Determination of Coumarin-type Anticoagulants in Human Plasma by HPLC–Electrospray Ionization Tandem Mass Spectrometry with an Ion Trap Detector

Manfred Kollroser1* and Caroline Schober2

Background: Coumarin-type anticoagulants are used for the long-term treatment and prevention of thromboembolic disorders. The identification of these drugs is crucial in patients with an increased prothrombin time of unknown origin. The aim of this study was to develop a sensitive and specific method for the simultaneous determination of phenprocoumon, acenocoumarol, and warfarin in human plasma by HPLC-electrospray ionization tandem mass spectrometry.

Methods: After addition of the internal standard, p-chlorowarfarin, plasma samples were extracted using Oasis® MCX solid-phase extraction cartridges. The compounds were separated on a Symmetry C18 column (Waters) with a mobile phase of acetonitrile–1 g/L formic acid (75:25 by volume) at a flow rate of 0.5 mL/min.

Results: Extraction and separation of the three drugs and the internal standard were accomplished in 9 min. The overall extraction efficiency was >89% for all three compounds. The limits of detection were 1 µg/L for phenprocoumon and warfarin and 10 µg/L for acenocoumarol. Regression analysis of the calibration data revealed good correlation ($r^2 \approx 0.995$) for all compounds. Within-run accuracies for quality-control samples were ±1% to 7% of the target concentration, with CVs <9%.

Conclusions: The proposed method enables the unambiguous identification and quantification of phenprocoumon, warfarin, and acenocoumarol in both clinical and forensic specimens. This method combines a new, extremely fast chromatographic analysis, which is especially advantageous for clinical laboratories.

The coumarin-type anticoagulants acenocoumarol, phenprocoumon, and warfarin have been used for the prophylaxis and treatment of thromboembolic disorders for >50 years (1). Coumarins are vitamin K antagonists and inhibit the hepatic synthesis of blood clotting factors II (prothrombin), VII, IX, and X as well as of anticoagulant proteins C and S from precursor proteins (2). These precursors are activated by vitamin K (phytomenadione), which is initially reduced to its hydroquinonone form and is then reversibly converted to its 2,3-epoxide form. Coumarins block the enzymatic regeneration of the hydroquinonone form, reducing the amount of biologically active vitamin K and causing an accumulation of inactive precursors of the blood clotting factors. In addition, coumarins inhibit a variety of other vitamin K-dependent carboxylation reactions in the kidneys, the placenta, and bones.

Monitoring of these drugs in patients is usually done by their pharmacodynamic effects (prothrombin time). However, there are several clinical situations in which the unambiguous identification and quantification of the drugs themselves are essential. Confirmation of the presence of coumarin-type anticoagulants in biologic tissues is required in cases of increased prothrombin time, when there is no apparent cause for this condition, to confirm an accidental or intentional ingestion. Therapeutic plasma concentrations are 30–100 µg/L for acenocoumarol (3), 160–3600 µg/L for phenprocoumon (3), and 600–3000 µg/L for warfarin (4). Exceeding the therapeutic window of these drugs triggers unwanted bleedings. The determination of anticoagulant plasma concentrations facilitates diagnosis and allows for the effective treatment of severe intoxication. Furthermore, anticoagulant serum concentrations can also be helpful in distinguishing noncompli-

1 Institute of Forensic Medicine, Karl-Franzens University Graz, Universitätsplatz 4, A-8010 Graz, Austria.
2 Institute of Molecular Biology, Biochemistry, and Microbiology, Karl-Franzens University Graz, Heinrichstrasse 31a, A-8010 Graz, Austria.
*Author for correspondence. Fax 43-316-380-9635; e-mail manfred.kollroser@kfunigraz.ac.at.
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ance from genuine anticoagulant resistance. Hence, the unambiguous identification and quantification of acenocoumarol, phenprocoumon, and warfarin require a specific and sensitive method that is suitable for routine analysis.

