Determination of Phenprocoumon, an Anticoagulant, in Human Plasma

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Phenprocoumon is extracted from acidified plasma, the organic phase evaporated, and part of the residue, in ethanol, is quantitatively applied to a thin-layer plate. After separation, the quantity of phenprocoumon is assayed by fluorescence densitometry in situ. Results are reproducible to about 2.5%. The lower limit of detection is 0.1 mg/liter, which makes the method fully applicable to human plasma, because therapeutic concentrations range from 1 to 3 mg/liter. Seven determinations can be made within 3 h. For toxicological purposes, a qualitative analysis can be done in a shorter time, because the phenprocoumon spots are visible under ultraviolet light at 254 nm.

The effect of coumarin anticoagulants is usually measured by determination of a biological variable such as prothrombin time. Although this test is easier to perform than the chemical assays used to determine the concentrations of these drugs in blood and may give more direct information on the dosage regimen, there are clinical situations in which information on coumarin concentrations themselves is of value:

(a) for toxicological purposes, to find out whether a given coagulation disturbance is the result of coumarin intake;
(b) to determine whether lack of anticoagulant effect is the result either of noncompliance by the patient, or of malabsorption of the drug, or of hereditary resistance to coumarin congeners; and
(c) to study the pharmacokinetic behavior of the drug, from information on concentrations in plasma.

Most pharmacological work on coumarins has been done with warfarin and dicoumarol; only few pharmacokinetic data are available for another coumarin congener, phenprocoumon ("Liquamar," Organon; "Marcoumar," Hoffmann-La Roche). This gap may be due to the lack of a sensitive and specific procedure for determination of phenprocoumon in human plasma.

Here, we describe a sensitive method for simultaneously identifying and measuring phenprocoumon in plasma.

The accuracy is increased by the use of internal standards added to blank plasma, as a compensation for extraction variation.

Materials and Methods

Chemicals. All chemicals were of analytical grade, and were purchased either from E. Merck A. G., Darmstadt, G.F.R., or from British Drug House Ltd., Poole, England.

3-(1-Phenyl-propyl)-4-oxycoumarin (Phenprocoumon) was kindly supplied by Hoffmann-La Roche B. V., Mijdrecht, The Netherlands.

Glassware was cleaned with dichromate-sulfuric acid before use.

Thin-layer chromatography was performed on silica gel thin-layer plates, 0.25 mm in thickness without fluorescence indicator (Merck 5721), after using a Hamilton Repeating Dispenser equipped with a 50-μl Luer Tip syringe and a Teflon cannula for quantitative application of the sample. The developing fluid consisted of chloroform/methanol (97/3 by vol).

Fluorescence densitometry was performed directly on the thin-layer plate with a Vitatron TLD 100 Flying Spot densitometer with attached recorder and integrator.

Fluorometry. For fluorometric assay of phenprocoumon (1), we used a Zeiss spectrophotometer PMQ II with fluorescence unit ZFM 4 in the so-called B-formation. The excitation wavelength was 313 nm, from a Hanau St 41 mercury lamp. The slit width was 0.9 mm. All emission light with a wavelength above 390 nm was measured with use of a cut-off filter FL 39.

Patient plasma was prepared from 4.5 ml of venous blood that was drawn into plastic tubes containing 0.5 ml of 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES)-buffered sodium citrate, 0.13 mol/liter (2).

Thrombotest determination. The biological effect of phenprocoumon was measured at the Thrombosis

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Service Leiden (Head: Prof. Dr. E. A. Loeliger) as the Thrombotest time (3), expressed in seconds (normal value, about 40 s; therapeutic value, about 140 s).

**Procedure**

**Extraction.** To 1 ml of plasma in a Sovirel culture tube (11 × 16 mm) with a Teflon-lined screw cap add 1 ml of 1 mol/liter HCl/acetone (9:1). After mixing, add 7 ml of chloroform. Treat in the same way a calibration series containing 0, 0.5, 1.0, 2.0, and 3.0 mg of phenprocoumon per liter added to blank plasma. Mechanically shake the tubes for 15 min and centrifuge at maximal speed in a clinical centrifuge for 10 min. Aspirate the upper layer with a Pasteur pipette and loosen the protein precipitate at the interface. Filter the contents of the tubes in pointed tubes through Whatman No. 1 PS phase separating filter paper that has been washed thoroughly with chloroform. Rinse the culture tubes with 1–2 ml of chloroform with which the filters are washed. Then evaporate the filtrate in a hot waterbath under a stream of nitrogen, and dissolve the residue in 100 μl absolute ethanol.

