II.3.5 Bromisovalum

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Introduction

Bromisovalum (α-bromoisovalerylurea, bromovalerylurea, Brovarin) (*Figure 5.1)* has long been being used as a hypnotics or sedative since many years ago. It is not only prescribed as an ethical drug, but also contained in some analgesic- antipyretics and hypnotics being sold as over-the-counter drugs. Because of the easiness of getting it, bromisovalum is one of the most important drugs in poisoning in Japan.

The analysis of bromisovalum is being made by GC [1, 2], GC/MS [3], HPLC [4, 5] and LC/MS [6–8]. Because of its thermolability, HPLC or LC/MS is more recommendable than GC or GC/MS to obtain good reproducibility. In this chapter, three kinds of methods for extraction of bromisovalum from blood and urine and its HPLC analysis are presented.

Reagents and their preparation

- Bromisovalum (Nippon Shinyaku Co., Ltd., Kyoto, Japan, Wako Pure Chemical Industries, Ltd., Osaka, Japan and other manufacturers) is dissolved in methanol to prepare 1 mg/mL standard solution.
- Phenytoin (internal standard, IS[a], Wako Pure Chemical Industries and other manufacturers) is dissolved in methanol to prepare 1 mg/mL standard solution.

HPLC conditions

Column: a reversed-phase column (CAPCELL-PAK C_{18} MG[b], 250 × 3 mm i. d., particle diameter 5 μm, Shiseido, Tokyo, Japan); mobile phase: acetonitrile/8 mM KH_2PO_4 solution (35:65, v/v)[c]; detection wavelength: 210 nm; flow rate: 0.8 mL/min; column temperature: 40 °C.

Figure 5.1

bromisovalum apronalide 2 – bromohexanoylurea[a]

Bromisovalum and its related compounds.

Procedures

i. Extraction with an Extrelut column [9]

i. A 1-mL (or g) aliquot of a specimen (whole blood, serum or urine) is mixed with 5 μL phenytoin solution (IS, 1 mg/mL) and 1 mL of 0.1 M hydrochloric acid solution [d] in a centrifuge tube.
ii. The mixture is vortex-mixed for 10 s.
iii. It is centrifuged (4 °C, 2,500 rpm, 15 min) to obtain a supernatant fraction.
iv. A 2.5-g aliquot of Extrelut[e] (Merck, Darmstadt, Germany) is packed in a glass column (about 15 cm × 15 mm i. d.).
v. The above supernatant fraction is poured into the column and left for 20 min.
vi. Bromisovalum and IS are eluted with 7 mL ethyl acetate; the eluate is evaporated to dryness under a stream of nitrogen.
vii. The residue is dissolved in 100 μL of the mobile phase; a 10-μL aliquot of it is injected into HPLC.

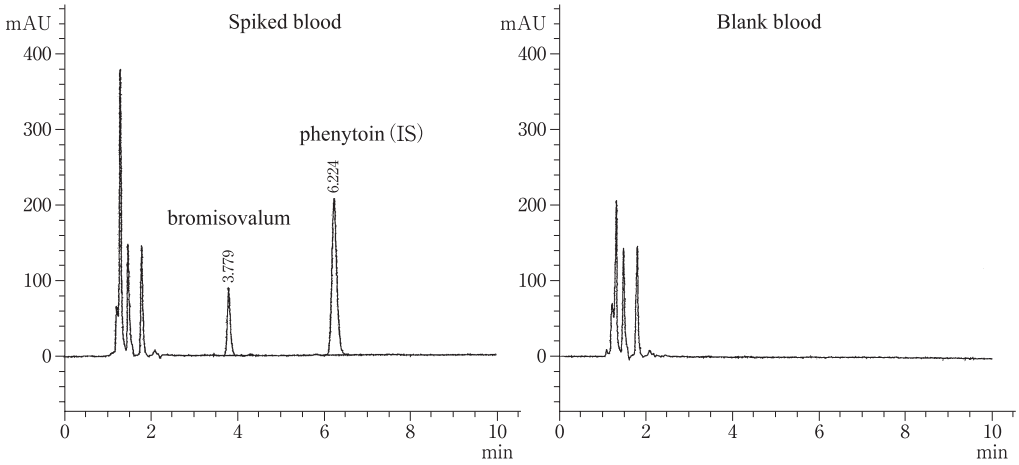
ii. Extraction with a Sep-Pak C_{18} cartridge [1]

i. A 1-mL (or g) aliquot of a specimen is mixed with distilled water (9 mL for a whole blood specimen; 4 mL for serum and urine specimens) and 5 μL phenytoin solution (IS, 1 mg/mL) in a centrifuge tube.
ii. The mixture is vortex-mixed for 10 s.
iii. It is centrifuged (4 °C, 2,500 rpm, 15 min) to obtain a supernatant fraction.
iv. A Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) is activated by passing 5 mL of dichloromethane/methanol (9:1), 5 mL acetonitrile and 10 mL distilled water.
v. The above supernatant fraction is poured into the Sep-Pak cartridge, washed with 10 mL distilled water and eluted with 3 mL of dichloromethane/methanol (9:1).
vi. After removal of a small amount of the upper layer (aqueous phase) of eluate with a Pasteur pipette, the organic eluate is evaporated to dryness under a stream of nitrogen.
vii. The residue is dissolved in 100 μL of the mobile phase, and a 10-μL aliquot is injected into HPLC.

