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Bromadiolone Poisoning: LC–MS Method and Pharmacokinetic Data

ABSTRACT: Poisoning with superwarfarins, like bromadiolone, is a growing public health problem, and the mortality is high. Pharmacokinetic data on bromadiolone in humans are however scarce, and there are no reports following repeated exposures to bromadiolone. We have developed a method for quantification of bromadiolone in whole blood, using liquid chromatography–mass spectrometry (LC-MS). The analytical method is reported. Limit of detection was 0.005 mg/L and limit of quantification was 0.01 mg/L. The concentrations of bromadiolone in whole blood and plasma in serial samples from a 62-year-old woman were measured. The half-life of bromadiolone in blood was estimated to be about 6 days in the initial phase of elimination and about 10–13 days in the terminal phase. The mean plasma/blood ratio of bromadiolone was 1.7 ± 0.6. Stability testing of bromadiolone in whole blood samples after two cycles of freeze and thaw revealed that bromadiolone concentrations decreased.

KEYWORDS: forensic science, forensic toxicology, bromadiolone, superwarfarins, liquid chromatography–mass spectrometry, poisoning

Warfarin was patented in 1948 as a rodenticide, but because rats developed resistance, more potent derivatives of warfarin were developed, namely superwarfarins or second-generation anticoagulant rodenticides (1,2). The superwarfarins are considered to be about 100 times more potent than warfarin on a molar basis, with strong binding affinity, greater absorption due to increased fat-solubility, and long-acting effects (2–4). A number of superwarfarin products, e.g., bromadiolone (Fig. 1), are sold over-the-counter and are readily available in stores and homes (1,3).

It is well known that superwarfarins cause a prolonged anticoagulant effect by inhibiting the carboxylation of vitamin K–dependent factors (II, VII, IX, and X) in the liver, and treatment of superwarfarin poisoning requires administration of vitamin K1 for several months (1,3,4). Poisoning with these products, whether accidental or intentional, is a growing public-health problem (2,5) and up to 20% mortality has been reported (3). Cases may be associated with accidental exposure (often children), suicide attempts, or Munchausen syndrome, and may be difficult to diagnose (3).

Only a few laboratories perform analysis of superwarfarins and the possibility of detecting superwarfarins in blood samples has been limited, although some methods are reported (1,6–8). To the best of our knowledge, there are only two reports on the kinetics of bromadiolone metabolism in humans (7,8), but the half-lives reported vary from 50 h (7) to 140 h (8) in the two studies. These half-lives are however much shorter than half-lives reported from animal studies, where, for example, 120 days is reported for dogs (9). No previous studies have measured the concentration in humans after repeated exposure to bromadiolone.

To investigate a patient repeatedly exposed to bromadiolone, an analytical method was developed for the quantification of bromadiolone. The method was based on liquid chromatography–mass spectrometry (LC–MS) with an electrospray ionization (ESI) interface. Serial blood samples from a woman who was repeatedly exposed to bromadiolone had been collected and the half-life of bromadiolone was estimated. The plasma/blood ratio and the stability of bromadiolone in whole blood after storage at −20°C, including thaw and freeze cycles, were also investigated.

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Materials and Methods

Chemicals

Bromadiolone was supplied by Promochem (LGC Promochem, Borås, Sweden) and cyclobarbitone (internal standard) by Chemische Fabrik Berg GmbH (Bitterfeld, Germany). Acetonitrile (far UV HPLC) was purchased from LAB-SCAN (Dublin, Ireland), AnalaR® ammonium formate from BDH Laboratory Supplies (Poole, England), and formic acid from Merck (VWR International AS, Oslo, Norway). De-ionized water was obtained from a Milli-Q UF Plus water purification system (Millipore, Bedford, MA). Human whole blood was supplied by Blood Bank at Ullevål University Hospital, Oslo, Norway. Formate buffer, 10 mM, was prepared from a stock solution 50 mM pH 4 in water.

Preparation of Standard Stock Solution

Stock solution of bromadiolone was prepared in methanol and working standards in water. Calibration samples were prepared from whole blood spiked with working standard solutions (concentration range 0.03–1.1 mg/L). Control samples were prepared separately using the same procedure and analyzed together with calibrators and samples from the patient.

