Acute Bromadiolone Intoxication

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**Abstract**

A 55-year-old man came to the hospital with a bleeding wound on his tongue. The coating of his tongue was green, and his sputum was red. Because an increased international normalized ratio-value was measured, a blood sample was sent to our laboratory with the suspicion of coumarin intoxication. Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis confirmed the poisoning was by bromadiolone, with its maximum serum concentration at 440 pg/L. The analysis of further samples resulted in a calculated elimination half-life of 140 h. The analytical method described was developed for the determination and quantitation of bromadiolone using LC-MS. This method is suitable for the simultaneous identification and quantitation of 10 indirect anticoagulants in human serum, which include five superwarfarins (brodifacoum, bromadiolone, difenacoum, difethialone, and flocoumafen) as rodenticides licenced in Germany and five other vitamin K antagonists (acenocoumarol, coumatetralyl, coumachlor, phenprocoumon, and warfarin). The method is based on an acidic (pH 4.2) liquid-liquid extraction followed by LC-ESI-MS analysis. Analytical separation was carried out using an Atlantis C18 column (2.1 x 20 mm, 3 μm). The mobile phase consisted of methanol/0.1% formic acid; the flow rate was 0.6 mL/min, and the time needed for analysis was 5 min. The lower limit of quantitation was 5 pg/L (signal-to-noise > 10).

**Introduction**

4-Hydroxycoumarins, which are derived from the woodruff flavor coumarine and 1,3-indandione, belong to the group of indirect anticoagulants (Figure 1). 4-Hydroxycoumarins are also called vitamin K antagonists because they reduce the synthesis of vitamin K depending factors that are necessary for blood coagulation. For example, the synthesis of the clotting factors II, VII, IX, and X and the proteins C and S are affected. Because of the half-life of the already circulating clotting factors, the anticoagulative effect appears after a latency period of several days after ingestion (1). Coumarinic derivatives are absorbed from the gastrointestinal tract, its half-life is relatively long, and the plasma protein binding high (3). Some coumarinic derivatives are absorbed into breast milk (2). They are mainly excreted as metabolites (glucuronides and sulfates) in the urine and feces (3).

Some vitamin K antagonists are used therapeutically as anticoagulants (acenocumarol, phenprocoumon, and warfarin, see Table I).

The same anticoagulative effect is used for pest control (4–6). Because of the increasing resistance of several rat species to this group of anticoagulants, the so-called “second-generation anticoagulants”, superwarfarins, were developed. They are distinguished because of their high effectiveness (Table I) at low doses. The half-life is long, which leads to a long lasting pharmacodynamic effect (7). Parallel to the growing commercial distribution of superwarfarins, the number of human and animal poisoning instances are increasing (8). In 2004 the Poison Control Centre of Berlin registered 110 cases involving suspicious anticoagulant ingestion. In Germany, the compounds are also found in commercial animal baits in a concentration between 0.0025% and 0.79%. There are baits with more than one active substance available, such as Racumin Plus (coumatetralyl and colecalciferol) or Celaflor and Brumolin (difethialone and sulfachinoxalin) (9). 1,3-Indandiones such as diphacinon, pindon, and valon are not licensed in Germany (9).

![Figure 1. Structural relationship between vitamin K and its antagonists.](image-url)
There are 24 products containing bromadiolone available in Germany (10). The symptoms of intoxication are bleeding tendencies. Cause of death is internal hemorrhage and hemorrhagic shock (1,4,11). The bioavailability of bromadiolone is approximately 50%. The maximum plasma concentration in mammals is reached approximately 6–9 h after ingestion. Bromadiolone, as well as its two major metabolites, and other similar products are mainly excreted in the feces (urine: < 1% after 96 h) (4). According to the toxic compounds list (German Gefahrstoffverordnung), bromadiolone is classified as very toxic (T+) (4,12).

The most important parameter for diagnosis is the international normalized ratio (INR)-value. The INR-value is independent of the changing sensitivity of the thromboplastin preparations used for diagnosis. Immediate treatment of intoxications includes decontamination and antidote administration (vitamin K₁) if necessary. In life threatening bleeding cases, the defective clotting factors must be substituted (1,3).