Several analytical methods have been developed for the determination of anticoagulants. Gas chromatography–mass spectrometry (MS)\(^3\) is a sufficiently sensitive and specific method for the detection and quantification of coumarin-type anticoagulants; its major disadvantage, however, is the need for time-consuming derivatization before analysis (5–8). Several HPLC methods have been described that use either ultraviolet (UV) (9–14) or fluorescence detection (11, 15, 16). However, the shortcoming of HPLC with UV or fluorescence detection is unreliability with regard to selectivity. The coupling of HPLC to MS with atmospheric pressure ionization leads to a much more specific and sensitive analytical technique. A second stage of mass analysis, tandem MS (MS/MS), further enhances specificity and provides an improved signal-to-noise ratio compared with single-stage MS. One liquid chromatography (LC)–MS application has been described for the determination of coumarin-type anticoagulants in biologic specimens by use of thermospray and particle beam interfaces (17). These interfaces have several limitations, such as low sensitivity or reduced universality, whereas atmospheric pressure ionization techniques, electrospray ionization (ESI), and atmospheric pressure chemical ionization have become the gold standards.

The purpose of this work was to exploit the high selectivity and sensitivity of an ion trap detector operated in MS/MS mode with an ESI interface for the detection and quantification of acenocoumarol, phenprocoumon, and warfarin in human plasma. The limitations of alternative gas chromatography and HPLC methods were overcome by combining a new simple solid-phase extraction (SPE) procedure with the power of a benchtop MS/MS system.

**Materials and Methods**

**REAGENTS**

Acenocoumarol and phenprocoumon were generous gifts from Novartis AG (Basel, Switzerland) and from Roche AG (Basel, Switzerland), respectively. Warfarin and p-chlorowarfarin (internal standard; IS) were supplied by Sigma-Aldrich GmbH. HPLC-grade acetonitrile and analytical grade concentrated formic acid were obtained from Promochem GmbH. Isopropanol, ammonia (33%), methylene chloride, and orthophosphoric acid (85%) were of analytical grade and were purchased from Merck KGaA. A MilliQ® Plus water purification system was used to obtain purified water for the HPLC solvent (Millipore GesmbH). Oasis® MCX SPE cartridges were supplied by Waters GesmbH.

**LC-ESI-MS/MS**

The LC-MS/MS analyses were performed with a TSP LC system consisting of a vacuum degasser, a P4000 quaternary pump, an AS3000 autosampler, a UV6000LP diode-array UV detector, and a Finnigan LCQDUO quadrupole ion trap mass spectrometer equipped with an ESI source (Finnigan MAT) run by XCALIBUR 1.2 software.

HPLC separations were performed on a Symmetry C18 (5-μm, 150 × 3.0 mm i.d.; Waters) HPLC column operated at ambient temperature and protected by a Symmetry C18 Sentry guard column (5-μm, 20 × 3.9 mm i.d.; Waters). Each 4-min chromatographic run was carried out at a flow rate of 0.5 mL/min with a mobile phase of acetonitrile–1 g/L formic acid (75:25 by volume).

Operating conditions for the ESI source, used in the positive ionization mode, were optimized by constantly adding a mixture of acenocoumarol, phenprocoumon, warfarin, and the IS, each at a concentration of 1 mg/L, in methanol to the HPLC flow by a syringe pump via a T-connector in the infusion mode. The signal was optimized on the total ion current in MS mode, producing a transfer capillary temperature of 270 °C, a spray voltage of 4.5 kV, and a sheath gas flow of 80 units (units refer to arbitrary values set by the LCQ software). At the same time, the selection of ions and the collision voltages were optimized using LCQ software.

In the MS/MS experiments, the protonated precursor molecular ions [M+H]\(^+\) of acenocoumarol (m/z 354.1), phenprocoumon (m/z 281.1), warfarin (m/z 309.1), and the IS (m/z 343.1) were selected and fragmented by helium gas collision in the ion trap at a relative collision energy of 35%. The mass spectra resulting from these fragmentations were acquired in the full scan mode from m/z 100 to m/z 450. Several product ions were observed for each compound. The most abundant product ions, m/z 163 for acenocoumarol, m/z 203 for phenprocoumon, m/z 163 for warfarin, and m/z 163 for the IS, respectively, were extracted and chosen for quantification.