To an aliquot of 450 μL of whole blood, 50 μL internal standard solution (4.7 mg/L cyclobarbitone in water), and 500 μL cold acetonitrile (ACN) were added. The samples were immediately agitated on Whirlmixer for 1 min and thereafter deep-frozen (−20°C) for a minimum of 10 min. The samples were centrifuged at 3900 × *g* at 4°C for 10 min. The ACN layer was transferred to the autosampler vials.

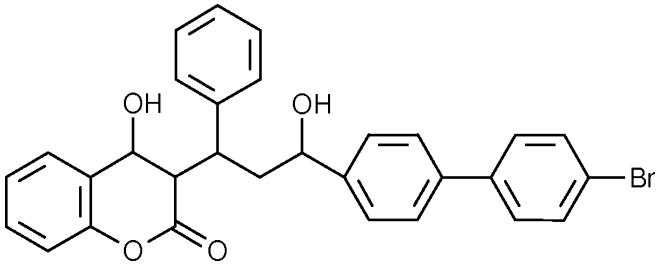

FIG. 1—*The molecular structure of bromadiolone.*

LC–MS

The analyses were performed on a LC–MS system obtained from Waters (Waters 2690 Separation Module and ZQ 2000 single hexapole mass spectrometer with an ESI interface; Waters Corp., Milford, MA) equipped with XTerra® Phenyl HPLC column (2.1 × 150 mm) 3.5 μm particles and from Waters (Milford, Ireland). The mobile phase consisted of 90% 10 mM formate buffer pH 4 and 10% acetonitrile. The separation was achieved by gradient elution starting with a mobile phase of 10% acetonitrile subsequently increased to 90% acetonitrile. The running time was 15 min.

The ESI was operated in negative mode for detection of bromadiolone and cyclobarbitone at the following mass to charge ratios (*m/z*): bromadiolone *m/z* 527.10 and cyclobarbitone: *m/z* 235.20. The retention times were 9.8 and 5.4 min, respectively. Peak height ratios were used for quantifications.

Blood Samples

Sixteen blood samples from a 62-year-old woman poisoned with bromadiolone were collected in Vacutainer® tubes (BD Diagnostics, Franklin Lakes, NJ) containing sodium fluoride as preservative and heparin as anticoagulant, and sent to the Norwegian Institute of Public Health for analysis. The samples were stored in a refrigerator until analysis. The woman could not explain how or when the exposure of bromadiolone had taken place. She has given her written consent to let us publish the analytical findings from her blood samples. The blood samples were taken at the hospital where the patient was treated, and sent to our laboratory the same day by delivery. The mean time from sampling to analysis was 5 days, with 1–8 days variation, except for one sample that was analyzed 17 days after sampling. All the samples were analyzed for bromadiolone, warfarin, and salicylic acid. The samples used to estimate plasma/blood ratio of bromadiolone were centrifuged on arrival at the institute. The concentration of bromadiolone was measured in both whole blood and plasma samples. Five of the whole blood samples containing bromadiolone were reanalyzed after two freeze and thaw cycles, to investigate stability after storage in a freezer at −20°C. For estimating the bromadiolone half-lives, only the results from the first analysis were used.

Calculations

Elimination half-lives ($t_{1/2}$) were estimated from the concentrations of bromadiolone in samples 3–4, 8–9, 12–13, and 14–15 (Table 1), using the formula $C_2 = C_1 \times 2^{\Delta t/t_{1/2}}$ (10). C_1 is the concentration in the first of the two samples and C_2 is the second concentration. Δt is the time between these two samples. The samples were selected because the concentration of bromadiolone was declining from the first to the second sample. The plasma/blood ratio of bromadiolone has been estimated from the samples where bromadiolone was measured in both plasma and whole blood (n = 10).

TABLE 1—*The estimated half-lives (days) from the samples with declining concentrations of bromadiolone.*

Estimated half-lives (days)	Sample number	Days between samples
5.6	3–4	17
9.8	8–9	19
27.2	12–13	36
12.8	14–15	50

Results and Discussion

Validation of Method

Quantitative results were obtained by peak-height measurements. Limit of detection (LOD) for bromadiolone in whole blood was 0.005 mg/L and limit of quantification (LOQ) was 0.01 mg/L. These values were determined as a mean of background noise + three standard deviations (SD) and +10 SD, respectively. Day-to-day variations were 20% (0.05 mg/L) and 11% (0.5 mg/L) (n = 10), and intra-day variations for the same concentration levels were 14.6% and 6.9%, respectively (n = 10). The calibration curve was linear in the concentration range up to 1.0 mg/L.

