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Plasma metabolic profiling analysis of toxicity induced by brodifacoum using metabonomics coupled with multivariate data analysis

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ABSTRACT

Brodifacoum is one of the most widely used rodenticides for rodent control and eradication; however, human and animal poisoning due to primary and secondary exposure has been reported since its development. Although numerous studies have described brodifacoum induced toxicity, the precise mechanism still needs to be explored. Gas chromatography mass spectrometry (GC–MS) coupled with an ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) was applied to characterize the metabolic profile of brodifacoum induced toxicity and discover potential biomarkers in rat plasma. The toxicity of brodifacoum was dose-dependent, and the high-dose group obviously manifested toxicity with subcutaneous hemorrhage. The blood brodifacoum concentration showed a positive relation to the ingestion dose in toxicological analysis. Significant changes of twenty-four metabolites were identified and considered as potential toxicity biomarkers, primarily involving glucose metabolism, lipid metabolism and amino acid metabolism associated with anticoagulant activity, nephrotoxicity and hepatic damage. MS-based metabonomics analysis in plasma samples is helpful to search for potential poisoning biomarkers and to understand the underlying mechanisms of brodifacoum induced toxicity.

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1. Introduction

Brodifacoum(3-[3-(40-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro-1-naphthyl]-4-hydroxycoumarin) is one of the most commonly used rodenticides around the world [1,2]. It belongs to the class of second generation anticoagulant rodenticides, which are long-acting, fat soluble, and 100 times more potent than the first generation anticoagulant rodenticide warfarin. Brodifacoum is preferred over other anticoagulants because it kills rats after a single feeding [3]. However, non-target vertebrates are potentially susceptible to the toxicity of brodifacoum when it is consumed directly (primary exposure) or indirectly (secondary exposure) [4,5]. Dowding et al. reported a mortality of 50–60% in New Zealand dotterels over a 3-month period following aerial broadcast of brodifacoum bait compared to the normal mortality rate of 6–9% per year for that population [6]. The use of brodifacoum for rat removal on another seabird island poses a clear risk of secondary poisoning to avian scavengers [7].

Human brodifacoum exposure, intentional or accidental, can cause severe toxicity, resulting in intracranial, nasal, gingival and buccal, gastrointestinal, vaginal, thoracic, pulmonary, and adrenal hemorrhage, as well as hemoperitoneum, hematuria, muscle bleeding, and death [8,9]. Brodifacoum has been shown to inactivate vitamin K1 2,3-epoxide reductase and vitamin K quinone reductase, leading to an increase of the inactive form of vitamin K, i.e., vitamin K1 2,3-epoxide. Thus, the carboxylation of glutamic acid residues to gamma-carboxy glutamic acid (Gla) in substrate proteins of clotting factor (II, VII, IX, and X) precursors is inhibited in the liver [10]. Brodifacoum liver disposition data suggest that following the initial liver accumulation, brodifacoum redistributes to reach a steady state of liver distribution between days 2–7, and no redistribution is seen from the microsomal fraction. Brodifacoum is slowly eliminated via a two compartment model, with a terminal half-life ranging from 20 to 62 days in humans [9]. After a long-term exposure of brodifacoum, it takes more than eight months to be eliminated from the body, despite the use of an effective antidote (vitamin K) [8,11]. Therefore, the biological processes of brodifacoum induced toxicity still requires further investigation to illuminate its mechanism and to identify reliable biomarkers.

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Metabonomics has recently attracted increasing interest in the fields of toxicology, since it involves the determination of changes in the levels of endogenous metabolites in biological samples that caused by physiological stimuli or genetic injuries [12]. Metabonomics has been employed to investigate the toxicity of rodenticide as well as pesticides [13–17]. Deguchi et al. [13] investigated the effect of warfarin on plasma metabolites by untargeted metabonomics. Two novel molecules, palmitoylethanolamide (PEA) and stearoylethanolamide (SEA), were identified from the same family, and the ethanolamide family possibly stimulates the coagulation pathway to increase thrombin in plasma. The consequent anticoagulant effect by ethanolamide reductions might be minor compared to the warfarin anticoagulant effect on vitamin K dependent coagulation factors. Zheng et al. [18] evaluated anticoagulant effect of Danggui Sini decoction. The previous studies have initially investigated anticoagulant mechanism of warfarin or herbal medicine by metabonomic method. However, it is still lacking of the evaluation of brodifacoum toxicity from molecular level.

Recent developments in metabonomics technologies are typically based on gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS) and nuclear magnetic resonance spectroscopy. These techniques provide not only direct information on metabolism, but also the potential reciprocal relationship between metabolic networks and the underlying mechanisms of toxicity. In particular, integration of GC–MS and LC–MS could provide the possibility of determination of broader set of metabolites than using only one technique. Combining multiparallel technologies in metabonomics applications has become indispensable, thus aiming at a comprehensive metabolome coverage [19]. Therefore, a GC–MS and LC–MS/MS method based on a metabonomic strategy was developed to screen and identify metabolic perturbations associated with brodifacoum induced toxicity in rat plasma.

