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Simultaneous determination of bromvalerylurea and allylisopropylacetylurea in human blood and urine by gas chromatography–mass spectrometry

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Abstract

We devised a sensitive and simple method to simultaneously determine bromvalerylurea and allylisopropylacetylurea in human blood and urine by gas chromatography–mass spectrometry. Bromvalerylurea and allylisopropylacetylurea were extracted using an Extrelut® column with an internal standard, 2-bromohexanoylurea, followed by derivatization with heptafluorobutyric anhydride. The derivatized extract was submitted to GC–MS analysis of EI-SIM mode. The calibration curves of both compounds were linear in the concentration range from 0.01 to 10 μg/ml in both blood and urine samples. The lower limits of detection of bromvalerylurea and allylisopropylacetylurea were 0.005 and 0.005 μg/ml, respectively. This method proved most useful in accurately identifying these drugs in blood and urine from an autopsied individual.

Keywords: Bromvalerylurea; Allylisopropylacetylurea

1. Introduction

Bromvalerylurea (bromisovalum, α-bromoisovalerylurea) and allylisopropylacetylurea (Fig. 1) are widely used in Japan as sedatives and hypnotics, and are also used for analgesic and antipyretic properties. As these drugs are readily available at drug stores and as poisoning by these drugs has occurred frequently in Japan, toxicological analyses are needed.

Determination of bromvalerylurea in human sam-

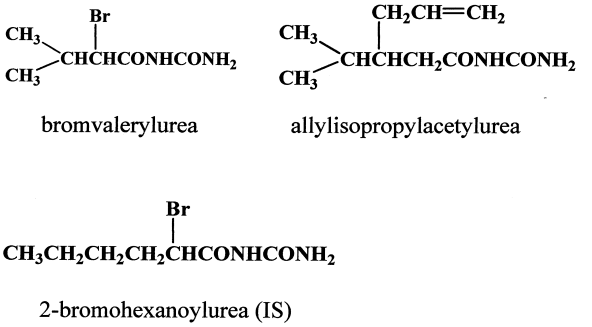

Fig. 1. Chemical structures of bromvalerylurea, allylisopropylacetylurea and 2-bromohexanoylurea (I.S.).

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ples had been done using gas chromatography (GC) [1] and gas chromatography–mass spectrometry (GC–MS) [2,3] with use of a packed or wide bore capillary column. However, GC methods have drawbacks such as thermal instability and decomposition of the drug, and special care was required for choosing the column temperature. When a narrow bore capillary column was used with similar conditions, it gave only decomposed peaks, in addition to thermal instability.

High-performance liquid chromatography (HPLC) with UV detection [4–7] is therefore used in toxicological laboratories. Although this technique provides a stable analysis of bromvalerylurea, the specificity was unsatisfactory compared with findings using GC–MS. As bromvalerylurea is rapidly metabolized and quickly disappears in the blood circulation, detection of the drug using HPLC can be made only when a large amount of the drug has been ingested and if sample collections are taken at an early stage. Therefore, the proof of taking this drug was often unsuccessful, especially in forensic cases.

To overcome these problems, several LC–MS methods with particle beam [8] and frit-FAB [9,10] interfaces have been presented for the analysis of bromvalerylurea. However, such equipment is not always available in many laboratories.

Furthermore, there is only one HPLC method reported for the analysis of allylisopropylacetylurea, the sensitivity of which is not so high [4].

Okada and Ohashi [11] reported a highly sensitive GC method to examine bromvalerylurea after derivatization of the drug by trifluoroacetic anhydride. We have now made use of their acylation technique and developed an even more sensitive and selective GC–MS method for the simultaneous determination of bromvalerylurea and allylisopropylacetylurea in human blood and urine.

2. Experimental

2.1. Reagents

Bromvalerylurea was obtained from Nippon Shinyaku (Osaka, Japan) and allylisopropylacetylurea was provided by Itami Seiyaku (Imazu, Japan). 2-Bromohexanoyl bromide was purchased from Aldrich (Milwaukee, WI, USA). Extrelut® NT Refill Pack was purchased from Merck (Darmstadt, Germany). The powder (2.0 g each) was packed in a 1.5 cm diameter glass column, and each sample was directly applied to the column without any conditioning steps. Heptafluorobutyric anhydride was purchased from Wako Pure Chemical Industries (Osaka, Japan). Ethyl acetate, purchased from Wako, was distilled prior to use. The other chemicals were of analytical reagent grade.

2.2. Biological samples

Whole blood and urine samples obtained from healthy volunteers were kept at −20 °C until analysis.

