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Short communication

Determination of plasma bromvalerylurea and its main metabolite by a simple high-performance liquid chromatographic method and quantitation of bromide by energy dispersive X-ray spectrometry in carbon tetrachloride-intoxicated rats

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Abstract

In the present study, small volumes of plasma were used for the measurement of bromvalerylurea (BVU), its metabolite, 3-methylbutyrylurea (MVU), and bromide in carbon tetrachloride (CCl_4)-treated rats by HPLC–UV and energy dispersive X-ray spectrometry. A liquid–liquid extraction system was also investigated. BVU and MVU were extracted from 100 μl plasma samples in a single-step involving deproteination with 1 *M* hydrochloric acid using ethenzamide as internal standard. Samples were separated by HPLC in an acetonitrile–8 m*M* potassium dihydrogenphosphate buffer (35:65, v/v) mobile phase at a flow-rate of 0.4 ml/min on a 15 cm octadecylsilyl column at room temperature. Analytes were detected at a wavelength of 210 nm. The limits of quantitation for BVU, MVU and bromide are 0.1, 0.1 and 50 μg/ml, respectively. The intra-day accuracies over the range of concentrations were 95.8 to 121.1%, 97.2 to 119.7% and 96.2 to 105.8% for BVU, MVU and bromide, respectively. The inter-day accuracies were 97.7 to 115.1%, 98.3 to 111.6% and 98.3 to 102.9% for BVU, MVU and bromide, respectively. The absolute recoveries using *tert*.-butyl methyl ether are 96–98% for BVU and 95–98% for MVU. The decline in the plasma concentrations of BVU in olive oil-treated rats fitted a one-compartment model and the plasma MVU level reached a peak at around 1.5–2 h and then decreased gradually. The elimination of BVU in CCl_4 (1 ml/kg)-treated rats was delayed and MVU production was less than that in the olive oil-treated group. However, there was no difference in the plasma levels of bromide between CCl_4-treated rats and control rats. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Bromvalerylurea (α-bromisovalerylurea, BVU) (Fig. 1) is widely used as a mild hypnotic or sedative drug in Japan, and many acute poisoning (overdose, etc.) and fatal cases (suicide, homicide, etc.) have been observed [1].

At first, BVU is rapidly metabolized by two major routes in the liver: one pathway is the production of

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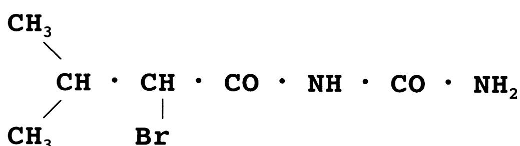

Fig. 1. Structure of bromvalerylurea (α-bromoisovalerylurea).

3-methylbutyrylurea (MVU) by reductive debromination, the other pathway is conjugation by glutathione *S*-transferase in man [2] and animals [3–6]. Recently, Oka et al. [7] have reported that a reconstituted cytochrome P450 (P450 or CYP) system containing NADPH-P450 reductase, and CYP1A1 or CYP2B1 exhibited debrominating activity toward this hypnotic. These results indicate that a P450 system may play an essential role in the microsomal debromination of BVU. The bromide produced by the reductive debromination of BVU has a sedative effect on patients suffering from withdrawal or convulsions [8,9]. There is no report of any changes in BVU metabolism in patients with liver disease.

The determination of BVU and its metabolites is usually carried out by gas chromatography (GC) [10], gas chromatography–mass spectrometry (GC–MS) [11], high-performance liquid chromatography (HPLC) [12,13] and high-performance liquid chromatography–mass spectrometry (LC–MS) [14]. HPLC is the most popular method for determining polar and ionic compounds like BVU and its metabolites.

In the present paper, we report changes in the plasma concentrations of BVU, MVU and bromide in carbon tetrachloride (CCl_4)-treated rats using HPLC–UV (BVU and MVU) and energy dispersive X-ray spectrometry (bromide) in a pre-clinical study.