iii. Liquid-liquid extraction [5]

i. A 1-mL (or g) aliquot of a specimen (whole blood, serum or urine) is mixed with 5 μL of phenytoin solution (IS, 1 mg/mL) and 1 mL of 0.1 M hydrochloric acid solution in a centrifuge tube.
ii. A 3-mL volume of *tert*-butyl methyl ether [f] (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan and other manufacturers) is added to the above mixture and vortex-mixed for 2 min.
iii. It is centrifuged (4 °C, 2,500 rpm, 15 min).
iv. The organic phase is transferred to a glass vial, and evaporated to dryness under a stream of nitrogen.
v. The residue is dissolved in 100 μL of the mobile phase, and a 10-μL aliquot is injected into HPLC.

Figure 5.2



HPLC chromatograms for blood extracts in the presence and absence of bromisovalum and IS. The concentration of bromisovalum spiked into whole blood was 5 μg/mL.

Assessment and some comments on the methods

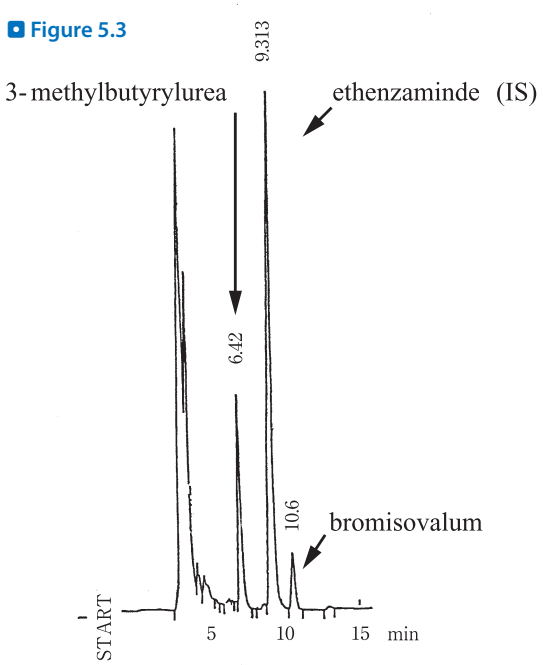
Figure 5.2 shows HPLC chromatograms for whole blood specimens, which had been extracted with an Extrelut column. The peaks of bromisovalum and IS appeared at 3.77 and 6.22 min, respectively, without any interfering peak. The calibration curve showed excellent linearity in the range of 0.1–10 μg/mL; recovery rates were 60–80 %. When ethenzamide is used as IS, it appears before bromisovalum; when 2-bromohexanoylurea is used as IS, it appears after phenytoin. By extraction with Extrelut or Sep-Pak C_{18}, there is occasionally a case in which bromisovalum is not separated from other basic drugs, when they are ingested simultaneously. By the liquid-liquid extraction, the basic drugs can be removed efficiently; but coloration of the organic phase takes place to some extent, when whole blood is analyzed.

Figure 5.3 shows an HPLC chromatogram of the extract of rat plasma obtained 2 h after intraperitoneal administration of bromisovalum (30 mg/kg) [5]. Because of the different HPLC conditions, the retention times were somewhat different; but a de-bromo-metabolite (3-methyl butyrylurea) appeared before ethenzamide.

As over-the-counter drugs containing bromisovalum, Rislon (100 mg bromisovalum per tablet, Sato Pharmaceutical, Tokyo, Japan) and Wutt (83 mg bromisovalum, 50 mg apronalide and 8.3 mg diphenhydramine hydrochloride per tablet, Itami Pharmaceutical, Shiga, Japan) can be mentioned. Apronalide (*Figure 5.1*) contained in Wutt appears at 5.07 min under the present HPLC conditions and thus can be an indicator of ingestion of Wutt.

In some analgesic-antipyretics, bromisovalum is also contained together with acetaminophen and ethenzamide. Therefore, when bromisovalum is detected, various possibilities of concomitant ingestion of other drugs should be taken into consideration.

Figure 5.3



HPLC chromatogram for the extract of rat serum obtained 2 h after intraperitonal injection of bromisovalum (30 mg/kg). HPLC conditions; column: Symmetry Shield RP_{18}, 15 cm × 4.6 mm i. d., particle diameter 3.5 µm, Waters; mobile phase: acetonitrile/8 mM KH_2PO_4 solution (35:65, v/v); detection wavelength: 210 nm; flow rate: 0.4 mL/min.

Toxic and fatal concentrations

Fatal blood bromisovalum concentrations in poisoning with bromisovalum only were reported to be 44.0–93.8 µg/mL by Hishida [10], 67–134 µg/mL by Maguchi [11] and 114 µg/mL by Kojima et al. [12]. In the fatal cases of multiple drug ingestion, blood bromisovalum concentrations were reported to be 37 µg/mL by Terada et al. [13], 23.6 µg/mL by Matsubara et al. [14], and 31.5 and 40.8 µg/mL by Yashiki et al. [15].