2.3. Preparation of internal standard (I.S.)

2-Bromohexanoylurea previously synthesized in our laboratory as I.S. for the analysis of bromvalerylurea by particle beam LC–MS was also used in this study [8].

2.4. Standard solutions

Bromvalerylurea and allylisopropylacetylurea (5.0 mg each) were dissolved in methanol and the volume was adjusted to 5 ml, to obtain a concentration of 1000 ng/μl. This solution was further diluted in methanol to 100, 10 and 1 ng/μl. 2-Bromohexanoylurea (5.0 mg) was dissolved in methanol, in the same manner.

2.5. Extraction and derivatization procedure

The method of extracting bromvalerylurea reported by Okamoto et al. [12] was modified as follows. One gram of whole blood or urine sample was mixed with 1 ml of 0.01 *M* hydrochloric acid and 1 μl I.S. solution (1 μg 2-bromohexanoylurea) in a centrifuge tube (10 ml). The mixture was vortex-mixed and centrifuged at 850 *g* for 10 min. The supernatant was applied to an Extrelut® NT column. After standing for 20 min, bromvalerylurea,

allylisopropylacetylurea and I.S. were eluted with 7 ml of ethyl acetate. The eluate was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl of ethyl acetate, and 20 μl of heptafluorobutyric (HFB) anhydride were added to the solution for HFB derivatization. The mixture was kept at 60 °C for 30 min. The mixture was gently evaporated under a stream of nitrogen and just before the complete evaporation of the mixture 70 μl of ethyl acetate were added. A 2-μl aliquot of the solution was injected onto a GC–MS apparatus.

2.6. GC–MS conditions

The apparatus used was a Hewlett-Packard 5989A GC–MS system. A HP-1 fused-silica capillary column (12 m×0.2-mm I.D., 0.33-μm film thickness) coated with 100% dimethylpolysiloxane stationary phase was used. Splitless injection mode was selected with a valve off-time of 2 min. The GC–MS conditions were as follows: the initial temperature 60 °C was held for 2 min, the temperature was programmed to 200 °C at a rate of 20 °C/min, then to 300 °C at a rate of 30 °C/min; this temperature being maintained for 1 min. Injection port and transfer line temperatures were set at 130 and 280 °C, respectively. Helium with a flow-rate of 1 ml/min was used as the carrier gas.

The selected ion monitoring (SIM) mode was used. The following ions were used as quantifier and qualifier ions: m/z 335 and 162 for bromvalerylurea, m/z 295 and 96 for allylisopropylacetylurea and m/z 335 and 254 for I.S., respectively.

2.7. Preparation of calibration curves

Whole blood and urine samples were prepared so as to contain bromvalerylurea and allylisopropylacetylurea at concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/ml, each containing 1 μg I.S. These samples were extracted in the same manner as described above. The calibration curve was obtained by plotting the peak-area ratio of bromvalerylurea derivative (or allylisopropylacetylurea derivative) to I.S. derivative versus the amount of bromvalerylurea (or allylisopropylacetylurea).

3. Results and discussion

3.1. Extraction procedure

Several procedures using single solvent [5], Sep-Pak® cartridge [1] and Extrelut® columns [12] have been reported for extraction of bromvalerylurea. We first extracted bromvalerylurea and allylisopropylacetylurea with methylene chloride or *tert.*-butyl methyl ether under acidic conditions. However, colorless extracts were not obtained and many interfering peaks appeared on the chromatogram, especially for the whole blood samples. This problem was overcome using an Extrelut® NT column. The colorless clean extracts gave sharp and symmetrical peaks of bromvalerylurea and allylisopropylacetylurea.

3.2. Derivatization procedure

Trifluoroacetylation (TFA) and heptafluorobutylation (HFB) were examined for the derivatization of bromvalerylurea and allylisopropylacetylurea. As HFB derivatives gave sharper peaks than did TFA derivatives for both compounds, HFB derivatization was selected.

3.3. Selection of internal standard

To obtain a satisfactory reproducibility for determination of bromvalerylurea and allylisopropylacetylurea, the use of I.S. of a similar structure to these drugs is essential. The I.S. used in this study, 2-bromohexanoylurea, had similar properties with those of bromvalerylurea and allylisopropylacetylurea, on the extraction, separation and mass spectrometric analysis. Therefore, this I.S. was considered to be suitable for the determination of bromvalerylurea and allylisopropylacetylurea by GC–MS.

