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A SPECIFIC AND SENSITIVE METHOD FOR THE DETERMINATION OF THE ANTICOAGULANT PHENPROCOUMON IN PLASMA

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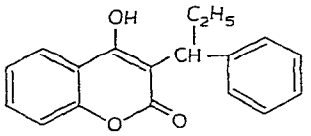
SUMMARY

A specific thin-layer chromatographic assay for phenprocoumon has been developed with a sensitivity of 5 ng/ml of plasma, using only 0.2 ml. This sensitivity is more than 20 times higher than that of the published methods. The drug is extracted from acidified plasma, an aliquot of the extract is applied to a silica-gel thin-layer plate and separated from interfering substances. The quantity of phenprocoumon is determined by fluorescence densitometry in situ. The standard deviation of the whole procedure is less than ± 3%. The new procedure permits pharmacokinetic studies with low doses of phenprocoumon to be performed on volunteers. Furthermore, due to the high sensitivity of the method, it is possible to determine the free drug fraction of this highly protein-bound substance in the plasma of patients. It was shown that, in the therapeutic concentration range, phenprocoumon is bound by about 99.5% to the plasma proteins. Since the assay is simple and quick to perform, a large series of plasma samples can be analysed without any problems.

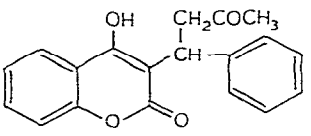
INTRODUCTION

The coumarin derivative phenprocoumon (3-[1'-phenylpropyl]-4-hydroxycoumarin, active principle of Marcoumar®) is an orally administered anticoagulant, which is structurally related to warfarin. Several analytical procedures have been described in the last few years for the determination of this drug, such as fluorimetry [1], gas chromatography [2—5] and thin-layer chromatography (TLC) [6, 7]. All these assays have a sensitivity of 100—200 ng/ml of plasma. This sensitivity is sufficient for the determination of therapeutic plasma levels of phenprocoumon. However, for extensive pharmacokinetic studies with volunteers, rather high doses of the drug have to be administered to obtain measurable plasma concentrations over a sufficient period of time [1, 2, 8, 9]. Due to the high activity of phenprocoumon, such quantities involve risks for the volunteers. Therefore, an assay for the drug in plasma

Phenprocoumon



Warfarin



with a considerably higher sensitivity was desirable to reduce this danger.

Phenprocoumon is strongly bound to the plasma proteins [1, 8, 10, 11]. For the determination of the free drug in plasma of patients, the sensitivity of the published methods is not sufficient and erroneous results could occur, i.e. due to the large variation of the sample blank.

To facilitate study of the pharmacokinetics after single oral and i.v. administration of phenprocoumon and to determine the non-protein-bound drug in plasma of patients, a sensitive and specific TLC procedure was developed for the measurement of the intact substance.

MATERIALS AND METHODS

Reagents

All chemicals must be of analytical grade and were purchased either from Merck (Darmstadt, G.F.R.) or from Fluka (Buchs, Switzerland).

Buffer solutions

Phosphate buffer (pH 7.4): add 22 ml of 1 *N* HCl to 24 g of Na_2HPO_4, dilute to 1000 ml with distilled water and adjust the pH to 7.4 with 0.1 *N* HCl or 0.1 *N* NaOH.

Acetate buffer (pH 4.0): dissolve 33 g of $CH_3COONa \cdot 12H_2O$ in 900 ml distilled water, adjust the pH to 4.0 with acetic acid and dilute to 1000 ml.

Thin-layer chromatography

The TLC was performed on silica gel 60 F_{254} 0.25 mm precoated thin-layer plates from Merck, measuring 20 × 20 cm. The thin-layer plates had to be cleaned with the solvent methanol—triethylamine (80:20) by developing for 15 cm. The plates were then dried for 5 min at 140° and cooled to room temperature. The lower edges of the plates were impregnated to 3 cm with the mixture pentane—triethylamine (100:10). This impregnation of the application zone is necessary to stablize phenprocoumon on the silica gel. After evaporation of pentane at room temperature, the samples were applied with 10-μl pipettes (Desaga, Model 130083). The developing fluid consisted of chloroform—methanol—triethylamine (95:15:5).

