

LC-MS/MS analysis of brodifacoum isomers in rat tissue

Zane Z. Hauck, Douglas L. Feinstein, and Richard B. van Breemen*

Department of Medicinal Chemistry and Pharmacognosy

University of Illinois College of Pharmacy

833 S. Wood Street

Chicago, IL 60612, USA

*Corresponding author

Richard B. van Breemen

Department of Medicinal Chemistry and Pharmacognosy

University of Illinois College of Pharmacy

833 S. Wood Street, M/C 781

Chicago, Illinois 60612

Tel: 312-996-9353

Fax: 312-996-7107

Email: breemen@uic.edu

Abstract

Brodifacoum (BDF) is a second generation anticoagulant rodenticide structurally related to warfarin but containing two chiral centers. Highly stable, BDF can contaminate food and water supplies causing accidental poisoning of humans and non-target animals. To determine the distribution of BDF isomers in serum and tissues, a quantitative method was developed and validated according to FDA guidelines based on high performance liquid chromatography-tandem mass spectrometry. A single liquid-liquid extraction step provided recoveries exceeding 93%. Reversed phase chromatographic separations required less than 6 min, and quantitative analysis utilized a triple-quadrupole mass spectrometer equipped with negative ion electrospray and selected reaction monitoring. The standard curve had a linear regression coefficient of 0.999 and intra- and inter- assay variations of less than 10%. The chromatographic method enabled the resolution and measurement of pairs of BDF diastereomers in commercial materials as well as in rat tissues. This method is suitable for measuring BDF exposure as well as basic science studies of the distribution and elimination of BDF diastereomers to various tissues.

Keywords

Brodifacoum; superwarfarin; diastereomers; LC-MS/MS

Introduction

Following a 1920s outbreak of an unusual bleeding disorder in cattle in the United States and Canada attributed to silage from sweet clover, a botanical anticoagulant was identified in 1940 as 4-hydroxy coumarin (1). First synthesized in 1948 at the University of Wisconsin, this compound was named warfarin (Wisconsin Alumni Research Foundation and *-arin* from coumarin) and later received approval for use as a rodenticide in the United States as well as for clinical use to prevent blood clots. After extensive use as a rodenticide, mutations in vitamin K 2,3-epoxide reductase complex 1 (the main target of warfarin) reduced the effectiveness of warfarin in controlling rat populations (1).

Second generation coumarin anticoagulants, known as “superwarfarins,” contain a hydrophobic substituent that increases biological half-life as well as binding affinity to the target vitamin K 2,3-epoxide reductase complex 1 by up to 100-fold (2,3). After ingestion of a lethal dose, superwarfarins typically cause death in rodents 1-2 weeks due to internal hemorrhage. The long half-lives of superwarfarins as well as their high potencies increase the risk that they might contaminate water or food and harm non-target wildlife as well as humans (4-7).

Brodifacoum, 3-[3-[4-(4-bromophenyl)phenyl]-1,2,3,4-tetrahydronaphthalen-1-yl]-4-hydroxychromen-2-one, (BDF, figure 1) is a hydroxycoumarin and second-generation superwarfarin anticoagulant rodenticide that is characterized by a long half-life, persistence in tissues, and tendency to be excreted unchanged (3,8). BDF accumulates in and damages the liver (~90% of ingested amount) but has been found in many other organs including brain, kidney and lungs (8). Over 10,000 people, mostly children, are exposed to BDF or other superwarfarins annually in the United States (9). The majority of BDF poisoning cases are accidental but some include attempted suicides. Like other superwarfarins, BDF primarily functions by inhibiting the

vitamin K 2,3-epoxide reductase complex 1, interrupting the vitamin K cycle and preventing clot formation (2,9). The current treatment for BDF poisoning is daily vitamin K₁ (phyloquinone) administration, but due to the long half-life of BDF, vitamin K supplementation may be required for an extended time, and interruption of treatment can result in recurrence of symptoms (3,9).