A number of analytical methods for the determination of vitamin K antagonists in animals are described in the literature (13–15). Some procedures deal with the determination of a single compound using high-performance liquid chromatography–fluorescence detection (HPLC–FL) (16) and their metabolites (2) or with the separation and detection of the enantiomers (17,18). Multicompound analyses in humans (number of active substance) are based on HPLC (4,5,19,20), HPLC–FL (31), HPLC–UV/FL (5,8,22,23), HPLC–diode-array detection (DAD) (13,24), HPLC–atmospheric pressure chemical ionization–MS (5,25), as well as HPLC–ESI–MS–ion-trap (3,26), LC–thermospray–MS (4,27) or gas chromatography–MS (4,28).

We developed a rapid method for the determination of vitamin K antagonists with a simple extraction procedure, which allows the broad substance screening at a high selectivity using two masses for identification and quantitation without previous derivatization. The substances, which are covered by the method, are shown in Figures 2 and 3.

### Table I. Some Pharmacological Data of the Analytes (4,10)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Therapeutical Level (µg/L)</th>
<th>Elimination Half-Life</th>
<th>LD₅₀ Rat Oral (mg/kg KG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acenocoumarol</td>
<td>30–90</td>
<td>10 h</td>
<td>-</td>
</tr>
<tr>
<td>Coumachlor</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coumatetralyl</td>
<td>-</td>
<td>-</td>
<td>approx. 30 (m)*</td>
</tr>
<tr>
<td>Phenprocoumon</td>
<td>1000–3000</td>
<td>120–150 h</td>
<td>-</td>
</tr>
<tr>
<td>Warfarin</td>
<td>300–3000</td>
<td>37–50 h</td>
<td>approx. 60–320 mg</td>
</tr>
<tr>
<td>Brodifacoum</td>
<td>-</td>
<td>-</td>
<td>0.4–0.8 (m)</td>
</tr>
<tr>
<td>Bromadiolone</td>
<td>-</td>
<td>26–58 h</td>
<td>approx. 1.3</td>
</tr>
<tr>
<td>Difenacoum</td>
<td>-</td>
<td>28 days</td>
<td>approx. 2.0</td>
</tr>
<tr>
<td>Difethialone</td>
<td>-</td>
<td>2–3 days</td>
<td>approx. 0.6</td>
</tr>
<tr>
<td>Flocoumafen</td>
<td>-</td>
<td>158–261 days</td>
<td>approx. 0.3</td>
</tr>
</tbody>
</table>

* m = male.

### Case History

A 55-year-old man came to the hospital with a bleeding wound on his tongue. The coating of his tongue was green, and the color of his was sputum red. He complained about headache and stomachache, a sore throat, and muscle pain. His blood pressure was 120/70 mm Hg and heart rate 83 beats/min. Within the scope of the coagulation testing, a considerable INR-value increase was measured without a conscious intake of anticoagulatives. Within two days, the INR-value increased to 10. Detailed inquiries showed that neither the patient nor anyone in his surroundings was prescribed anticoagulative...
medicine. Initially, the patient was administered clotting factors (Beriplex™) and vitamin K₁ (i.v.) every morning. The next day a blood sample was sent to our laboratory for analysis because of the suspicion of coumarin intoxication.

Materials and Methods

Because intoxication by a coumarinic derivative was suspected, the serum sample was analysed using the described LC-MS method after liquid–liquid extraction (1-chlorbutane, buffer pH 4.2). In addition, systematic toxicological analysis was performed (29).

Chemicals

All chemicals, reagents, and solvents were of analytical grade. Potassium dihydrogen phosphate and 1-chlorbutane were obtained from Fluka (Darmstadt, Germany). Acetone, methanol, and formic acid were from Riedel de Haen (Seelze, Germany); acenocoumarol was from Novartis (Nürnberg, Germany); brodifacoum and coumachlor were from Sigma-Aldrich (Seelze, Germany); flocoumafen was from BASF (Ludwigshafen, Germany); phenprocoumon was from Salutas (Magdeburg, Germany); bromadiolone, coumatetralyl, difenacoum difethialone, and warfarin were from Ehrenstorfer (Augsburg, Germany). The internal standard (IS), 7-acetoxy-6-(2,3-dibromopropyl)-4,8-dimethylcoumarin, was obtained from Sigma-Aldrich.

Serum sample preparation

A total of 0.5 mL serum, 0.1 mL potassium buffer (pH 4.2), and 0.4 mL extraction reagent [50 μL IS (c = 1 mg/mL in acetone) dissolved in 50 mL 1-chlorbutane] were mixed in a 1.5-mL eppendorf cup for 2 min. The sample was centrifuged for 2 min at 15,000 g and 0.2 mL of the organic phase was evaporated to dryness under a stream of nitrogen at 30°C. The residue was redissolved in 120 μL of methanol.