2. Experimental

2.1. Chemical and reagents

Brodifacoum (0.5%) was obtained from the Tianjin Tianqing Chemical Co., Ltd. (Tianjin, China). Water was filtered through the Milli-Q system (Millipore, Molsheim, France). HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Ammonium acetate was purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA), and ethylacetate was of analytical grade. The brodifacoum standard was purchased from AccuStandard (New Haven, CT, USA).

2.2. Animal experiments and sampling

All animal experiments were approved by the Ethics Committee of Institute of Forensic Sciences, Ministry of Justice, PRC, and complied with the relevant national legislation and local guidelines. A total of 40 male Wistar rats weighing approximately 200 ± 20 g were obtained from Shanghai Slac Laboratory Animal Technology Co., Ltd. (Shanghai, China). Handling was performed in accordance with the relevant national legislation and local guidelines. The animals were housed at room temperature from 21 to 23 °C with 40% humidity and under a 12 h light/dark cycle. They were fed with commercial aseptic food and given drinking water ad libitum. After one week of acclimatization, the rats were randomly divided into four groups (n = 10): low-dose (2.7 μg/kg bw/day, 1/100 LD_{50}), middle-dose (6.8 μg/kg bw/day, 1/40 LD_{50}), high-dose (13.5 μg/kg bw/day, 1/20 LD_{50}), and control (tap water) groups. As reported in the literature, the acute oral half-lethal dose(LD_{50}) of brodifacoum is 0.27 mg/kg [20]. Treatments were given daily for 14 consecutive days. The rats were weighed once a week. On the last day, blood samples were collected from the intraorbital angular vein, using EDTA as an anticoagulant, and then divided into two parts: one was used to measure brodifacoum levels by LC–MS/MS and the other one was centrifuged at 3000 rpm for 10 min [21]. The resulting plasma samples were stored at −80 °C prior to metabonomics analysis.

2.3. Sample preparation

Ethyl acetate (3 mL) was added to each blood sample (1.0 mL). After vortexing the mixtures for 1 min, the mixtures were centrifuged at 2500 rpm for 3 min. The organic layer was transferred into 5 mL glass tubes and was evaporated to dryness at 55 °C. The residue was reconstituted in 100 μL of methanol and 5 μL of the residue was used for blood brodifacoum measurement.

To remove proteins and obtain small molecule metabolites, 100 μL of plasma was extracted with methanol containing four chemical standards that allowed the monitoring of exaction efficiency. The extraction mixture was shaken vigorously for 2 min followed by centrifugation to precipitate proteins. The resulting supernatant was covered, split into three aliquots and dried under nitrogen. For UPLC–MS/MS, the dried extract was reconstituted in acidic (formic acid) or basic (ammonium bicarbonate) solvents for positive and negative ionization, respectively. For GC–MS, the dried extract was derivatized (silylation) under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA) [22,23].

2.4. LC–MS/MS condition for blood brodifacoum measure

LC–MS/MS analysis was performed according to our previous work [24]. The blood samples were analyzed on an Acquity UPLC (Waters, Milford, MA, USA) coupled to an API 4000 QTRAP mass spectrometer (Applied Biosystems/MSD Sciex, Toronto, Canada) equipped with an electrospray ionization source. The column used was a Waters XBridge C18 column (50 mm × 2.1 mm i.d., 5 μm) fitted with an end-capped C18 guard column (12.5 mm × 2.1 mm i.d., 5 μm); the analysis was conducted at room temperature with a mobile phase of (A) 10 mM ammonium acetate and (B) methanol. The mobile phase gradient elution was 60% A/40% B (v/v) at 0 min, held at 40% B for 1 min, increased to 85% from 1 to 2 min, held at 85% B until 4.5 min, and returned to 40% B at 5 min. The flow rate was 0.2 mL/min. The 4000 Q TRAP mass spectrometer was operated in the negative ionization mode. The optimized conditions were as follows: ion spray voltage, −3.5 kV; source temperature, 500 °C; nebulizing gas, 60 psi; turbo spray gas, 65 psi; curtain gas, 25 psi; collision-activated dissociation, medium; multiple reaction monitoring (MRM) mode; declustering potential, −95 V. Qualification transitions for brodifacoum were m/z 521.1 > 134.9 and m/z 521.1 > 187.0, and m/z 521.1 > 134.9 was used as a quantification transition.

2.5. Untargeted metabonomic analysis

Each plasma extract was analyzed using a global untargeted mass spectrometry-based platform integrating both GC–MS and UPLC–MS/MS analyses [23,25]. The LC–MS/MS portion of the platform was based on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo-Finnigan LTQ mass spectrometer that consisted of an electrospray ionization (ESI) source and a linear ion-trap (LIT) mass analyzer. The extracts were analyzed using both acidic positive ion-optimized conditions and basic negative ion-optimized conditions in two independent injections using separate Waters ACQUITY UPLC BEH C18 columns (100 mm × 2.1 mm i.d., 1.7 μm) heated to 40 °C. For acidic positive ion-optimized analysis, extracts were reconstituted in 50 μL of water/methanol containing 0.1% formic acid and

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