Fluorescence measurement

The fluorescence of phenprocoumon on thin-layer plates can be used directly for quantitative analysis. The measurements were performed with a Zeiss chromatogram spectrophotometer PMQ II.

Standard solutions

Standard solutions in organic solvents: 20 mg of phenprocoumon were dissolved in 10 ml of acetone (stock solution I). One ml of this solution was diluted to 100 ml with acetone (stock solution II). Further dilutions for checking the extraction yield were prepared by dilution of this stock solution II with ether.

Since phenprocoumon shows some instability in organic solvents, the standards in organic solvents cannot be stored longer than 1—2 weeks.

Standard solutions in plasma: 10.0 mg of phenprocoumon were dissolved in 0.5 ml 2.5% sodium hydroxide and diluted with phosphate buffer (pH 7.4) to 20 ml. Four ml of this stock solution were diluted with distilled water to 10 ml (= 200 μg/ml). One ml of this solution was mixed with blank plasma and brought to 20 ml (plasma standard containing 10 μg/ml of phenprocoumon). Further plasma standards containing 5 μg down to 5 ng/ml plasma were prepared by diluting aliquots of this plasma standard with blank plasma. The plasma standards were stored in portions of 1—2 ml at — 20°. No decomposition of phenprocoumon was observed in plasma stored at — 20°.

PROCEDURE

Extraction

The following procedure was used for concentrations of 0.1—1 μg/ml plasma. Pipette 0.2 ml of plasma to be analysed, 0.05 ml of acetate buffer (pH 4) and 0.5 ml of isopropylchloride into 5-ml stoppered centrifuge tubes. Shake for 5 min on a reciprocating shaker, and centrifuge for 5 min at 700 *g*. Along with eight plasma samples, process three standard plasmas (i.e. 1, 0.5 and 0.1 μg/ml) according to the expected plasma levels.

Apply 20 μl (two times 10 μl) of the extracts to a thin-layer plate prepared as described above. The distance from the bottom and side-edges of the plate should be 1.5 cm, between the spots 1.4 cm. For localization of phenprocoumon after separation, place 5 μl of the stock solution II to a boundary point.

The quantities of plasma and solvents used for extraction depend on the expected plasma levels. The relevant data are given in Table I.

TABLE I

AMOUNTS OF PLASMA AND ISOPROPYLCHLORIDE TO BE USED FOR THE DETERMINATION OF PHENPROCOUMON

Expected concentration range of phenprocoumon in plasma (μg/ml)	Amount of plasma to be extracted (ml)	Amount of isopropylchloride (ml)	Volume to be applied to the thin-layer plate (μl)	Plasma standards to be used (μg/ml)
1—10	0.1	1	10	1, 5, 10
0.1—1	0.2	0.5	20	0.1, 0.5, 1.0
0.005—0.1	0.2	0.5	40	0.01, 0.05, 0.1

Chromatography

Develop the thin-layer plate in a jar lined with filter paper to achieve vapour saturation. Equilibrate with the mobile phase (chloroform—methanol—triethylamine, 95:15:5) for about 10 min before use. Developing distance: 8 cm from application points. Dry the plate for 3 min at room temperature and then for 1 min in a drying oven at 30—35°. Mark the position of the phenprocoumon zone under shortwave UV light (254 nm).

Fluorescence densitometry

Scan the chromatogram with a Zeiss chromatogram spectrophotometer. We used a model PMQ II with the following instrument settings: mercury lamp, excitation wavelength 312 nm; entrance diaphragm 6 mm, slit-width 1 mm; secondary filter M365 nm; ordinate 2 or 4 times extended, damping 1, scanning speed 10 cm/min, paper speed 10 cm/min.

Calculation

In the concentration range 0.1—10 ng per spot the peak heights of the fluorescence signals are directly proportional to the quantity applied. The linear regression curve of the plasma standards used is determined with the aid of a suitable calculator. With this regression curve the concentrations of the plasma samples to be determined are calculated according to the fluorescence peak-heights measured.

RESULTS

Analytical variables

Extraction. The recovery of phenprocoumon added to blank plasma was 95% and higher in the concentration range 0.005—10 μg/ml using the extraction conditions mentioned above. Therefore, the addition of an internal standard for compensation of the extraction variation is not necessary.

