tablet or tablet–capsule. The period between ingestion of the two formulations was 2 weeks.

RESULT AND DISCUSSION

Analytical procedure

The chromatographic system used (reversed-phase ion-pair partition), is not temperature sensitive and room temperature can be used throughout, but the column
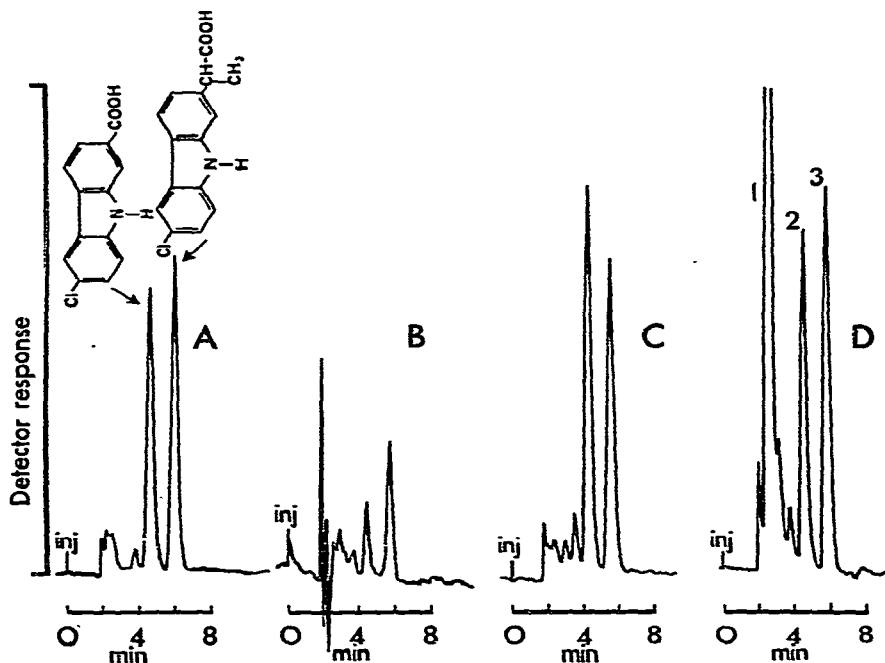

Fig. 2. Chromatograms from analysis of plasma samples. A, standard sample (307 ng/ml of compound 1); B and C, UV and fluorescence detector responses, respectively, to the 30-min sample from a normal subject after administration of 100 mg; D, 60-min sample from a plasma containing salicylate. Peaks: 1, salicylate; 2, compound 2; 3, compound 1.

must be equilibrated with the mobile phase at a flow-rate of 1.5 ml/min for 45 min before use. With a counter ion concentration of 10 mM, the water content of the mobile phase must be 25–30%, depending on the number of theoretical plates in the column, in order to achieve a good resolution and a retention time of about 6 min.

Fig. 2A shows a typical chromatogram from a standard sample of compound 1, Fig. 2B shows the chromatogram obtained with plasma from a patient when a UV detector (LDC Spectromonitor) was used at the absorption maximum of 238 nm, and Fig. 2C shows the chromatogram obtained with fluorimetric detection with excitation at 300 nm for the same plasma sample. The results show a higher sensitivity accompanied by increased specificity for the fluorimeter.

The absorption and luminescence properties of compound 1 are well documented[1,5] and the fluorescence spectrum for compound 1 in the mobile phase is in accordance with the spectrum obtained by De Silva *et al.* in ethanol and 1% glacial acetic acid[1]. The fluorescence was linear over a wide range of concentrations (Fig. 3).

The simple sample pre-separation makes it possible to analyze at least 150 samples in 3 days. The recovery of the drug from plasma varied from 84 to 94% (Table I). The error of the method was calculated for two plasma levels (Table II).
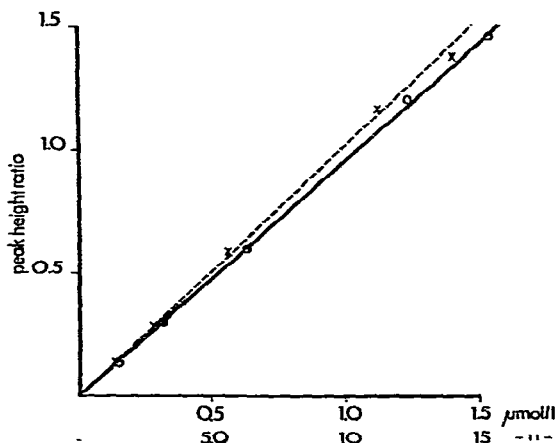

Fig. 3. Standard curves: peak-height ratio of compound 1 to compound 2 *versus* concentration of compound 1. Amounts extracted: ×, 0.14–1.4 μmol/l from 1 ml of plasma; O, 1.54–15.4 μmol/l from 100 μl of plasma.