The chemical structure of BDF (figure 1) contains two chiral centers thereby forming four stereoisomers (10). These pairs of diastereomers have different chemical and physical properties and likely have different biological activities as observed for the biological activities and routes of metabolism of the *R* and *S* isomers of warfarin (11). Furthermore, BDF from different sources might contain different ratios of isomers. For example, Cort, *et al.* (10) used proton NMR to show that 9 of 10 commercial BDF products contained a small excess of the (*S,R*)/(*R,S*) stereoisomers. Analytical methods are needed to measure BDF in serum and tissues that can not only separate BDF from matrix compounds, but also differentiate the BDF diastereomers. Whether using UV, fluorescence or mass spectrometric detection, none of the current chromatographic methods separate any of the BDF diastereomers (12-21). Here, we report a method using high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) to separate pairs of BDF diastereomers while providing high sensitivity and accuracy for the determination of BDF in rodent tissues.

Experimental

Chemicals and Reagents

BDF, difenacoum (figure 1) and HPLC-grade acetone, acetonitrile and dichloromethane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared using a Milli-Q water purification system (Millipore, MA, USA).

Sample Collection

Adult male Sprague-Dawley rats (180-220 g, Harlan Laboratories, Indianapolis, IN, USA) were given single doses of BDF (0.5 mg/kg; ethanol/methanol/water 1:1:2; v/v/v) by gavage. Animals were euthanized following the guidelines of the American Veterinary Medical Association (22). The tissues were removed, placed immediately into liquid nitrogen and stored at -80°C until extraction. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Tissue extraction

Tissue specimens were thawed, and 100 mg samples were homogenized in 50 µL of saline using a polypropylene pestle. The BDF analog difenacoum (figure 1) (5 µL of a 100 µg/mL solution) was added to each sample as an internal standard. Each sample was extracted by adding 445 µL of 30% (v/v) acetone in dichloromethane (total volume 500 µL) and sonicating in a water bath for 10 minutes. After chilling on ice for 15 minutes to precipitate proteins, samples were centrifuged at 13,000 x g for 10 minutes. Each supernatant (400 µL) was removed, transferred to a 1.5 mL microcentrifuge tube and evaporated to dryness using vacuum centrifuge. Samples were stored at -80°C until analysis, when they were reconstituted in 25% acetone in water.

Preparation of standards and quality control (QC) samples

Stock solutions of BDF and difenacoum were prepared in acetone (1 mg/mL) and stored at -20°C. Working solutions ranging from 1 ng/mL to 1 µg/mL in acetone were prepared from stock solution and stored at -20°C. The final calibration standards were prepared in 445 µL

acetone, 50 μ L saline and 5 μ L of a difenacoum solution (100 μ g/mL) at BDF concentrations of 0.1, 0.5, 1, 2, 5, 10, 25, 50, and 100 ng/mL. Quality control (QC) samples (low, medium, high) were prepared identically to the standards at final concentrations of 1, 10 and 50 ng/mL.

Chromatography

Separations were carried out using a Shimadzu (Kyoto, Japan) Nexera chromatography system equipped with an Agilent Poroshell 120 EC-C₁₈ column (2.1 mm x 150 mm, 2.4 μ m). BDF was eluted using a 6-min linear gradient from 50% to 100% acetonitrile containing water and held for 1 minute at a flow rate of 0.55 mL/min. The column was regenerated by switching to 100 % acetone over 30 seconds and holding for 2 minutes. This was followed by re-equilibration at 50% acetonitrile for 7-min before the next injection. The injection volume was 10 μ L, and the column temperature was 40 °C. Data acquisition and integration were carried out using Shimadzu Lab Solutions software.

Tandem mass spectrometry

LC-MS/MS was carried out using a Shimadzu LCMS-8050 triple quadrupole mass spectrometer equipped with negative ion electrospray and operated at unit resolution. Nitrogen was used for nebulization at a flow rate of 3.0 L/min and as a drying gas at 5 L/min. The ion source capillary temperature was 300°C, the vaporizer temperature was 400°C and the heat block temperature was 100°C. BDF was measured using collision-induced dissociation with selected reaction monitoring (SRM). Argon was used as the collision gas at a pressure of 230 kPa. The SRM transitions for BDF were m/z 523 to 135 (quantifier) and m/z 523 to 187 (qualifier); and the transitions for the internal standard difenacoum were m/z 443 to 293 and m/z 443 to 135 for the quantifier and qualifier, respectively. The SRM dwell time was 25 msec.