**CALIBRATORS AND QUALITY-CONTROL SAMPLES**

Stock solutions of acenocoumarol, phenprocoumon, warfarin, and the IS were prepared by dissolving 1 mg of the analyte in 1 mL of methanol. The stock solutions of acenocoumarol, phenprocoumon, and warfarin were diluted with methanol to obtain working solutions containing the respective substances at 1, 10, and 100 mg/L. The stock solution of the IS was also diluted in methanol to a final concentration of 10 mg/L. To prepare calibration samples, the appropriate volumes of working solution were added to drug-free plasma to obtain acenocoumarol concentrations of 25, 50, 100, 200, 300, 400, and 600 μg/L and phenprocoumon concentrations of 50, 100, 200, 400, 1600, and 4000 μg/L. For warfarin, calibration samples at concentrations of 500, 1000, 2000, 3000, 4000, and 5000 μg/L were prepared.

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\(^3\) Nonstandard abbreviations: MS, mass spectrometry; UV, ultraviolet; LC, liquid chromatography; ESI, electrospray ionization; SPE, solid-phase extraction; and IS, internal standard.
were similarly prepared. Three replicate analyses were performed for each calibrator to evaluate linearity. The calibration curves were constructed by linear regression using the peak-area ratios of acenocoumarol, phenprocoumon, and warfarin, respectively, to the IS, plotted against the corresponding concentrations.

A similar dilution procedure was used to prepare separate working solutions containing 10 and 100 μg/L acenocoumarol, phenprocoumon, and warfarin. These solutions were added to drug-free plasma to make quality-control samples containing 50, 100, and 300 μg/L acenocoumarol; 200, 2000, and 3500 μg/L phenprocoumon; and 1000, 2000, and 4000 μg/L warfarin.

**SPE Procedure**

One milliliter of each calibrator, quality-control plasma sample, or patient sample was pipetted into labeled glass tubes; 20 μL of orthophosphoric acid and 10 μL of the diluted IS solution (10 mg/L) were then added to each sample. The tubes were vortex-mixed.

SPE was performed using Oasis MCX cartridges (1 cm³/30 mg), a vacuum manifold device, and a vacuum source. SPE cartridges were conditioned and equilibrated with 1 mL of methanol and 1 mL of water. Supplemented, acidified specimens were applied to cartridges and passed through the beds at a constant flow rate of 1 mL/min. Cartridges were washed with 1 mL of 0.1 mol/L HCl. Analytes were eluted with 1 mL of methanol, and 20 μL of the eluate was injected directly into the chromatographic system.

**Extraction Efficiency**

The extraction efficiency was determined experimentally at concentrations of 30, 90, and 300 μg/L for acenocoumarol; 150, 1000, and 3000 μg/L for phenprocoumon; and 500, 1500, and 3500 μg/L for warfarin. The extraction efficiency for the IS was determined at a concentration of 100 μg/L. The absolute extraction recoveries were evaluated by comparing the analyte peak areas obtained from the supplemented plasma samples to those obtained from the corresponding unextracted calibrators prepared at the same concentrations (n = 5).

**Ion Suppression**

The degree of ion suppression that could be attributed to the sample matrix was estimated in a separate set of experiments. Blank plasma samples were extracted as described above, and the derived methanolic eluants were supplemented with 90 μg/L acenocoumarol, 1000 μg/L phenprocoumon, 1500 μg/L warfarin, or 100 μg/L IS. Additionally, the coumarin drugs or IS were added to pure methanol at the same concentrations. Ion suppression was calculated by comparing analyte peak areas obtained from pure methanolic preparations with those obtained from supplemented plasma samples after extraction (n = 5).