Quantitation

For quantitation, serum was spiked at six concentrations of each analyte (5, 15, 25, 50, 150, and 250 μg/L). All calibration samples were stored frozen at -18°C until analysis. Quantitation followed the internal standard method. Precision and accuracy for each determined compound was measured using in-house quality control samples (spiked matrix, each analyte c = 100 μg/L).

LC parameters

The HPLC was equipped with a binary pump (LC-10 ADVP), a system controller (SCL-10 A), a solvent degasser (DGU-10 ADVP), an autosampler (SIL-10 ADVP), an oven (CTO-10 ASVP), and a UV-detector (SPD-6 A, Shimadzu, Duisburg, Germany). An Atlantis C18 (2.1 x 20 mm, 3 μm, Waters) analytical column was used. The oven temperature was 40°C. The mobile phase consisted of (solvent A) methanol and (solvent B) a mixture of methanol/0.1% formic acid (10:90, v/v) pumped at a flow rate of 0.6 mL/min. The following gradient was used: 0–0.7 min, 95% B; 0.7–1.1 min, 50% B linear; 1.1–3.2 min, 6% B linear; 3.2–3.8 min, 6% B; and 3.8–4.2 min, 95% linear. Injection volume was 50 μL.

MS parameters

The MS (MS-2010-System) was obtained from Shimadzu (Duisburg, Germany). The ESI source was operated with a spray voltage of 4.5 kV. Nitrogen was used at a flow-rate of 4.5 L/min. Block- and curved dissolution line-temperature were set at 300°C. All other settings were default from the standard-tuning. The MS apparatus was operated in the positive- and negative-ion detection mode with a focus on the masses [m/z 1 = (-)ESI, m/z 2 = (+)ESI]: acenocoumarol, 353 and 352; brodifacoum, 523 and 521; bromadiolone, 527 and 525; coumachlor, 343 and 341; coumatetralyl, 292 and 291; difenacoum, 445 and 443; difethialone, 539 and 537; flocoumafen, 543 and 542; phenprocoumon, 280 and 279; warfarin, 308 and 307; and IS [ESI(+): 431 and 433], at a detector voltage of 1.9 kV. Shimadzu LCMSolution Software version 2.05 was used for data acquisition.

Results and Discussion

This report presents a novel screening method for the simultaneous identification and quantification of 10 vitamin K antagonists by LC–MS. The chosen conditions for the analysis allowed a fast elution of the substances within 5 min. The general conditions (mobile phase, analytical column, and ESI-source) corresponded to the LC–MS standard configuration in our laboratory; thus, no extra time for set up was needed.

Quantitation

The following linear regression data resulted (R² ± SD; n = 3): acenocoumarol (0.998 ± 0.002); coumachlor (0.996 ± 0.001); coumatetralyl (0.997 ± 0.001); phenprocoumon (0.998 ± 0.001); warfarin (0.993 ± 0.003); brodifacoum (0.995 ± 0.003); bromadiolone (0.994 ± 0.003); difenacoum (0.993 ± 0.003); difethialone (0.990 ± 0.007); and flocoumafen (0.994 ± 0.004).

The lower limit of quantitation of each analyte was determined to be 5 μg/L (S/N > 10), and the upper limit of quantitation (ULOQ) was 250 μg/L. Because of the high sensitivity of the method, the ULOQ was not suitable for the quantitation of phenprocoumon and warfarin within the therapeutic drug monitoring range (therapeutic level may be up to 3000 μg/L). For the quantitation of these substances, the sample must be diluted with coumarin-free serum.

Accuracy and precision

Accuracy and precision of the method was determined from replicate analyses of spiked QC serum samples at 100 μg/L for each compound. Accuracy was determined by injecting one QC sample twice. Intraday variation was estimated by injecting three different QC samples during one working day. Interday variation was estimated using six different samples during consecutive days. The degree of precision and accuracy of the LC–MS method is summarized in Table II.

To avoid a possible misidentification, two masses were
used. Within the scope of the method validation, 12 drug-free human serum samples of six different sample tubes were successfully tested for the absence of coumarins or disturbing signals.

