Journal of Chromatography, 620 (1993) 273–277
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7077

Short Communication

Sensitive high-performance liquid chromatographic method for the determination of coumarin in plasma

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(First received September 22nd, 1992; revised manuscript received July 27th, 1993)

ABSTRACT

A high-performance liquid chromatographic method was developed for the determination of coumarin in plasma at low concentrations. The method involves a single-step extraction of the alkalinized sample with hexane and subsequent evaporation of the organic phase in the presence of hydrochloric acid to collect and concentrate the coumarin. Analysis of the acidic phase was performed on a C_8 column and coumarin was detected by measuring the UV absorbance at 275 nm. The limit of detection was 0.3 $\mu g\ l^{-1}$. The assay was used to study the evolution of concentrations of coumarin in one volunteer after oral administration of a single 10-mg dose.

INTRODUCTION

Coumarin (5,6-benzo-2-pyrone) is a naturally occurring substance widely used at low doses (5–10 mg day^{-1}) as a vascular protectant in the treatment of venous insufficiency symptoms and more recently at high doses (100–400 mg day^{-1} and more) as a macrophage stimulant in the reduction of high protein oedemas [1–3]. It has also been tested as an immunostimulant in malignant neoplasmas [4–7], in addition to cytostatic agents.

Methods for the determination of coumarin in foodstuffs, plant extracts and galenical formulations have been extensively published, but very few have dealt with its determination in biological fluids. To our knowledge, only spectrofluorimetry for coumarin and 7-hydroxycoumarin in blood [8] and thin-layer chromatography [9] and high-performance liquid chromatography (HPLC) for their metabolites in urine [10,11], have previously been proposed. These methods were primarily designed for pharmacokinetic or toxicological studies after administration of high doses (0.5–25 mg kg^{-1}) of coumarin used in lymphoedema or cancer therapy. The main problem in determining coumarin in blood is the very low level of the circulating intact compound, which is due to an extensive first-pass effect (95–99%) with transformation into hydroxylated and conjugated metabolites [12].

The aim of this work was to develop a sensitive HPLC method for the determination of coumarin in plasma after oral administration of a low dose (10 mg). This would allow pharmacokinetic

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0378-4347/93/$06.00

studies of coumarin at doses close to those used in venous insufficiency.

EXPERIMENTAL

Chemicals

Coumarin was generously provided by Boots-Pharma Labs. (Courbevoie, France) and flunitrazepam, the internal standard, by Roche Labs. (Neuilly-sur-Seine, France). HPLC-grade acetonitrile and analytical-reagent grade potassium monophosphate, tetraethylammonium chloride and hexane were obtained from Merck (Darmstadt, Germany), analytical-reagent-grade hydrochloric and phosphoric acid from Prolabo (Paris, France) and Tris buffer (pH 8) from Syva-Biomérieux (Dardilly, France). Ultra-pure water was prepared using a Milli-Q reagent water system (Millipore, St.-Quentin Yvelines, France).

HPLC equipment

Chromatography was performed using a Varian (Sunnyvale, CA, USA) Model 5000 equipped with a 20-μl universal loop injector (Valco Instruments, Houston, TX, USA), a variable-wavelength Spectroflow 783 UV detector (Kratos, Ramsey, NJ, USA) and a Linseis L600 strip-chart recorder (Bioblock Scientifics, Illkirch, France). Separation and analysis were carried out on a reversed-phase system with a C_8 column (LiChrospher 100 RP-8, 250 mm × 4 mm I.D., 5 μm; Merck) linked to a C_8 precolumn, 35 mm × 4 mm I.D., packed with a Vydac reversed phase (Varian).

Chromatographic conditions

The mobile phase was acetonitrile–0.01 *M* phosphate buffer (pH 3) (60:40), containing 0.02 *M* tetramethylammonium chloride and was filtered through a 0.45-μm Millipore filter before use. The solvent flow-rate was 1.2 ml min^{-1} and the chromatographic system was operated at room temperature. The eluent was monitored by measuring the UV absorbance at 275 nm at a sensitivity of 0.001 or 0.002 AUFS and the recorder chart speed was 0.25 cm min^{-1}.

Preparation of standard solutions

Stock standard solutions of coumarin and the internal standard, flunitrazepam, were prepared by dissolving the appropriate amounts in methanol to give 1 g l^{-1} solutions. They were stored in glass flasks at 4°C. Calibration standards were prepared by diluting the appropriate volume of stock standard coumarin solution in drug-free plasma to give final concentrations of 0, 0.625, 1.25, 2.5, 5 and 10 μg l^{-1}. A flunitrazepam working standard solution was obtained by diluting the stock standard solution in methanol to give a final concentration of 1 mg l^{-1}.