**Results**

**Chromatography and Mass Spectra**

All of the compounds investigated gave protonated precursor molecular ions [M+H]⁺ in the MS mode. The
major ions observed were \( m/z \) 354.1 for acenocoumarol, \( m/z \) 281.1 for phenprocoumon, \( m/z \) 309.1 for warfarin, and \( m/z \) 343.1 for the IS, respectively (Fig. 1). The product ion chromatograms and the corresponding full-scan product ion spectra of acenocoumarol, phenprocoumon, warfarin, and the IS extracted from supplemented plasma are depicted in Fig. 2. The most intense product ions observed in the MS/MS spectra were \( m/z \) 163 for acenocoumarol, \( m/z \) 203 for phenprocoumon, \( m/z \) 163 for warfarin, and \( m/z \) 163 for the IS.

As shown, the retention times of acenocoumarol, warfarin, phenprocoumon, and the IS were 2.35, 2.53, 2.82, and 2.98 min, respectively. The total HPLC-MS/MS analysis time was 4 min per sample. No interferences of the analytes were observed because of the high selectivity of the MS/MS technique.
The product ion chromatograms and corresponding spectra obtained from an extracted plasma sample of a patient with an increased prothrombin time, who repeatedly denied taking coumarin anticoagulants, are depicted in Fig. 3. Whereas warfarin and acenocoumarol were clearly excluded, phenprocoumon was unambiguously identified and was quantified as 2990 μg/L.

The HPLC-MS/MS analysis of an extracted plasma sample of another patient, who showed bleeding from the urinary tract during therapy with acenocoumarol, is shown in Fig. 4. The measured plasma acenocoumarol concentration was 201 μg/L.

**METHOD VALIDATION**

The extraction recoveries of acenocoumarol, phenprocoumon, and warfarin are presented in Table 1. The recovery of each compound was >89% at all concentrations tested. The extraction efficiency of the IS was 96.4% ± 5.9% at a
concentration of 100 μg/L. Regression analysis of the calibration data showed satisfactory linearity over the concentration range investigated. The correlation coefficients ($r^2$) for all three calibration lines were $\approx 0.995$, indicating a linear relationship between the peak-area ratio and the concentration of each compound up to 600 μg/L for acenocoumarol, 4000 μg/L for phenprocoumon, and 5000 μg/L for warfarin. The slopes and intercepts, the standard deviations of the slope and the intercept, and the coefficients of correlation were as follows: for acenocoumarol, $y = (0.0036 \pm 0.0001)x + (0.0082 \pm 0.0159)$, $r^2 = 0.999$; for phenprocoumon, $y = (0.0275 \pm 0.0009)x + (2.7930 \pm 1.5134)$, $r^2 = 0.996$; for warfarin, $y = (0.0124 \pm 0.0004)x + (8.6128 \pm 1.3193)$, $r^2 = 0.995$.

To evaluate the accuracy and precision of the assay, we analyzed quality-control samples containing acenocoumarol at 50, 100, and 300 μg/L; phenprocoumon at 200, 2000, and 3500 μg/L; and warfarin at 1000, 2000, and 4000 μg/L. The results are summarized in Table 2. To determine intraassay accuracy and precision, we performed five replicate analyses at each of the above concentrations. Interassay accuracy and precision were determined at the same concentrations over a period of 20 days, with two replicates per specimen and two analytical runs per day.

The within-run CVs for acenocoumarol, phenprocoumon, and warfarin were $\approx 8.6\%$. The total CV for all drugs was $< 14\%$. The accuracies, referred to as the percentage of target in Table 2, were determined by comparing the mean calculated concentrations with the target concentrations of the analytes in the quality-control samples. The within-run and total accuracies for all analytes were within 7.2% and 11.0%, respectively, of the target values.