Possible interference by several other drug groups was tested, including benzodiazepines, analgesics, antidepressants, anticonvulsants, and antipsychotics. No interfering drugs were detected.

Bromadiolone was detected in 13 of the 16 blood samples when the analyses were performed within a few days after sampling (Fig. 2). The concentrations of bromadiolone in plasma were always higher than the concentrations in the corresponding whole blood samples. The mean plasma/blood ratio was estimated to 1.7 ± 0.6. It is important to be aware of the difference between the concentrations of bromadiolone in plasma and whole blood when comparing different analytical results.

From the present report, the half-lives of bromadiolone in a 62-year-old woman are estimated to be 6 and 10–13 days based on the concentrations measured at different time points (Table 1). The estimated half-life of 6 days from sample 3 to 4 (Table 1) might represent the initial α-phase of elimination. The concentration of bromadiolone in sample 3 is very high and probably indicates a

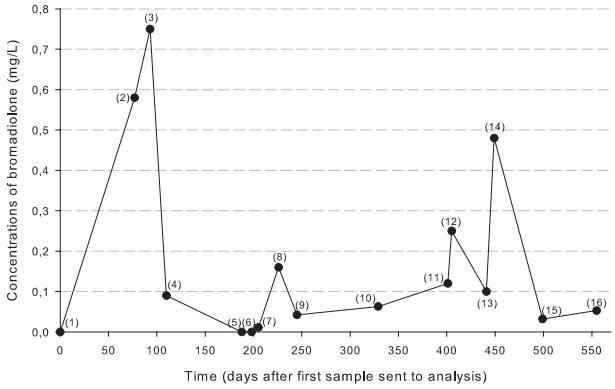

FIG. 2—*Concentration–time profile of bromadiolone for all the samples received from the patient.*

recent exposure. The rapid decline in concentration makes it unlikely that ingestion has taken place between these samples. Calculation of the elimination half-life in a 55-year-old man is reported to be 140 h or about 6 days (8), and this is consistent with the α-phase in the present report. These half-lives are, however, much shorter than reported from animal studies (9). Excluding the two reports where pharmacokinetics in humans are investigated (7,8), only single measures of bromadiolone concentration have been reported in cases of poisoning. A 27-year-old woman with menorrhagia, easy bruising, mild epistaxis, and extensive petechiae associated with scratching, had a bromadiolone concentration in serum of 40 ng/mL (11). The TIAFT list reports that a toxic concentration of bromadiolone is 20 ng/mL in serum (http://www.tiaft.org/index.php).

Some kinetic data in humans are available for brodifacoum, another superwarfarin with similar properties to bromadiolone (4). After poisoning with brodifacoum, a terminal half-life of 31 days has been reported in a 26-year-old woman (12) and 25 days in a 37-year-old man (6).

The concentrations of bromadiolone in the samples 8–9 and 14–15 (Table 1) may represent a later phase of elimination, with an estimated terminal half-life of 10–13 days. Animal studies have reported biphasic elimination from the liver with an initial rapid phase of 2–8 days, and a slower phase with a half-life of 170 days (http://www.inchem.org/). Bromadiolone is lipophilic, and repeated exposures might cause storage of bromadiolone in the body, resulting in delayed elimination. This phenomenon is well known, for example, in different cannabis preparations, where chronic use can be detected for several weeks after cessation (13). Our findings may be in concordance with biphasic elimination, indicating that bromadiolone distribution in the body may follow at least a two compartment model, especially when there is repeated intake of bromadiolone. If ingestion has taken place in-between the samples, our estimate would, however, be too long. The half-life estimated to be 27 days in samples 12–13 is so much longer than the other estimates, that new ingestion of bromadiolone in-between the two samples is suspected.