3.4. Determination of bromvalerylurea and allylisopropylacetylurea in whole blood and urine by GC–MS

EI mass spectra of HFB derivatives of bromvalerylurea, allylisopropylacetylurea and I.S. gave major fragment ions at m/z 335, 333, 254, 162 for bromvalerylurea, m/z 295, 169, 96, 81 for allyliso-

propylacetylurea and m/z 335, 333, 254, 178 for I.S. Each ion was examined, and ions of m/z 335, 295 and 335 were selected as quantifier ions and those of m/z 162, 96 and 254 were selected as qualifier ions for bromvalerylurea, allylisopropylacetylurea and I.S., respectively.

Fig. 2 shows SIM chromatograms of derivatized extracts from whole blood spiked with 1 μg/ml each of bromvalerylurea, allylisopropylacetylurea and I.S. and from blank whole blood. Each peak was clearly separated on the chromatograms with retention times of 4.97, 4.82 and 5.67 min, respectively. There were no interfering peaks on the chromatograms of blank samples. In urine samples the same chromatograms were obtained as shown in Fig. 3. The calibration curves were linear in the concentration range from 0.01 to 10 μg/ml in whole blood and urine samples. Linear regression analyses gave the equations, $y = 1.1265x - 0.1079$ (blood) and $y = 0.991x + 0.055$ (urine) for bromvalerylurea, and $y = 1.0256x - 0.0121$ (blood) and $y = 0.9112x + 0.0448$ (urine) for allylisopropylacetylurea with the correlation coefficients exceeding 0.99 (x = the analyte concentration (μg/ml), y = peak area ratio). The lower limits of detection for bromvalerylurea and allylisopropylacetylurea in whole blood and urine samples, at a signal-to-noise ratio of 3, were 0.005 μg/ml for bromvalerylurea, and 0.005 μg/ml for allylisopropylacetylurea. The sensitivity was ~20 times higher than that seen using the HPLC method [4,5] for bromvalerylurea. The absolute recoveries of bromvalerylurea and allylisopropylacetylurea in whole blood and urine at two different concentrations, 0.1 and 1 μg/ml, were determined by comparing the peak areas of derivatives of bromvalerylurea or allylisopropylacetylurea in samples with those in standard solutions. The calculated recoveries for bromvalerylurea and allylisopropylacetylurea were 51.4% and 60.3% in whole blood and 36.6% and 29.2% in urine, respectively. Within-day precision of