Extraction of coumarin from plasma samples

In a 10-ml glass-stoppered centrifuge tube, 200 μl of Tris buffer (pH 8) and 25 μl of the internal standard solution were added to 1 ml of plasma sample. The resulting solution was stirred for 1 min with 6 ml of hexane on a Heidolph Top-Mix 94323 shaker (Bioblock Scientifics) and centrifuged. The organic layer was transferred into a 5-ml glass centrifuge tube containing 50 μl of 0.2 *M* HCl and evaporated at 40°C under dry nitrogen, while avoiding as much as possible any loss of acidic phase. The hydrochloric solution was used as the sample solution.

Quantification

The standards were extracted along with the unknown samples according to the same procedure. Peak-height ratios of coumarin to flunitrazepam were used to construct the calibration graphs. All calibration graphs were calculated by least-squares linear regression analysis of peak-height ratio *versus* coumarin concentration. The concentration of coumarin in the unknown samples was calculated from the equations of these calibration graphs.

Extraction recovery and precision

Two controls were prepared from plasma spiked with 1.25 and 5 μg l^{-1} of coumarin. The extraction efficiency was calculated by comparing the peak-height ratios of these controls with those of directly injected coumarin and flunitrazepam. The within-day precision was determined

by analysing each control five times on the same day. The day-to-day precision was established by analysing the interpolated values of the standards on calibration graphs obtained from seven different assays.

Pharmacokinetic application

At 9 a.m. a fasting healthy male volunteer was given 20 ml of an oral solution of sweet clover coumarinic extracts (Esberiven®, Boots-Pharma Labs.), containing 0.5 mg ml^{-1} of coumarin, *i.e.* 10 mg. Blood samples were collected in heparinized tubes 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 190, 240 and 300 min after administration. The blood was rapidly centrifuged after collection and the plasma was stored at −20°C before being analysed by the HPLC procedure.

RESULTS AND DISCUSSION

Performance of the HPLC system

For optimum UV detection, the wavelength of the detector was set at 275 nm, which was close to the λ_{max} of coumarin and flunitrazepam. Fig. 1 shows typical chromatograms of extract from plasma from an untreated subject, an extract from the same plasma spiked with diluted stock solutions and from a patient plasma sample drawn 6 min after a 10-mg oral dose of coumarin. The peaks of both coumarin and flunitrazepam were well resolved and their symmetry was satisfactory. The retention times were 5.5 min for coumarin and 9 min for flunitrazepam and their resolution was > 1 (3.75). There were no interfering peaks and the calibration graphs were linear over the concentration range examined.

Recovery, precision, calibration and sensitivity

Table I illustrates the recovery obtained from spiked plasma; the extraction efficiency was about 80% for both coumarin and flunitrazepam.

Repeated assays of spiked samples indicated that the reproducibility of the procedure was satisfactory over the calibration range (Table II). Day-to-day coefficients of variation (C.V.) of values of interpolated standards ranged from 13.8% at 0.625 $\mu g\,l^{-1}$ and 15.8% at 1.25 $\mu g\,l^{-1}$ to 1.1% at 10 $\mu g\,l^{-1}$. The within-day C.V. for samples spiked at 0.625 $\mu g\,l^{-1}$ was 9.4% but always below 5% from 1.25 to 10 $\mu g\,l^{-1}$.