TABLE I

EXTRACTION RECOVERIES

Results obtained by adding the internal standard after the extraction procedure and comparison with a non-extracted methanol standard series. Each figure represents the mean of 4 observations.

Amount of compound 1 (μmole/l)		Recovery (%)
Added	Found	
1.54	1.37	89.5
3.07	2.59	84.3
6.17	5.50	89.1
12.3	11.2	91.0
15.4	14.4	93.5

TABLE II

EXPERIMENTAL ERRORS FOR THE DETERMINATION OF COMPOUND 1 IN PLASMA

Determined by duplicate analysis. $S = \sqrt{\dfrac{\Sigma d^2}{2n}}$, where d is the difference between the duplicate analysis.

Range ($\mu mole/l$)	n	Mean ($\mu mole/l$)	Error(s) of the method ($\mu mole/l$)	% of mean
0.14– 1.4	12	0.30	0.034	11.4
1.54–15.4	95	10.9	0.460	4.21

Specificity of the method

For routine clinical use, the specificity of the method was checked for some substances with antiinflammatory activity. Phenylbutazone, indometacin, phenacetin and naproxene did not interfere. Salicylates, on the other hand, are detectable under the chromatographic conditions used but appear just after the front in the chromatogram (Fig. 2D). The metabolite 6-chloro-α-hydroxy-α-methylcarbazole-2-acetic acid (compound 3, Fig. 1) does not interfere under the conditions used. It gives intense fluorescence on excitation at 300 nm and, after several injections of a large amount of this metabolite, the baseline will rise slightly. The columns have been used for more than 300 injections with only a small decrease in the theoretical plate number.
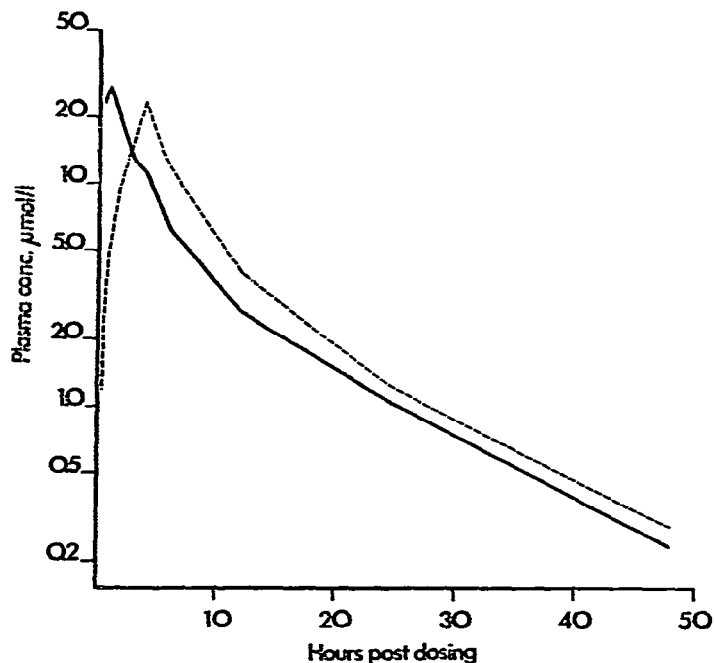

Fig. 4. Plasma level fall-off curve of a subject after administration of 75 mg of compound 1 as tablet (solid line) and as capsule (broken line).

In vivo study

The plasma level fall-off curves ranged from a maximum of 15.6–43.1 μmoles/l after 1–4 h to 0.073–0.555 μmole/l after 48 h for capsules, and from a maximum of 14.8–30.0 μmoles/l after 0.5–4 h to 0.036–0.475 μmole/l after 48 h for tablets. A plasma level fall-off curve is shown in Fig. 4. There was no consistent difference in plasma level attributable to the form of galenic preparation.

The variation of the peak level is probably due to the fact that the substance is poorly dissolved in the acidic medium and is therefore dependent on the acidity in the stomach and the stomach emptying time. Probably the irregularities in the first part of the plasma concentration curve can be explained by the same mechanism. The intra- and inter-individual variations of the "peak time", if due to this mechanism, will make any conclusion on the relationship between the galenical preparation and resorption time irrelevant.

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