2. Experimental

2.1. Reagents and materials

The BVU used as a standard was of pharmaceutical grade (Nippon Shinyaku, Kyoto, Japan) Ethenzamide (2-ethoxybenzamide) (internal standard; I.S.) was purchased from Aldrich (Milwaukee, WI, USA). The major metabolite (MVU) of BVU was kindly provided by Dr. H. Tsuchihashi (Osaka Prefectural Police Headquarters, Osaka, Japan). The chemicals, CCl_4 and potassium bromide, were purchased from Wako (Tokyo, Japan). The mobile phase was prepared by mixing deionized water obtained using a Milli-Q system (Millipore, Bedford, MA, USA) and HPLC-grade organic solvent.

2.2. Extraction procedure

One hundred μl I.S. (10 μg/ml), 100 μl 1 *M* hydrochlolic acid and 3 ml *tert*.-butyl methyl ether were added to 0.1 ml plasma (or standard aqueous solution) in 15-ml culture tubes. After vortex mixing for 2 min, the tubes were centrifuged at 1200 *g* for 5 min and the aqueous phase removed by aspiration. The organic phase was transferred to a clean conical tube and evaporated in a water bath at about 40°C under a gentle stream of nitrogen. The residue was dissolved in 100 μl mobile phase and 50 μl injected into the HPLC apparatus.

2.3. Standard solutions and calibration

A standard stock solution containing BVU and its major metabolite, MVU, was prepared in methanol at a concentration of 1 mg/ml of each compound and this remained stable for at least 2 months at −20°C. Plasma standards were prepared containing 0.1, 0.5, 1, 5 and 10 μg/ml of each compound by diluting appropriate aliquots of stock solution with drug-free plasma. A calibration curve was obtained by linear regression of the peak-height ratio versus concentration. The plasma concentration of bromide was also measured following preparation of solutions containing 10, 50, 100, 500 and 1000 μg/ml and a calibration curve was obtained by linear regression.

2.4. Apparatus

The HPLC equipment consisted of a pump (Model CCPS, Tosho, Tokyo, Japan) and a variable-wavelength UV detector (Model UV-8020, Tosho). The separation was achieved using a C_{18} reversed-phase column (Symmetry Shield RP_{18}, 15 cm×4.6 mm I.D., 3.5 μm; Waters). The mobile phase was composed of acetonitrile–8 m*M* potassium dihydrogen-

phosphate buffer (35:65, v/v) and the flow-rate was 0.4 ml/min. The absorbance of the eluent was monitored at 210 nm. All instruments were operated at ambient laboratory temperature (ca. 23°C).

The levels of bromide in 200 μl plasma at each time period were measured using potassium bromide as a standard by the energy dispersive X-ray spectrometry (Model EDX-700, Shimadzu, Kyoto, Japan). The sample was placed on special filter paper (ST-30) attached to an O-ring.

2.5. Animal study

Male Sprague–Dawley (250–300 g) rats, 8–9 weeks of age, were obtained from CLEA Japan (Tokyo, Japan). The rats were kept in an air-conditioned room (25±1°C, 50–60% humidity) with a 12-h light–dark cycle (08:00–20:00) and given free access to commercial rat chow (Oriental-MF, Tokyo, Japan) and water.

The rats were fasted overnight in the metabolic cage, given free access to water and injected intraperitoneally with 30 mg/kg BVU. Normal control rats were given olive oil (2 ml/kg). CCl_4 (1 ml/kg) was dissolved in olive oil and administered orally 24 h prior to the oral administration of BVU. Blood samples (about 0.4 ml) were obtained from the jugular vein at 0.25, 0.5, 1, 1.5, 2 and 4 h after intraperitoneal administration of BVU.

2.6. Data analysis

2.6.1. Pharmacokinetics

To obtain the pharmacokinetic parameters for BVU, the elimination half-life ($t_{1/2}$) was calculated as $t_{1/2}=0.693/K$, where K is the elimination rate constant, as assessed by applying logarithmic regression analysis to the terminal portion of the serum concentration profile. The area under the serum concentration–time curve (AUC) was determined up to 4 h and was extrapolated to infinity using K. The total body clearance (Cl) of BVU was determined as Cl=Dose/AUC. The apparent volume of distribution (V_d) was calculated from $V_d=\mathrm{Cl}/K$.