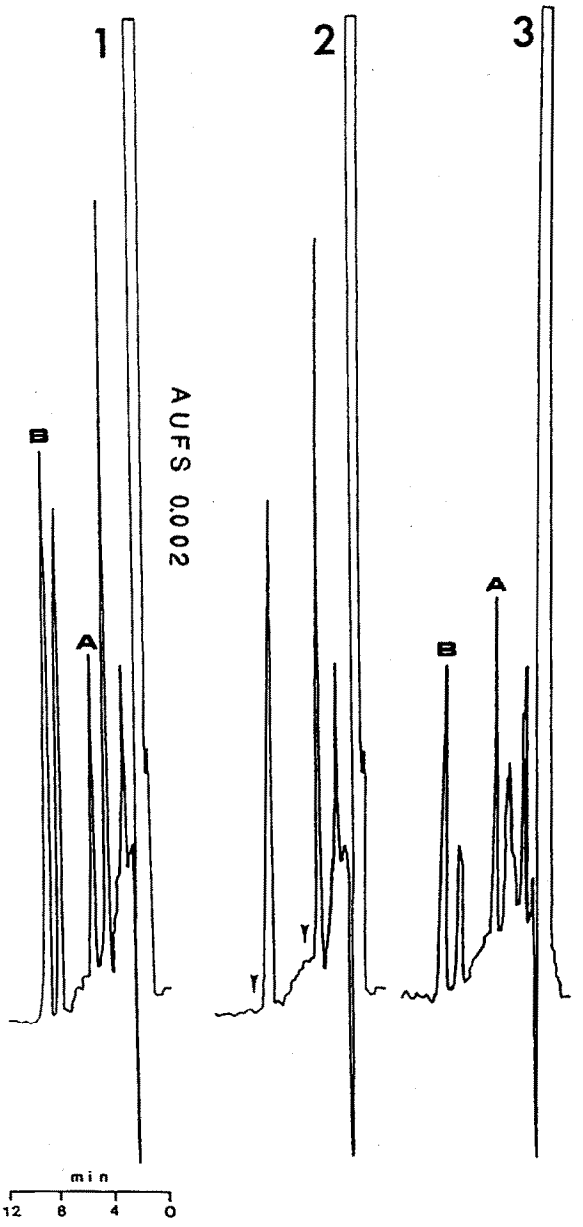

Fig. 1. Chromatograms of plasma extracts. (1) Drug-free plasma spiked with (A) 5 $\mu g\,l^{-1}$ of coumarin and (B) 25 $\mu g\,l^{-1}$ of flunitrazepam; (2) drug-free plasma; (3) sample obtained from a patient 6 min after a single oral dose of 10 mg of coumarin.

It must be emphasized that the evaporation step is most critical for the quality of recovery

TABLE I

RECOVERY OF COUMARIN FROM SPIKED PLASMA SAMPLES

Compound	Concentration added ($\mu g\ l^{-1}$)	Recovery (%) (n = 5)	Standard deviation (%)
Coumarin	1.25	81.3	6.8
	5.00	80.6	3.3
Flunitrazepam	25.00	77.1	3.7

and precision: coumarin, with a melting point of 68–70°C, is a volatile compound which sublimes at room temperature in the solid state. It was therefore necessary to evaporate the hexane phase in dilute hydrochloric acid, which would trap the coumarin, while evaporating as little of the aqueous phase as possible.

Calibration graphs of concentration against peak-height ratios were linear with correlation coefficients over 0.995. The detector response was found to be consistently linear over the range 0–10 $\mu g\ l^{-1}$, and preliminary studies showed the same linearity over the range 0–125 $\mu g\ l^{-1}$. The limit of detection for coumarin was calculated to be 0.3 $\mu g\ l^{-1}$ for a signal-to-noise ratio of 2.

Pharmacokinetic experiment

A plot of coumarin plasma concentration *versus* time after oral administration of 10 mg of coumarin is shown in Fig. 2. The maximum concentration (C_{max}) was found as soon as 5 min after a single oral administration and reached 5.81 $\mu g\ l^{-1}$. The coumarin concentration then declined rapidly and fell beneath the limit of detection at 240 min. The fitting of these concentrations to a two-compartment model with oral absorption gave values of 4 min for the distribution half-life and 72 min for the terminal half-life, while the mean residence time was 100 min. These data correspond with those reported in previous studies [13–16].

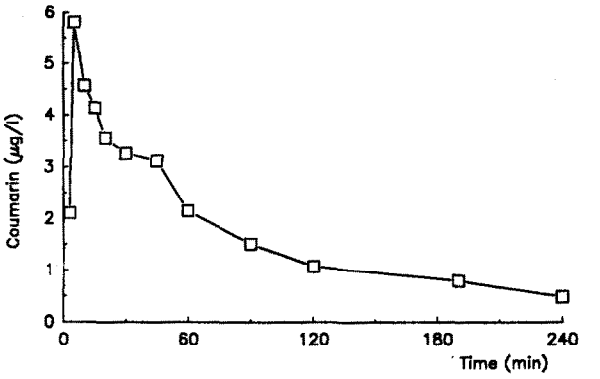

Fig. 2. Kinetics of coumarin after a single oral dose of 10 mg to a fasting healthy male volunteer.

TABLE II

WITHIN-DAY AND DAY-TO-DAY REPRODUCIBILITY FOR COUMARIN-SPIKED PLASMA SAMPLES

Coumarin ($\mu g\ l^{-1}$)	Within-day C.V. (%) (n = 5)	Day-to-day C.V. (%) (n = 7)
0.625	9.4	13.8
1.25	3.1	15.8
2.5	3.9	7.3
5	4.7	4.9
10	1.8	1.1

CONCLUSIONS

This method presents the lower detection limit for the determination of coumarin in plasma. It offers the advantages of high precision in low concentration ranges, an overall run time of about 10 min and easy execution, despite the necessity for carefully monitoring the evaporation step. Hence it seems well adapted to pharmacokinetic studies over a wide range of therapeutic doses. A simple adaptation of the sensitivity of the detector should allow one to deal with higher concentrations than those tested here.

ACKNOWLEDGEMENTS

We thank Dr. Eric Leutenegger (Boots-Pharma) for helpful suggestions, Jacky Denis for valuable technical assistance and Mrs. Liliane Haine-Boviere for expert secretarial assistance.

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