The limit of detection was established by serial extraction of plasma samples containing decreasing concentrations of acenocoumarol, phenprocoumon, and warfarin. The limit of detection was 1 μg/L for phenprocoumon and warfarin and 10 μg/L for acenocoumarol. In all cases, the signal-to-noise ratio was $> 7:1$. The limit of quantification (defined as a CV $\leq 20\%$ and an accuracy within $\pm 20\%$ of the true value) was 50 μg/L for phenprocoumon, 500 μg/L for warfarin, and 25 μg/L for acenocoumarol, respectively.

As a part of the method validation, the change in the efficiency of ionization that could be attributed to the

<p>| Table 1. Extraction efficiencies (n = 5) for acenocoumarol, phenprocoumon, and warfarin. |
|---------------------------------------------|----------------|-------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Added, μg/L</th>
<th>Recovery, %</th>
<th>SD, %</th>
<th>Added, μg/L</th>
<th>Recovery, %</th>
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<td>30</td>
<td>93.3</td>
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<td>150</td>
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<tr>
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<td>±5.7</td>
<td>1000</td>
<td>94.6</td>
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<td>1500</td>
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<tr>
<td>300</td>
<td>99.7</td>
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<td>93.2</td>
<td>±4.0</td>
<td>3500</td>
<td>102.1</td>
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* ACC, acenocoumarol; PPC, phenprocoumon; WAF, warfarin.
The purpose of these investigations was to develop a specific and sensitive assay for the simultaneous determination of the most frequently administered oral coumarin-type anticoagulants. HPLC-ESI-MS/MS has several advantages for the analysis of coumarin-type anticoagulants. The combination of HPLC (under the isocratic conditions described) with ESI-MS/MS leads to very short retention times and yields both high selectivity and sensitivity. ESI is a “gentle” ionization technique that produces high mass-to-charge \([M+H]^+\) precursor ions with minimal fragmentation of the analytes. No interferences of the analytes were observed because acquisition was in the precursor ion selection mode followed by fragmentation (Fig. 2). The processes that occur in the ion trap detector can be broken down into the following steps: ionization of the molecules, storage of the ions formed in the ion source, selection of ions of a single mass-to-charge ratio (precursor ions) and ejection of all other ions, collision-induced dissociation of the precursor ions, and detection of the product ions formed. Compared with LC-MS, this technique produces a higher signal-to-noise ratio, which is hardly, if at all, affected by the matrix. Hence, analytical background noise has substantially less influence on product ion chromatograms and mass spectra obtained by the LC-MS/MS technique than on those generated by LC-MS.

The most intense product ions in the MS/MS spectra of acenocoumarol and the IS (Fig. 2) can be explained by the loss of the \(p\)-nitrophenyl-3-oxobutyl group or the \(p\)-chlorophenyl-3-oxobutyl group, respectively. For warfarin, the loss of the phenyl-3-oxobutyl group is suggested. For phenprocoumon, the loss of the phenylpropyl ring is proposed. The main advantage of the LC-MS/MS technique compared with the so-called collision-induced dissociation LC-MS interface, where analyte fragments are formed from any substance eluting from the HPLC column, is that there are no uncertainties as to the origin of the fragments observed in the product ion spectra. In comparison with a triple-quadrupole mass spectrometer, the ion trap has the advantage that full-scan product ion spectra can be acquired without any loss of sensitivity.

In conclusion, the presented assay is the first HPLC-ESI-MS/MS method that allows for the simultaneous determination and quantification of acenocoumarol, phenprocoumon, and warfarin in biologic specimens. The simple mixed-mode SPE procedure provides a highly efficient sample clean up with excellent recoveries. The combination of HPLC and MS/MS with an ESI interface leads to specificity and sensitivity of drug identification, which is crucial in both clinical and forensic applications. The ion trap mass spectrometer enables MS/MS at an affordable price compared with a triple-stage quadrupole MS system.

References


5. Maurer HH, Arlt JW. Detection of 4-hydroxycoumarin anticoagulants and their metabolites in urine as part of a systematic toxicological analysis procedure for acidic drugs and poisons by