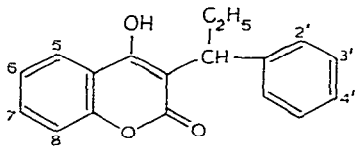
Stability of phenprocoumon on silica-gel thin-layer plates. It is a well-known fact that phenprocoumon decomposes very quickly on silica-gel thin-layer plates [7]. We found that the degradation rate of phenprocoumon on silica gel changed from one plate to another of the same batch. For quantitive densitometry it was therefore absolutely necessary to stabilize the drug on the silica-gel plates. This problem was solved by impregnating the starting zone with triethylamine. With this procedure the degradation of phenprocoumon could be almost completely abolished, but the samples still have to be applied to the silica-gel thin-layer plates within 10—15 min. The reason for the need for this stabilization is not known.

Chromatography. With the developing solvent chloroform—methanol—triethylamine (95:15:5) phenprocoumon has an R_F of 0.45. The R_F of some possible metabolites of phenprocoumon are given in Table II. The addition of triethylamine to the mobile phase considerably enhances and stabilizes the fluorescence of phenprocoumon. The chromatograms can still be scanned after some hours of storage at room temperature without a noticeable loss of sensitivity. Fig. 1 shows a scan of a thin-layer plate with a series of plasma standards, measured under the conditions described above.

TABLE II

THIN-LAYER CHROMATOGRAPHY OF PHENPROCOUMON DERIVATIVES

Solvent system: chloroform—methanol—triethylamine (95:15:5).



Compound	R_F
Phenprocoumon	0.45
5-Hydroxyphenprocoumon	0.39
6-Hydroxyphenprocoumon	0.23
7-Hydroxyphenprocoumon	0.14
8-Hydroxyphenprocoumon	0.28
2'-Hydroxyphenprocoumon	0.33
4'-Hydroxyphenprocoumon	0.21

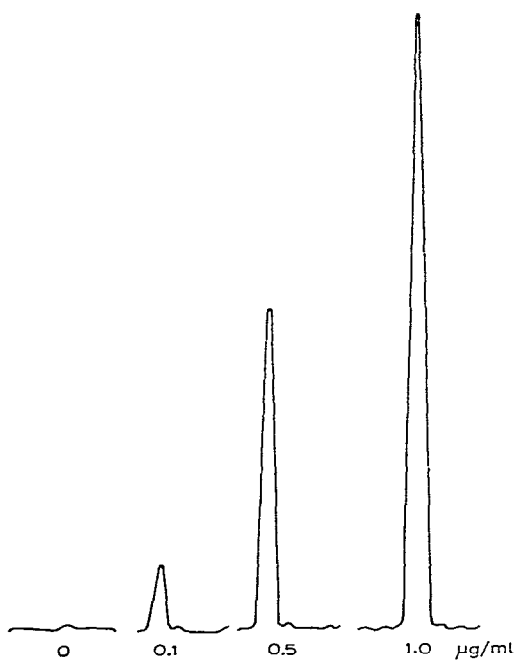


Fig. 1. Fluorescence densitometric scan of phenprocoumon plasma standards after extraction and thin-layer chromatography. Quantities: 0.2 ml plasma, 0.05 ml buffer pH 4, 0.5 ml isopropylchloride. 20 µl applied to the plate. Ordinate = arbitrary units.

Specificity. The extraction and chromatographic separation of the plasma extracts guarantee a high degree of specificity.

Sensitivity. The lower limit of detection of the procedure is about 5 ng/ml plasma. With less effort it should be possible to increase the sensitivity to about 1 ng/ml (i.e. the concentration of the plasma extract).

Reproducibility of the method. The same plasma samples were analysed at least twice on different days. With these results a relative standard deviation of the whole procedure could be calculated and was found to be less than ± 3% for concentrations down to 20 ng/ml plasma. For concentrations around 5 ng/ml this value rose to ± 10%.

Efficiency. The procedure for determination of phenprocoumon is very simple to perform. Since no time-consuming steps, such as back-extraction or evaporation, are needed, thirty or more analyses of unknown samples can be done in one day.