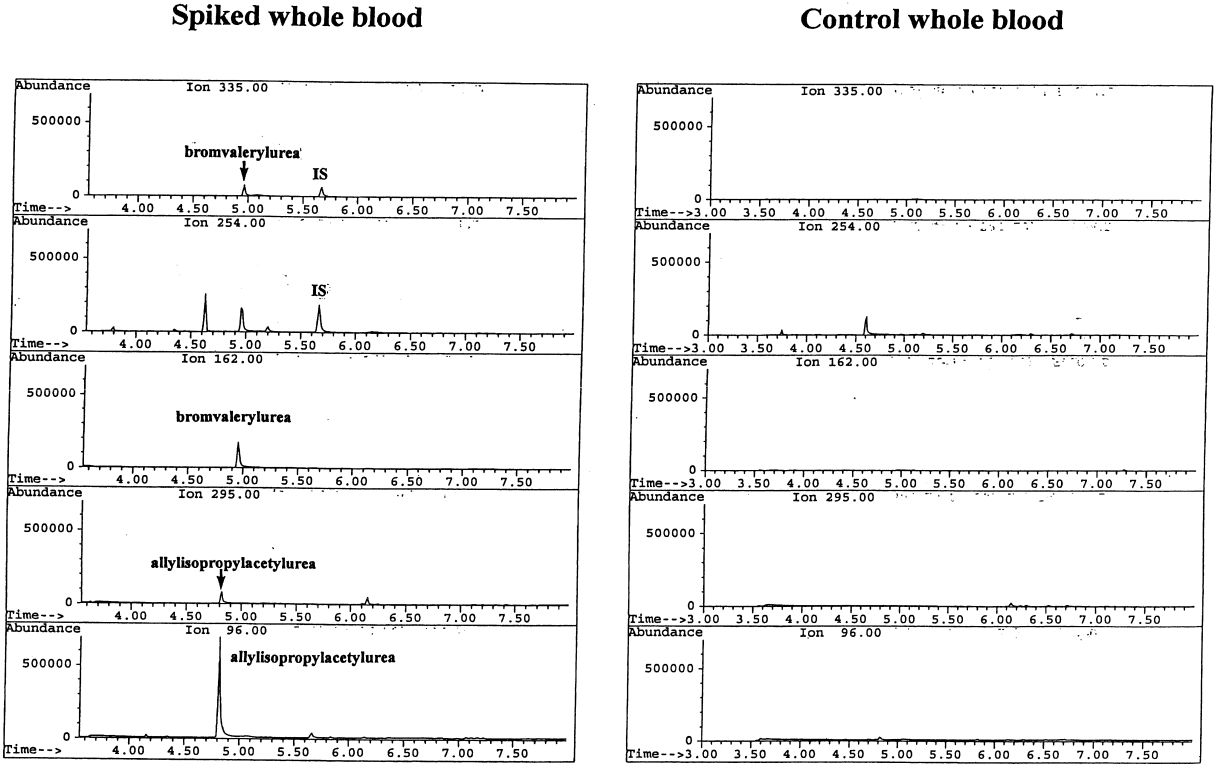

Fig. 2. SIM chromatograms of derivatized extracts from whole blood spiked with 1 μg/ml each of bromvalerylurea, allylisopropylacetylurea and I.S. (left), and from control whole blood (right).

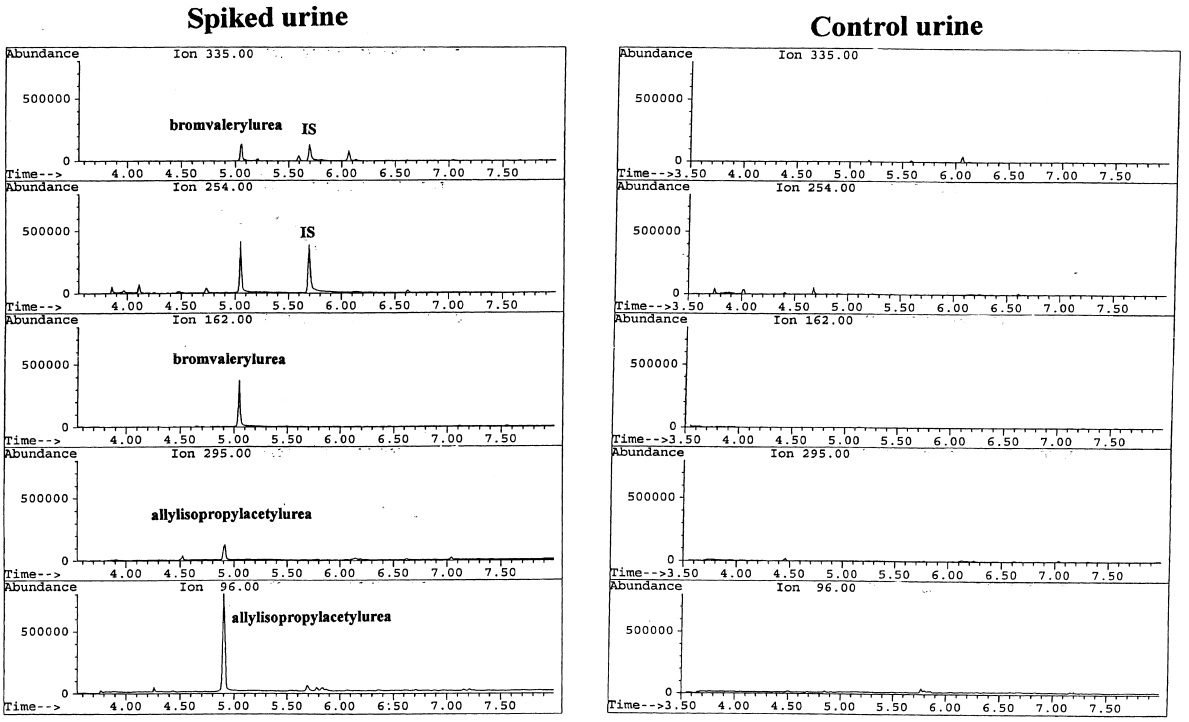

Fig. 3. SIM chromatograms of derivatized extracts from urine spiked with 1 μg/ml each of bromvalerylurea, allylisopropylacetylurea and I.S. (left), and from control urine (right).

this method in whole blood and urine at concentrations of 0.1 and 1 μg/ml is summarized in Table 1. The relative standard deviations ($n=5$) were 2.0 to 8.2% for bromvalerylurea and 4.4 to 14.9% for allylisopropylacetylurea.