2.6.2. Statistical analysis

All data is represented as means±SD (RSD, %). Statistical analysis was performed using the unpaired Student's *t*-test. Values were considered to be significantly different if $p<0.05$.

3. Results and discussion

3.1. Retention time

Fig. 2 shows chromatograms of BVU and its three metabolites separated using the C_{18} reversed-phase column. The retention times of MVU, I.S. and BVU were approximately 6.4, 9.4 and 10.6 min, respectively. BVU, MVU and I.S. were well separated. No interfering peaks appeared due to any endogenous material eluted after the drug. In this study, we did not detect the other two metabolites, [α-(cystein-S-yl)isovalerylurea (CVU) and α-(*N*-acetylcystein-S-yl)isovalerylurea (AcCVU)], because they would coelute with endogenouus interferences at the start of the chromatogram in any of the extracted plasma samples as shown in Fig. 2 (3) and (4).

3.2. Limits of quantification and concentration range

A wide range of concentrations was used for the analysis; 0.1–10 μg/ml for BVU and MVU and 50–1000 μg/ml for bromide. These concentration ranges represent blood concentrations commonly measured in pharmacokinetic studies and are also relevant in a clinical laboratory setting. Within each range, five different concentrations were measured.

The limit of quantification of BVU, MVU and bromide is the lowest concentration on the standard curve which can be measured with acceptable accuracy (a relative standard deviation, RSD, <5%). The lowest practical limit of quantification was 0.1 μg/ml for BVU and MVU, while the corresponding concentration of bromide was 50 μg/ml. This method was fivefold and twofold more sensitive than that for BVU reported by Matsubara et al. [12] and Miyauchi et al. [13], respectively.