Controlled studies of the pharmacokinetics of bromadiolone in humans would be too dangerous to perform in practice. Such information has to come from case reports, where some uncertainties must be expected. In the present report, the patient could not explain when the exposures of bromadiolone had taken place. The increase in concentration in several of the samples reveals that there have been repeated ingestions, making it difficult to make a concentration–time curve for elimination of bromadiolone. The different estimates of the half-lives in the present report are each based on only two concentrations, and must be considered as rough estimates.

The concentrations of bromadiolone when reanalyses were performed after two cycles of freeze and thaw are shown in Table 2.

TABLE 2—*Assessment of stability in whole blood for bromadiolone from five of the samples received.*

Concentration of Bromadiolone (mg/L) on Arrival	Concentration of Bromadiolone (mg/L) at Reanalysis	Days Between Arrival and Reanalysis	% Change in Concentration
0.12	0.098	201	−18
0.25	0.19	201	−24
0.10	0.059	165	−41
0.48	0.45	153	−6
0.032	0.025	83	−22

The change in concentration (%) is the concentration on arrival compared to the concentration at reanalysis after two freeze–thaw cycles.

TABLE 3—*Concentrations of bromadiolone, warfarin, and salicylic acid in whole blood.*

Sample No.	Bromadiolone (mg/L)	Warfarin (mg/L)	Salicylic Acid (mg/L)
1	n.d.	0.18	70.6
2	0.58	n.d.	n.d.
3	0.75	n.d.	n.d.
4	0.090	n.d.	118.2
5	n.d.	3.42	n.d.
6	n.d.	2.68	85.4
7	0.011	1.39	22.2
8	0.16	n.d.	33.6
9	0.042	n.d.	n.d.
10	0.063	n.d.	79.3
11	0.12	n.d.	n.d.
12	0.25	n.d.	n.d.
13	0.10	n.d.	n.d.
14	0.48	n.d.	34.4
15	0.032	n.d.	38.4
16	0.053	n.d.	30.0

n.d., not detected.

In cases of forensic toxicology, it is not unusual that new information leads to a request for reanalysis of blood samples a considerable time after sampling. It is thus of great importance to know the stability of the analyte investigated. The stability of bromadiolone has previously only been investigated in spiked samples, and only after 48 h at room temperature (7). In a laboratory setting, the samples are normally stored in a deep freezer (−20°C) when they are not analyzed, and if a reanalysis is requested, the sample is thawed before a new analysis is performed. We have measured the stability in five whole blood samples from the patient. The reanalyses were performed 83–201 days after the first analyses (Table 2). The bromadiolone concentration decreased by 6–41% in the samples. There was no systematic decrease in the bromadiolone concentrations during storage in the deep freezer. However, the results indicate that bromadiolone concentration might decrease substantially during storage, and caution must be taken when interpreting results from samples that have been stored.

The enzymes responsible for the metabolism of bromadiolone in humans are unknown, but phenobarbital is claimed to induce the metabolism of superwarfarins (3,9), as reported for several other substrates metabolized by the CytP450 enzymes (14). In some of the samples from the present study, warfarin and/or salicylic acid were detected in addition to bromadiolone (Table 3). We are not aware of how other drugs may interact with the distribution or elimination of bromadiolone.

In some of the samples, warfarin and/or salicylic acid were detected. The patient has denied ingestion of warfarin, but she uses salicylic acid for headache. Both warfarin and salicylic acid cause an increased bleeding tendency and this leads to an enhanced risk of serious bleeding conditions. Even a minor trauma can induce serious and deadly situations for the patient. Warfarin and salicylic acid are, however, not known to interact with the detection of bromadiolone in the blood specimens. Comparing the concentration–time profile of bromadiolone (Fig. 2) with the detection of warfarin and/or salicylic acid (Table 3), does not show an obvious pattern for drug ingestion or how the different drugs might interfere with detection of the others.

Conclusion

From the present analytical findings, the half-life of bromadiolone in a 62-year-old woman is estimated to about 6 days in the

initial phase of elimination and about 10–13 days in the terminal phase, and a possible two compartment model of distribution is suggested.

The initial half-life is in concordance with a previous report estimated in a 55-year-old man. These half-lives are, however, much shorter than previously reported from animal studies. LC–MS is a suitable method for rapid quantification of bromadiolone and other superwarfarins. In cases of uncertain coagulopathy, analysis of long-acting anticoagulants should be performed.

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