Validation

Linearity, LLOQ, and LLOD. The calibration curve was constructed using 6 calibration standards in duplicate. The area ratio of BDF to the internal standard was plotted against the BDF concentration, and linearity was determined using least squares linear regression analysis of the calibration curve using a weighting factor of $1/(\text{area ratio})$.

Selectivity and Matrix Effects. Blank rat tissues (brain, liver heart, lung, and kidney) were analyzed for interference at the BDF retention time of 5 min. A matrix effect study was carried out using low, medium, and high concentrations of BDF (1, 10 and 50 ng/mL) prepared in pure solvent or spiked into rat liver extract or brain tissue extract and then analyzed using LC-MS/MS.

Precision and Accuracy. Inter-assay and intra-assay precision and accuracy were calculated from the standard curve at three BDF concentrations, 1, 10 and 50 ng/mL. Intra-assay precision and accuracy were evaluated for the standard curve by multiple analyses for each point.

Recovery. The recovery of BDF following extraction of tissue was determined for three concentrations at the low, middle and high end of the standard curve.

Stability. Freeze-thaw stability of BDF in tissue after perfusion was determined over 3 cycles. Long-term stability in tissue at -80 °C was measured over 28 days. Short-term (24-hour) stabilities of BDF were determined at room temperature (27°C) and at the auto-sampler temperature (4 °C).

Results

Method Development

Experiments were carried out to optimize extraction recovery, chromatographic separation, and MS/MS measurement. Multiple extraction solvents and mixtures were investigated, and a combination of dichloromethane and acetone, as reported by Elmeros, *et al.* (7), were found to provide optimal recovery of BDF from rat tissues (see method validation details below). Several reversed phase HPLC and UHPLC columns and a variety of mobile phase conditions were evaluated for the separation of BDF isomers. Ultimately, a superficially porous HPLC column was selected that enabled baseline separation of pairs of BDF diastereomers (figure 2) in less than 6 minutes and durability for hundreds of sample injections. During this separation, the earlier eluting BDF peak was always larger than the second peak. The internal standard, difenacoum (figure 1), was also resolved into two pairs of diastereomers, and the earlier eluting peak was always smaller than the second peak (figure 2). Note that even when using a superficially porous HPLC column, acetonitrile but not methanol enabled the separation of pairs of BDF diastereomers (figure 2).

Positive ion (21) and negative ion electrospray (16) were evaluated for the mass spectrometric detection of BDF, and negative ion mode was found to provide lower limits of detection. Atmospheric pressure chemical ionization was also evaluated as an alternative ionization technique, but electrospray was found to provide more efficient ionization of BDF. Collision energies were optimized for collision-induced dissociation, and the quantifier (m/z 135) and qualifier ions (m/z 187) were the same as have been reported previously (166). Note that the heavy bromine isotopic peak of deprotonated BDF (m/z 523) was used as the precursor ion

instead of the lighter bromine isotope peak of m/z 521, as this peak produced higher signal-to-noise during LC-MS/MS.

Validation

Linearity and limits of detection and quantitation. The range of the standard curve was from 1 ng/mL to 50 ng/mL with a linear profile of $r^2=0.999$ (figure 3). The lower limit of detection (LLOD) was 0.1 ng/mL and the lower limit of quantitation (LLOQ) was 1 ng/mL (figure 2). The intra-assay and inter-assay accuracy, determined using the percentage difference from nominal values at low, medium, and high levels of BDF, ranged from 97% to 107% (Table 1). The precision was determined using the percentage relative standard deviation and was within 12% at all three levels of BDF (Table 1). Also within FDA guidelines (23), the inter-assay and intra-assay coefficients of variation were 3.1% and 7.3% for 1 ng/mL BDF, 2.7% and 0.2% for 10 ng/mL BDF, and 0.2% and 3.1% for 50 ng/mL BDF.

Selectivity, Matrix Effects and Recovery. Blank rat tissues (brain, heart, liver, lung, and kidney) were analyzed for interference over the 5.0 – 5.5 minute retention time range of BDF and the 4.2 – 4.8 min retention time range of the internal standard difenacoum. No interference was observed using the quantifier and qualifier SRM transitions of either BDF or difenacoum (figure 2). Matrix effect studies showed no ion suppression or enhancement when using LC0MS/MS to compare BDF samples prepared in extracts of rat brain or liver to those prepared in solvent (Table 3). Recovery experiments showed greater than 93% recovery of BDF at low, medium or high levels when using 30% (v/v) acetone in dichloromethane liquid extraction prior to LC-MS/MS analysis.