3.3. Precision

The intra-day variability was determined by

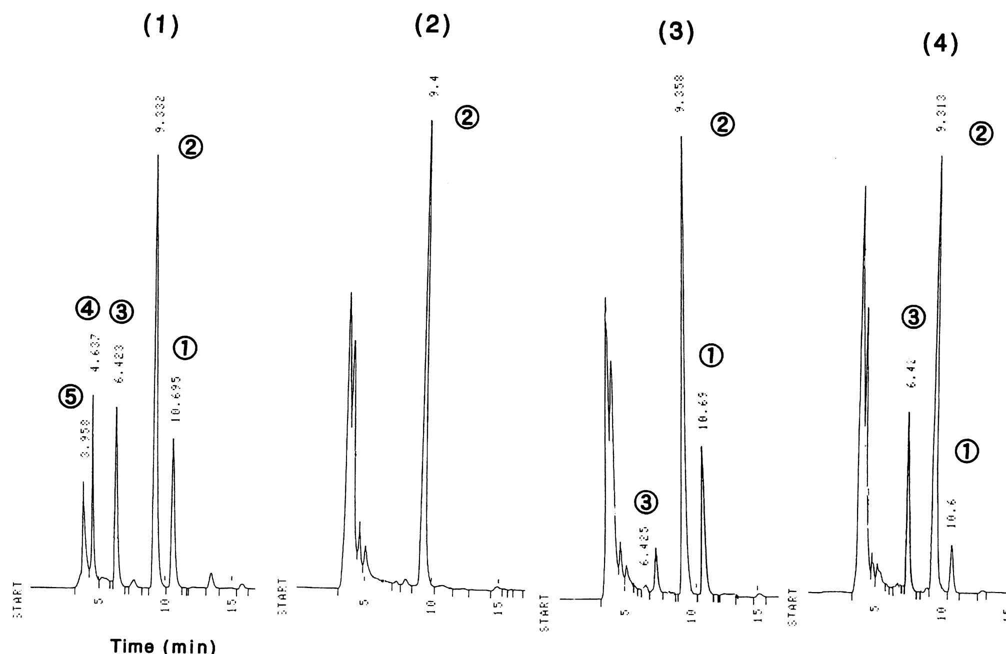

Fig. 2. Chromatograms of bromvalerylurea and its three metabolites extracted from rat plasma. Rat plasma spiked with AcCVU, CVU, MVU, I.S. and BVU. The concentrations are 10 μg/ml for MVU, I.S. and BVU. (2) Blank rat plasma. (3) Rat plasma obtained 2 h after intraperitoneal administration of BVU (30 mg/kg) to CCl_4-treated rats. (4) Rat plasma obtained 2 h after intraperitoneal administration of BVU (30 mg/kg) to normal control. Column: 150 mm×4.6 mm I.D., particle size 3.5 μm (RP_{18}), mobile phase: acetonitrile–8 m*M* potassium dihydrogenphosphate buffer (35:65, v/v), flow-rate: 0.4 ml/min, detection wavelength: 210 nm. All instruments and the column were operated at ambient laboratory temperature (ca. 23°C). 1=Bromvalerylurea (BVU), 2=ethenzamide (I.S.), 3=3-methylbutyrylurea (MVU), 4=α-(cystein-S-yl)isovalerylurea (CVU), 5=α-(*N*-acetylcystein-S-yl)isovalerylurea (AcCVU).

analyzing four sets of standards on the same day. For the determination of inter-day variability, a single set of standards was assayed on 3 consecutive days. Both the intra-day and inter-day variability were evaluated by spiking drug-free rat serum with five different concentrations of BVU and MVU, and calculating the RSD for each measurement. Over the range of concentrations of each compound, the intra-day RSDs were 1.8 to 9.6%, 1.3 to 9.8% and 1.5 to 9.7% for BVU, MVU and bromide, respectively. The inter-day RSDs were 1.4 to 8.5%, 0.9 to 8.8% and 1.2 to 8.5% for BVU, MVU and bromide, respectively.

3.4. Accuracy

Analytical accuracy was evaluated by measuring the variation between the added concentration and the measured concentration for the three analytes. The intra-day accuracy over the range of concentrations was 95.8 to 121.1%, 97.2 to 119.7% and 96.2 to 105.8% for BVU, MVU and bromide, respectively. The inter-day accuracy was 97.7 to 115.1%, 98.3 to 111.6% and 98.3 to 102.9% for BVU, MVU and bromide, respectively.

3.5. Absolute recovery

The absolute recovery using *tert*.-butyl methyl ether, ether [15] and ether–petroleum ether (1:1, v/v) [16] was measured at concentrations of 0.1, 0.5, 1, 5 and 10 μg/ml for BVU and its three metabolites. Plasma samples at each concentration were extracted and injected. Four samples of the same amount of compound in methanol were directly

injected and the concentrations were calculated. The absolute recovery was calculated by comparing the concentrations for direct injection of pure compounds with those for plasma samples. The absolute recoveries was 96–98% for BVU and 95–98% for MVU. The RSD ranged from 2.5 to 4.4%.

tert.-Butyl methyl ether was chosen as the extraction solvent (indicated absolute recoveries 95–98% for BVU and MVU) because the extraction efficiency was greater than that of ether or ether-petroleum ether (90–92% for BVU and MVU).

3.6. Linearity

The calibration curves (the ratio between the peak-height of the drugs analyzed and that of the I.S. versus amount of BVU and MVU) were obtained over the concentration range 0.1–10 μg/ml plasma. The r values for the curve were: $r=0.998$ for BVU ($y=0.04x-0.005$); $r=0.999$ for MVU ($y=0.05x-0.005$). The calibration curve of bromide was obtained over the concentration range 10–1000 μg/ml plasma. The r value for the curve was $r=0.997$ for bromide ($y=1.25x+11$). All the standard curves were linear with a correlation coefficient above 0.997 for each analyte, whether measured within days or between days. The slope of the peak-height ratio versus concentration curve showed little variability with an RSD of less than 3%, indicating good reproducibility. In addition, the retention time RSDs for all three analytes were between 0.20 and 0.47%.

As can be seen from the results of the intra-day and inter-day analyses, the present assay provides reasonable repeatability, precision, accuracy and recovery over the concentration range tested. In addition, this concentration range corresponds to the levels often seen in pharmacokinetic studies (the toxic plasma level of BVU is about 30–110 μg/ml) [12,17–19].