Analysis of samples

Phenprocoumon plasma levels after i.v. administration of the drug. With the method described above, plasma levels of phenprocoumon can be determined for a long period after a single i.v. or oral dose. The plasma concentrations of a volunteer who received a single dose of 3 mg phenprocoumon i.v. are given in Table III. These data demonstrate that, with this method, which has a sensitivity more than 20 times higher than the published ones, it is possible to study the pharmacokinetics of phenprocoumon with single doses of 3 mg. This low dose can be given to volunteers without taking any special precautions.

Free phenprocoumon in plasma. To illustrate the usefulness and the possibilities of the new procedure, the protein binding of phenprocoumon was determined by equilibrium dialysis. Spiked plasma and plasma of a patient

TABLE III

PLASMA CONCENTRATION OF PHENPROCOUMON AFTER A SINGLE i.v. DOSE OF 3 mg

Volunteer J.W.

Time after injection	Plasma concentration of phenprocoumon * (μg/ml)
Zero-plasma	0.0
5 min	0.800
10 min	0.434
20 min	0.319
30 min	0.265
45 min	0.204
1 h	0.180
2 h	0.160
4 h	0.144
8 h	0.140
1 day	0.122
2 days	0.110
4 days	0.091
6 days	0.072
8 days	0.060
16 days	0.038
24 days	0.031
32 days	0.024
64 days	0.012

*Mean of at least three determinations.

TABLE IV

PROTEIN BINDING OF PHENPROCOUMON IN BLANK PLASMA SPIKED WITH THE DRUG

Plasma concentration (μg/ml)	Free phenprocoumon (%)	Protein-bound phenprocoumon (%)
50	0.5	99.5
10	0.4	99.6
5	0.4	99.6
1	0.4	99.6
0.5	0.5	99.5

TABLE V

PROTEIN BINDING OF PHENPROCOUMON IN PLASMA OF A PATIENT RECEIVING 1.6 mg/day OF THE DRUG

Days after first medication	Plasma concentration of phenprocoumon (μg/ml)	Free phenprocoumon (%)	Protein-bound phenprocoumon (%)
12	1.75	0.5	99.5
16	1.80	0.4	99.6
23	1.90	0.4	99.6

treated with phenprocoumon were dialysed against phosphate buffer (pH 7.4). The data from this experiment are summarized in Tables IV and V.

DISCUSSION

A TLC procedure has been developed for the determination of phenprocoumon in plasma. The method has several important advantages compared with the published assays. These include high specificity and sensitivity, good reproducibility, the need for only small quantities of plasma for the analysis, and a low time requirement.

Until now for single-dose pharmacokinetics 20—50 mg of phenprocoumon have been administered to volunteers to give interpretable plasma levels [3, 8, 9]. These high doses are critical for volunteers since the drug is very active and haemorrhages could occur. Therefore, in some cases the antidote vitamin K was administered simultaneously [9], but it has not been proved that this comedication has no influence on the pharmacokinetic behaviour of phenprocoumon. The new procedure allows reliable pharmacokinetic studies with single doses of 3 mg which are harmless to volunteers. The high accuracy of the method makes it possible to measure even very low concentrations accurately, and to observe the plasma levels over a long period of time.

Free phenprocoumon could only be determined previously in spiked plasma samples in a concentration range that was much higher than the therapeutic one. Two important aspects could not be tested. Firstly it was not proved

whether or not the protein binding of phenprocoumon is higher in the therapeutic plasma levels. Also, it was not known if the protein binding is the same in spiked plasma as in plasma of patients medicated with the drug.

Our method gave us the possibility to answer both questions. In the range of 0.5—50 μg/ml plasma the protein binding of phenprocoumon is constant and amounts to 99.5% or more. Furthermore, there is no significant difference in the protein binding of phenprocoumon added to blank plasma and the protein binding of phenprocoumon in plasma of a patient medicated with this drug.

Since the described assay is simple, normal analytical-grade solvents can be used, and since it is not time-consuming, extensive pharmacokinetic studies with phenprocoumon are practicable. Eventually, future work can also answer the question of whether there is a correlation between the free phenprocoumon in plasma and the biological activity.

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