Stability. BDF was stable at room temperature for at least 24 hours, stable at -80°C for at least 1 month, stable in the reconstitution solvent at the autosampler temperature of 4°C for at least 24

hours, and stable for at least 3 freeze/thaw cycles (Table 2). Therefore, BDF was determined to be stable during and even beyond the storage and handling conditions required for this method.

Application of method to rat tissue analysis

Tissues were obtained from euthanized rats 24 hours after administration of BDF, and the levels of pairs of BDF diastereomers were measured in each specimen (figure 4). Liver contained the highest levels of BDF at 4.78 nmol/g (sum of both peaks of BDF diastereomers), which is consistent with a previous report (11), followed by lung tissue at 0.14 nmol/g. Rat brain contained lower but still measurable quantities of BDF consisting of 0.09 nmol/g in cerebellum and 0.07 nmol/g in frontal cortex. Pairs of BDF diastereomers were measured, and in each case, the first peak was larger than the second. The ratios of the peaks representing BDF diastereomeric pairs ranged from 1.95 ± 0.10 in lung tissue to 3.00 ± 0.07 for frontal cortex and cerebellum (figure 4). For comparison, the ratio of BDF diastereomeric pairs in the commercial-grade BDF that was administered to the rats was 1.18 ± 0.08 ($n = 20$).

Discussion

BDF has two chiral centers producing four stereoisomers that are separable as pairs of diastereomers (S,R/R,S; and R,R/S,S) using reversed phase chromatography. Nevertheless, previously published HPLC and UHPLC methods for BDF analysis (12-21) only reported one chromatographic peak for BDF. By optimizing the chromatographic mobile phase and elution parameters, we were able to separate pairs of diastereomers into two peaks with baseline separation (figure 2a). The earlier eluting peak of both the BDF administered to the rats and the BDF measured in rat tissues was always in greater abundance. Previous proton NMR analyses of

various commercial sources of BDF (10), which included the source used in this investigation, reported that the BDF (S,R/R,S) isomers were in excess; therefore, we assume that these isomers represent the peak eluting first at 5.10 minutes (figure 2).

LC-MS/MS measurements of BDF in rat tissues (figure 4) indicated that the relative amounts of the BDF (S,R/R,S) isomers to the (R,R/S,S) isomers in tissues were higher than they had been in the administered form of BDF. This suggests that the (S,R/R,S) isomers of BDF are more orally bioavailable in rats or that the BDF (R,R/S,S) isomers are eliminated more rapidly, perhaps through metabolism, as has been observed for the R and S isomers of warfarin (11). Selective metabolism of BDF diastereomers has been suggested by a US Environmental Protection Agency report indicating that one pair of BDF enantiomers forms glucuronic acid conjugates more readily than the other (24). Species differences with respect to the rates of elimination of specific BDF isomers have also been reported (25). Whether the different BDF enantiomers have different biological activity remains to be determined.

Conclusions

The toxicity of the superwarfarin rodenticide BDF is due in part to a long biological half-life. While treatment with vitamin K alleviates symptoms of BDF poisoning, that treatment does not eliminate BDF from tissue therefore necessitating long term treatment. The analytical method described here, validated to FDA standards, utilizes a single step liquid-liquid extraction to prepare the samples for LC-MS/MS quantitation. With a retention time of less than 6 minutes for BDF, this LC-MS/MS assay is equal to or faster than previous LC-MS/MS and UHPLC-MS/MS based assays for the analysis of BDF while still enabling the separation of pairs of BDF

diastereomers. The sensitivity, small sample size (100 mg), and fast analysis make this an attractive method for determining the effects and distribution of the different isomers of BDF in tissues.