**Chromatography.** Spot 30 μl of the ethanolic solution on a thin-layer plate (maximally 11 spots per 20 × 20 cm plate) and develop the plate until the solvent has ascended 10 cm from the starting point. After drying the plate, inspect it under an ultraviolet light source (254 nm). Mark the position and diameter of the phenprocoumon spots with a pencil at both vertical rims of the plate (RF 0.55).

**Densitometry.** Scan the plate with a Vitatron TLD 100 Flying Spot Densitometer. The instrument settings are:

- Mode: lin II +
- Level: e
- Zero: C 7
- Damping: 2
- Span: approx. 7
- Lamp: Hg

Diaphragm: 0.25 mm
Primary filter: 313 nm
Secondary filter: U 8 (410 nm)
Scanning speed: 1 cm/min
Recorder speed: 1 cm/min
Integrator sensitivity: position 6

Draw a calibration curve by plotting the number of integrator units vs. the concentration; calculate unknown concentrations by interpolation.

**Results**

**Analytical Variables**

The present method for determination of phenprocoumon in human plasma consists of three steps: extraction, chromatography, and densitometry.

**Extraction.** Several organic solvents and pH values were compared, to find conditions for maximal recovery. Of chloroform, dichloroethane, ethylacetate, n-heptane, petroleum ether (40–60 °C fraction), and toluene, the best results were obtained with chloroform. No phenprocoumon could be extracted from alkalinized plasma. Addition of one volume of 0.9 mol/liter HCl containing 10% acetone to one volume of plasma gave maximal results. The recovery for 3.0 mg of phenprocoumon per liter, added to blank plasma, amounted to 73 ± 1.9% (mean ± SE; n = 12). For lower concentrations, the same value was obtained. Recovery was not enhanced by reextracting the protein precipitate or the phase-separating filter paper.

Because extraction is not complete and the calibration curve not a straight line (see Figure 2), for each thin-layer plate a calibration series of at least four different concentrations of phenprocoumon in blank plasma is treated like patients’ plasma.

**Chromatography.** For thin-layer chromatography, the developing fluid chloroform/methanol (97:3) described by Daenens and Van Boven (4) was used. Under these conditions, phenprocoumon has an RF of 0.55.

Several drugs (Table 1) were tested for possible interference with the phenprocoumon assay.

**Densitometry.** When a thin-layer plate is scanned under the conditions described under Procedure, a series of peaks is obtained as shown in Figure 1. Figure 2 shows the calibration curve derived from this scan. Phenprocoumon concentrations in patients’ plasma are read by interpolation.

The lower limit of detection under the conditions mentioned above is about 0.1 mg/liter. The whole pro-

<p>| Table 1. Chromatographic and Fluorescence Characteristics of Some Drugs Investigated for Possible Interference with the Phenprocoumon Assay |</p>
<table>
<thead>
<tr>
<th>Compound</th>
<th>RF relative to phenprocoumon</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acenocoumarol</td>
<td>0.72</td>
<td>quenching</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>0.19</td>
<td>quenching</td>
</tr>
<tr>
<td>Butobarbital</td>
<td>0.57</td>
<td>quenching</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>0.41</td>
<td>reddish</td>
</tr>
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<td>Diazepam</td>
<td>0.90</td>
<td>yellow</td>
</tr>
<tr>
<td>Dicoumarol</td>
<td>0.52</td>
<td>white</td>
</tr>
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<td>Dicumoxane</td>
<td>0.00</td>
<td>white</td>
</tr>
<tr>
<td>Disopyramide</td>
<td>0.00</td>
<td>quenching</td>
</tr>
<tr>
<td>Ethylbiscoumacetate</td>
<td>0.00</td>
<td>white</td>
</tr>
<tr>
<td>Furosemide</td>
<td>0.00</td>
<td>white</td>
</tr>
<tr>
<td>Glafenin</td>
<td>0.09</td>
<td>yellow</td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>0.90</td>
<td>purple</td>
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<tr>
<td>Hydroquinidine</td>
<td>0.11*</td>
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</tr>
<tr>
<td>Methaqualon</td>
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</tr>
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<td>Nitrazepam</td>
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<tr>
<td>Paracetamol</td>
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<td>Phenacetin</td>
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<td>Phenobarbital</td>
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</tr>
<tr>
<td>Phenprocoumon</td>
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</tr>
<tr>
<td>Phenytoin</td>
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<td>quenching</td>
</tr>
<tr>
<td>Procainamide</td>
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<td>no effect</td>
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<tr>
<td>Propranolol</td>
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<td>yellow</td>
</tr>
<tr>
<td>Quinidine</td>
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<td>white</td>
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<td>Salicylic acid</td>
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</tr>
<tr>
<td>Triamterene</td>
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</tr>
<tr>
<td>Warfarin</td>
<td>0.82</td>
<td>purple</td>
</tr>
</tbody>
</table>