4. Practical application

We used this method to accurately identify bromvalerylurea and allylisopropylacetylurea in whole blood and urine from an autopsied individual who was found dead in a car after being missing for about 3 days. There was no evidence of drug intake in the car. As GC–MS screening revealed the presence of diphenhydramine from a urine sample, ingestion of over-the-counter sedative (WUTT®) containing bromvalerylurea, allylisopropylacetylurea and diphenhydramine was suspected and HPLC analysis was carried out. Although we found a large peak with retention time similar to allylisopropylacetylurea in a blood sample, interfering peaks overlapped the peak of bromvalerylurea in both

Table 1
Precision and accuracy of the method. Within-day variations for bromvalerylurea and allylisopropylacetylurea in human samples (RSD (%), $n=5$)

Samples	Concentration (μg/ml)			
	Bromvalerylurea		Allylisopropylacetylurea	
	0.1	1	0.1	1
Whole blood	8.2	4.2	14.9	9.7
Urine	6.1	2.0	11.5	4.4

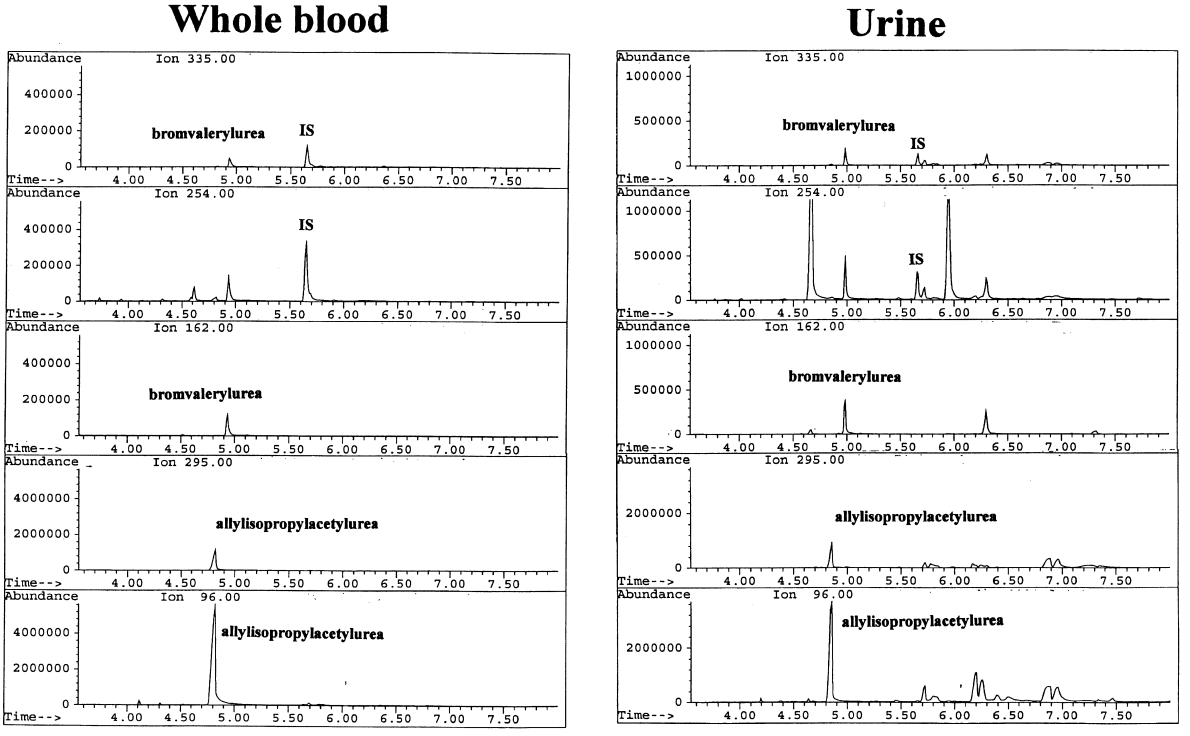

Fig. 4. SIM chromatograms of derivatized extracts from whole blood and urine samples of a man who was found dead in a car.

whole blood and urine samples, and identification of these drugs by HPLC was not feasible.

Fig. 4 shows the GC-SIM chromatograms of the derivatized extracts from whole blood and urine samples of the cadaver. As shown in Fig. 4, both drugs were clearly detected without any interfering peaks: the concentrations were 0.81 and 5.5 μg/ml for bromvalerylurea, and 41.4 and 39.8 μg/ml for allylisopropylacetylurea in whole blood and urine, respectively. Thus intake of these drugs was confirmed.

5. Conclusions

We developed a highly sensitive and selective GC–MS method for the simultaneous determination of bromvalerylurea and allylisopropylacetylurea in human blood and urine. This method should be useful in cases where a small dose is taken orally or samples are collected for testing even after a long time.

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