**Acute intoxication**

Corresponding to the suspected coumarin intoxication, the sample was analyzed with the described analytical method. In the first sample, a bromadiolone concentration of 440 μg/L was calculated (the sample was previously diluted with coumarin-free serum, 1:1, v/v). As one can see from the chromatograms (Figure 4), bromadiolone was clearly identified through its retention time (3.5 min) as well as the two mass peaks (m/z 527 and 525).

The actual calibrations showed linearity for all analytes within the range of 5–250 μg/L. The QC sample met the target level of 80–120 μg/L (± 20% of the rated value). Additional screening for basic substances (HPLC–DAD) (29) with the first sample of the patient identified no other substances.

In cooperation with the medical doctors from the hospital, six further serum samples were sent to our laboratory for the quantitation of bromadiolone. The concentration-time profile of the bromadiolone is illustrated in Figure 5.

Excluding the first measured value (assumed distribution phase), the half-life is approximately 140 h. The linearity of the curve demonstrated the high reliability and accuracy of the method. In all examined specimens, quantitation was possible and the limits of quantitation were sufficient.

The patient was continuously monitored in hospital (e.g., INR and red blood picture). The coagulation activity of the patient changed drastically within several hours after admission. Although vitamin K1 was given immediately, the INR increased to over 10. The time course of INR-value during hospitalization is shown in Figure 6.

When the patient was discharged, the INR value was at 1.55, and there was no sign of serious bleeding. Hemoglobin (1.50–1.61 g/L), erythrocytes (4.8–5.1 x 10¹²/L), and hematocrit (43–46%) were in normal ranges throughout hospitalization. It is worth mentioning that medical examination of his wife resulted in an INR value of 1 (normal value). The case was handed over to the police.

In the literature, few serum concentrations of rodenticide intoxications on the basis of vitamin K antagonists are given: brodifacoum 630 μg/L (30), 731 μg/L (31), and 710 μg/L (32) and difenacoum 600 μg/L (33) and 970 μg/L (11). There are two reports of intoxication with bromadiolone. In one case, a 27-year-old woman developed epitaxis and menorrhagia at a maximum serum concentration of 40 μg/L (34). In another case, chronic ingestion of bromadiolone pellets by two children, aged 2 and 3, which resulted in coagulopathy, with hematoma in one and hematrhosis in the other has been reported (35). Both chil-

| Table II. Accuracy and Precision of Each Analyte from Spiked Serum Samples |
|-----------------------------|------------------|------------------|------------------|
| Compound                  | Accuracy* (%) (n = 2) | Intraday Precision | Interday Precision |
|                            | 100 μg/L | [CVt (%), n = 3] | 100 μg/L | [CVt (%), n = 6] |
| Acenocoumarol             | 0.5      | 4.3              | 5.2              |
| Coumachlor                | 0.7      | 3.1              | 5.9              |
| Coumatetralyl             | 0.5      | 4.4              | 4.8              |
| Phenprocoumon             | 1.0      | 5.4              | 6.0              |
| Warfarin                  | 1.8      | 8.4              | 9.0              |
| Brodifacoum               | 5.2      | 13.3             | 14.9             |
| Bromadiolone              | 1.1      | 7.8              | 8.2              |
| Difenacoum                | 3.4      | 6.7              | 8.4              |
| Difethialone              | 3.7      | 9.5              | 11.7             |
| Flocoumafen               | 3.1      | 5.3              | 14.2             |

* Accuracy = average analyte concentration/known concentration x 100%.

* CV (%) = SD/mean x 100%.

Figure 4. Ion chromatograms of the serum sample containing bromadiolone (218 and 222 μg/L, previously diluted with coumarin-free serum 1:1), (–ESI, m/z 527 and 525.)
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Conclusions

A case of superwarfarin intoxication (bromadiolone) is reported. The maximum INR-value was above 10, and the maximum serum concentration of bromadiolone was 440 μg/L and, therefore, 11 times higher than in the case of the 27-year-old woman described in the literature (34). The measurement of several samples allowed the determination of the half-life of bromadiolone in the patient, which was 140 h. Upon the consequent treatment with vitamin K, the patient left the hospital after 22 days without any signs of serious bleeding during the time of hospitalization. Identification and quantitation was carried out with a sensitive and reliable LC–MS method that is capable of simultaneous determination of 10 vitamin K antagonists in human serum, including five superwarfarins.

The method is based on a simple, fast liquid–liquid extraction at pH 4.2. The time for analysis is five min.

References

20. F. Guan, A. Ishii, H. Seno, K. Watanabe, T. Kumazawa, and


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