* With tailing
procedure takes about 3 h. Seven unknown plasmas can be analyzed on one thin-layer plate.

Correlation with Results of Other Methods

Correlation with a fluorometric method. A fluorometric method for determination of phenprocoumon has been described (1), but this method is not specific, because salicylic acid, which can, like phenprocoumon, be extracted with organic solvents from acidified plasma, has fluorescence characteristics similar to those of phenprocoumon. Consequently, we found in blank plasma to which only sodium salicylate was added in a concentration of 1 mg/liter an apparent phenprocoumon concentration of 3 mg/liter by the method of Seiler and Duckert.

We compared our method with that of Seiler and Duckert (1) in citrated plasma from outpatients of the local Thrombosis Service. The correlation curve is given in Figure 3, which shows an excellent correlation between both methods, the densitometric procedure giving slightly higher values than the fluorometric assay. (In neither of these methods did we correct the phenprocoumon concentration for dilution of the plasma by the citrate anticoagulant.)

Correlation between plasma concentrations and Thrombotest values. Figure 4 shows that there is a poor correlation between steady-state concentrations of the drug in plasma and Thrombotest values in the 12 patients we have investigated.

Discussion

Our aim was to develop a specific, sensitive method for measuring phenprocoumon in human plasma. The specificity results from the combination of acid extraction, thin-layer chromatography, and fluorometry in situ at the appropriate excitation and emission wavelengths.

A correction can be made for the incomplete analytical recovery by preparing a calibration curve in blank
plasma. The reproducibility is 2.5% (derived from the standard error of the recovery experiments) and the lower limit of detection is 0.1 mg/liter or less.

Of the drugs investigated (Table 1) only griseofulvin, which has a $R_F$ value of 0.90 relative to phenprocoumon and has similar extraction and fluorescence characteristics, could disturb the determination of phenprocoumon when the spots are not kept as small as possible. This, however, is not an insurmountable problem when one uses the Hamilton Repeating Dispenser. Salicylate, which strongly interferes in the method of Seiler and Duckert (1), does not interfere in our procedure, because the two drugs have different chromatographic characteristics.

The interference of salicylate is the major disadvantage of the fluorometric procedure. If one assumes a volume of distribution of 150 ml/kg body weight and a plasma half-life of 6 h (5), ingestion of a single dose of 500 mg of aspirin results, after 36 h, in a salicylate plasma concentration of about 0.8 mg/liter. With the method of Seiler and Duckert (1) this plasma shows an apparent phenprocoumon concentration in the therapeutic range. Therefore, their procedure is not applicable for the toxicological differential diagnosis between salicylate- or phenprocoumon-induced coagulation disturbances.

In plasma of patients who were strongly advised not to take salicylate (the plasma was, however, not analyzed for salicylate), a good correlation was found between our method and the fluorometric assay. We have no explanation for the fact that the latter gives slightly lower values than the former.

The poor correlation between biological effect and plasma concentration, as shown in Figure 4, is in agreement with previous findings (6) for warfarin.

Future work may answer the question of whether there is a correlation between Thrombotest values and the nonprotein bound fraction of phenprocoumon.

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References