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Tables

Table 1. Intra-assay and inter-assay accuracy and precision for the quantitative analysis of BDF in rat tissue.

| BDF | Nominal value (ng/100 mg) | Intra Day (<i>n</i> =4) | | | Inter day (<i>n</i> =8) | | |
|--------|---------------------------------|--------------------------|----------------------------------|---------------|--------------------------|----------------------------------|---------------|
| | | CV% | Measured value (ng/100 mg) | Accuracy % | CV% | Measured value (ng/100 mg) | Accuracy % |
| Low | 0.5 | 8.3% | 0.54 | 102.4 | 6.5% | 0.53 | 91.3 |
| Medium | 10 | 1.5% | 10.46 | 101.6 | 2.0% | 10.48 | 108.4 |
| High | 50 | 1.0% | 50.65 | 99.1 | 1.7% | 51.52 | 102.6 |

Table 2. Long-term and short-term stabilities of BDF ($n=6$).

| BDF level (ng/100 mg tissue) | Treatment | % BDF remaining \pm RSD |
|---------------------------------|----------------------------|------------------------------|
| 1 | Autosampler (4°C) | 94.3 \pm 4.0% |
| | Freeze-thaw (3 cycles) | 94.0 \pm 3.4% |
| | Bench top (27°C, 24 hours) | 93.3 \pm 3.0% |
| | Long-term (-80°C) | 93.8 \pm 4.5% |
| 10 | Autosampler (4°C) | 94.7 \pm 3.6% |
| | Freeze-thaw (3 cycles) | 98.1 \pm 2.6% |
| | Bench top (27°C, 24 hours) | 95.8 \pm 4.2% |
| | Long-term (-80°C) | 98.0 \pm 1.9% |
| 50 | Autosampler (4°C) | 91.2 \pm 4.3% |
| | Freeze-thaw (3 cycles) | 99.1 \pm 1.0% |
| | Bench top (27°C, 24 hours) | 93.4 \pm 5.2% |
| | Long-term (-80°C) | 93.0 \pm 3.4% |

Table 3. Investigation of LC-MS/MS matrix effects for the analysis of BDF in rat tissue. Values for BDF spiked at low, medium or high concentrations into extracts of rat brain or liver tissue were indistinguishable from BDF in solvent.

| Matrix | Nominal BDF Concentration (ng/mL) | Measured BDF Concentration (ng/mL \pm RSD) | P Value (tissue and solvent) (<i>n</i>=3) |
|---------------|--|--|--|
| Brain | 1 | 0.96 \pm 0.06 | 0.46 |
| | 10 | 10.3 \pm 0.4 | 0.45 |
| | 50 | 49.1 \pm 0.8 | 0.39 |
| Liver | 1 | 1.04 \pm 0.05 | 0.29 |
| | 10 | 10.0 \pm 0.2 | 0.41 |
| | 50 | 50.1 \pm 1.0 | 0.47 |
| Solvent | 1 | 0.99 \pm 0.02 | - |
| | 10 | 10.1 \pm 0.1 | - |
| | 50 | 49.6 \pm 0.4 | - |

Figure Legends

Figure 1. Chemical structures of (A) brodifacoum (BDF); and (B) internal standard difenacoum.

Note that each compound contains two chiral centers and is synthesized commercially as racemic mixtures of each enantiomer.

Figure 2. Negative ion electrospray LC-MS/MS selected reaction monitoring (SRM) chromatograms of BDF and difenacoum internal standard, (A) 50 ng/mL standards using optimized mobile phase containing acetonitrile; (B) 50 ng/mL standards obtained using methanol instead of acetonitrile; (C) rat brain negative control from untreated animal showing detection of only internal standard; (D) BDF at the LLOQ (1 ng/mL); and (E) BDF [0.079 nmol/g tissue (peak 1) + 0.026 nmol/g tissue (peak 2) = 0.105 nmol/g tissue total] in cerebellum of treated animal 24 h after dosing.

Figure 3. Standard curve for BDF ($n=6$) obtained using LC-MS/MS with negative ion electrospray showing good linearity, dynamic range and sensitivity.

Figure 4. Comparison of BDF levels in various rat tissues 24 hours after BDF administration (nmol BDF/g tissue \pm RSD, $n = 4$). Pairs of BDF diastereomers were measured using LC-MS/MS at retention times of 5.10 minutes (open bars) and 5.35 minutes (diagonal bars), respectively. For comparison, the ratio of pairs of diastereomers in the BDF administered to the rats was 1.18 ± 0.08 ($n = 20$).