3.7. Animal study

Application of the present assay to determine the pharmacokinetics of BVU and MVU is shown in Table 1.

The BVU in the plasma of olive oil-treated rats was metabolized according to a one-compartment model and the concentration of the metabolite, MVU, reached a peak at around 1.5–2 h and then decreased gradually. The elimination of BVU in CCl_4 (1 ml/kg)-treated rats was delayed and MVU production in the CCl_4-treated group was less than in the olive oil-treated group (Fig. 3, Table 1). As shown in Fig. 3, however, the plasma levels of bromide were similar in CCl_4-treated rats and control rats.

Bromide is distributed predominantly in the extracellular fluid, and its excretion is very slow and

Table 1
Pharmacokinetic parameters following the intraperitoneal administration of bromvalerylurea to CCl_4-treated rats

Treatment	Control	CCl_4-treatment
$t_{1/2}$ (h)	0.86±0.37	1.25±0.23*
Cl (ml/min/kg)	1.57±0.31	1.12±0.33*
V_d (l/kg)	1.95±0.45	1.94±0.53

The rats were injected intraperitoneally with 30 mg/kg bromvalerylurea (BVU). Normal control rats were given olive oil (2 ml/kg). CCl_4 (1 ml/kg) was dissolved in olive oil and administered orally 24 h prior to the oral administration of BVU. $t_{1/2}$= elimination half-life, V_d=apparent volume of distribution, Cl= total body clearance, $n=4$, mean±SD, $*p<0.01$.

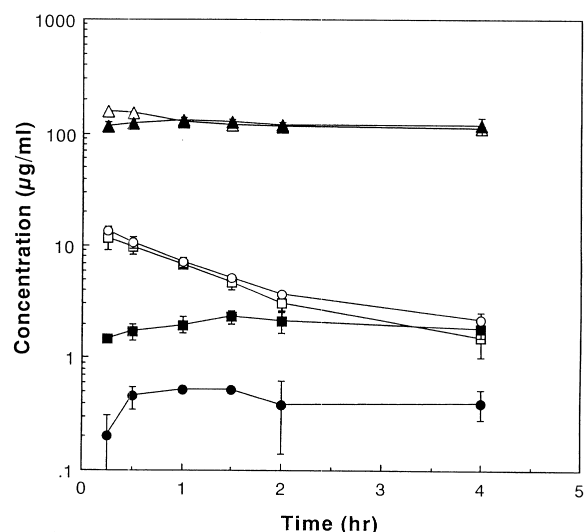

Fig. 3. Plasma concentration–time curves of bromvalerylurea and its major metabolite, 3-methylbutyrylurea, and bromide in rats. Bromvalerylurea: □=control, ○=CCl_4-treatment. 3-Methylbutyrylurea: ■=control, ●=CCl_4-treatment. Bromide: △=control, ▲=CCl_4-treatment.

solely by the renal tubules [20]. Maguchi [21] reported that the concentration of bromides in body fluids in acute intoxication ranged from 20 to 50 μg/ml, indicating that the BVU concentration was in the range of 55.8 to 140 μg/ml. Essen et al. [22] also found that the plasma BVU concentration of a patient admitted to hospital for poisoning was 90 μg/ml.

Recently, the technique of LC–MS was developed [14]. The detection limit of BVU and MVU using this method was 10 ng/ml. The sensitivity of our method is lower than this LC–MS method, and the sample volume is smaller. The sample volume used in our study is less than that in other reports (3–5 ml) [12–14]. In addition, the retention times of BVU and MVU in our method were shorter than those in the LC–MS method (about 20 min). This equipment is very expensive and, therefore, it is not available in many laboratories. The advantages of our HPLC procedure are as follows: (1) small sample volume; (2) no gradient elution required; (3) separation at room temperature; (4) the entire HPLC run requires ca. 10 min for each sample.

In this paper, we report the change in the plasma concentrations of BVU, MVU and bromide using small sample volumes in CCl_4-treated rats after BVU administration. We found that the production of MVU in CCl_4-treated rats was reduced compared with control rats, but the bromide level was unchanged.

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