Poisoning cases

Many cases of poisoning by bromisovalum were reported. In this section, representative clinical and medicolegal cases are presented.

a) Cases in clinical toxicology [16]

Case 1: a 26-year-old male ingested more than 3 g bromisovalum and his consciousness level was 300 (Japan Coma Scale) on arrival at a hospital. His clinical blood tests were: the maxi-

mum blood bromisovalum concentration, 235 μg/mL; bromide (Br) 1.4 mE/L (on day 2 of admission) and chloride (Cl), 151 mEq/L [g]. The half-life of bromisovalum was 12.6 h; that of bromide 92.7 h. The consciousness levels were in good parallel with blood concentrations of bromisovalum.

Case 2: a 29-year-old female ingested 20.4 g of bromisovalum. The maximum blood bromisovalum concentration was 117.3 μg/mL on arrival at a hospital; the concentration of chloride was 119 mEq/L. Her consciousness levels were improved according to the decrease in the bromisovalum levels. The chloride levels did not correlate with the consciousness levels.

Case 3: a 57-year-old female fell into cardiopulmonary arrest due to asphyxia, but was resuscitated by a rescue squad, and brought to a hospital. A 0.4-g aliquot of bromisovalum had been prescribed for her to sleep. Her blood bromisovalum concentration was 10.1 μg/mL on her arrival to the hospital; chloride concentration 177 mEq/L. Bromide concentrations decreased with a half-life of 58.3 h.

b) Medicolegal cases

Case 1 [12]: a 43-year-old housewife was missing. After 4 days, she was found dead in a shed located in a rice field. Her autopsy findings were: height, 151 cm; weight, 49 kg; mild subcutaneous hemorrhages observable in the chest, abdomen and extremities; and lung edema (left lung 460 g, right lung 440 g). Except these findings, neither severe injuries nor diseases were found. The stomach contents consisted of 14 g of white clayey substance and about 350 mL of aqueous solution. About 350 mL urine was present in her urinary bladder.

Analytical results: 3 g of bromisovalum was detected from the above white clayey substance; about 1 g of the same drug detected from the aqueous solution. Bromisovalum concentrations were 114, 140, 123 and 55 μg/mL or g in blood, the brain, liver and urine, respectively. It was diagnosed that the cause of her death was bromisovalum poisoning.

Case 2 [17]: human skeletal remains were discovered in a bush located in a suburban area of a big city. Next to the remains, three empty bottles, to which labels describing 100 tablets of bromisovalum had been attached, five unopened bottles containing the same tablets and a 1.5-L volume plastic bottle containing about a half volume of water were found. By dental findings, the remains were found to be a 46-year-old male who had been missing for 7 months. Using the femoral bone marrow, the analysis of bromisovalum was conducted by GC/MS and LC/MS. The drug was identified by the methods; its concentrations measured by LC/MS were 93.8 and 26.0 μg/g in the right and left femoral bones, respectively.

Notes

a) As an IS, ethenzamide can be used. For LC/MS analysis of bromisovalum, 2-bromohexanoylurea, showing very similar physicochemical properties, is most suitable as IS [7, 18, 19]. This compound can be easily synthesized with 2-bromohexanoyl bromide and urea: 5 g of 2-bromohexanoyl bromide (Aldrich, Milwaukee, WI, USA) and an equimolar amount of urea are placed in a 100 mL volume eggplant-shaped glass flask and warmed in a water bath to form a soft clay. After warm distilled water is added to the clay, solid sodium bicarbonate is gradually added to the mixture until the solution becomes alkaline with warming; this

procedure results in formation of white crystal powder, which is collected by filtration, and recrystallized in ethanol. The crystals thus obtained show a melting point at 133–135 °C. Kokatsu et al. [3] used 2-bromoisobutyrylurea as IS for analysis of bromisovalum by GC/CI-MS. 2-Bromoisobutyrylurea can be also synthesized by a similar method.

b) The column can be replaced by other reversed phase columns.

c) The pH of this solution is 4.75. When bromisovalum is analyzed by LC/MS, ammonium acetate buffer solution (10 mM ammonium acetate solution adjusted to pH 3.5 with formic acid) should be used as a volatile mobile phase [6].

d) For an old blood specimen, it may clot with strongly acidic solution. In such cases, 10 mM hydrochloric acid solution should be used.

e) The Extrelut powder should be well washed with ethyl ether and dried before use. Without such pretreatment, there is possibility that interfering impurity peaks appear in trace analysis of drugs. As a column for the Extrelut packing, a glass syringe can be used.

f) *tert*-Butyl methyl ether has a boiling point higher than that of diethyl ether, and does not contain peroxide compounds; it is thus suitable as a solvent for extraction.

g) When chloride is measured by the ion selective electrode (ISE) method, chloride may be overestimated in the presence of a high concentration of bromide [20].

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