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NEW BROMISOVAL (BROMURAL) METABOLITES IN HUMAN URINE: α-(CYSTEIN-S-YL)ISOVALERYLUREA, α-(N-ACETYLCYSTEIN-S-YL)ISOVALERYLUREA AND α-(CYSTEAMIN-S-YL)ISOVALERIC ACID

A. NIEDERWIESER*, B. STEINMANN** and ANA MATASOVIC
Medizinisch-Chemische Abteilung, Universitäts-Kinderklinik, CH-8032 Zürich (Switzerland)
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SUMMARY

Large amounts of α-(N-acetylcystein-S-yl)isovalerylurea (AcCVU) and α-(cystein-S-yl)isovalerylurea (CVU) and trace amounts of isovalthine (Ivt), N-acetylisovalthine (AcIvt) and α-(cysteamin-S-yl)isovaleric acid (CAVA) were found in human urine after administration of α-bromoisovalerylurea (Bromisoval, Bromural).

AcCVU and CVU were isolated from urine and identified by gas chromatography–mass spectrometry (GC–MS) as trimethylsilyl (TMS) derivatives, by MS of the methyl esters, by chemical degradation and by NMR (CVU only), IR and chromatographic comparison with synthetic reference compounds. Ivt, AcIvt and CAVA were identified by co-chromatography with pure reference compounds in several chromatographic systems. Syntheses of CVU, βCVU, AcCVU and CAVA are described.

CVU is hydrolysed under mildly alkaline conditions to give urea and a cyclic intermediate, 2-isopropyltetrahydro-1,4-thiazin-3-one-5-carboxylic acid (CITO), which was identified by GC–MS as the bis-TMS derivative. Under more drastic alkaline or acidic conditions, CITO, CVU and AcCVU are hydrolysed to give Ivt, an acidic sulphur amino acid.

Urinary excretion of CVU was measured quantitatively on an amino acid analyser. The sum of AcCVU and AcIvt was measured as N-acetylalanine (after desulphuration of the sample) by selected ion monitoring using isobutane chemical ionization and trideutero-AcCVU as an internal standard. After a single oral dose of 0.9 g of Bromisoval, two healthy adults excreted in urine 64 and 43 mole-%, respectively, in the form of the mercapturic acids AcCVU + AcIvt (mainly AcCVU, with only trace amounts of AcIvt) and 4.6 and 5.9 mole-% as CVU within 24 h. The excretion rate was maximal at about 5 h after drug intake with concentrations up to 12.5 mmole/l of AcCVU (+AcIvt) and 1.1 mmole/l of CVU found in a 3–6-h urine sample.

Hence, the dominating metabolic pathway of the xenobiotic Bromisoval is debromination by liver glutathione-S-transferases. The corresponding glutathione and cysteinylglycine derivatives can be expected to be present in bile.

* To whom correspondence should be addressed.
** Present address: Laboratory for Developmental Biology and Anomalies, NIH, Bethesda, Md. 20014, U.S.A.

We detected the urinary Bromisoval metabolites by chance in a woman who claimed not to have taken any drugs. It is possible that the freely available sedative α-bromoisovalerylurea is present in mixtures that are not signified as being drugs. Our findings make necessary the re-investigation of Japanese studies on isovalthinuria, which has been claimed to be induced by many compounds related to the cholesterol metabolism.

INTRODUCTION

During a study of sulphur amino acid metabolism in the mother of a cystathioninuric girl[1], several unknown compounds were detected in urine. Although she stated that she had not taken any drugs, it was proved by loading tests on normal controls that the compounds, mainly α-(cystein-S-yl)isovalerylurea (CVU) and its N-acetyl derivative, were metabolites of Bromisoval (α-bromoisovalerylurea), a freely available sedative and hypnotic, known since 1906[2]. As far as we know, these metabolites have not previously been described in a human, although they constitute at least about 70 mole-% of the metabolites. They indicate a glutathione (mercapturic acid) pathway of debromination and elimination, and may be of interest for the identification of Bromisoval intoxications. In this paper we describe the isolation from human urine, identification and properties of several of these metabolites, and loading tests with Bromisoval.

MATERIALS AND METHODS

Gas chromatography–mass spectrometry (GC–MS) was performed on a Micromass F-16 mass spectrometer (Vacuum Generators Micromass Ltd., Winsford, Great Britain) combined with a Carlo Erba Model 2101 AC gas chromatograph, over a jet separator. IR spectra were measured on a Perkin-Elmer Model 257 instrument and NMR spectra on a Varian XL-100. Quantitative amino acid analyses were performed on a Biocal Biochrom analyser using Aminex A-6 resin.

Ion-exchange chromatography was performed on Dowex 50W-X8 (200–400 mesh) with volatile buffers[3] and adsorption chromatography on Porapak Q (325 mesh) (Waters Assoc., Milford, Mass., U.S.A.), as previously described[3,4]. A Camag (Muttenz, Switzerland) apparatus was used for high-voltage electrophoresis (HVE) at 4000 V and 70 mA on Schleicher & Schüll paper No. 2043B with buffer of pH 1.9 (acetic acid–formic acid–water, 120:26:850, v/v/v) and buffer of pH 4.0 (0.05 *M* pyridinium acetate). Thin-layer chromatography (TLC) was performed on ready-to-use plates (Merck, Darmstadt, G.F.R.) in a trough-chamber with chamber saturation. Chromatograms (HVE and TLC) were stained for sulphur compounds with iodoplatinate[5,6] and a ninhydrin–copper–collidine reagent[7].

Hydrochloric acid (2 *M*) in methanol was prepared by addition of the appropriate amount of acetyl chloride to methanol at 0° and storing the mixture for at least 2 h at room temperature. Hexadeuteroacetic anhydride and trideuteromethanol were purchased from Stohler Isotope Chemicals (Innerberg, Switzerland) and Merck.

Trifluoroacetylation was performed with trifluoroacetic anhydride–methylene

chloride (4:1, v/v) at 60° for 15 min. Trimethylsilylation was performed with bistrimethylsilyltrifluoroacetamide–acetonitrile (1:1, v/v) at 100–130° for 1–4 h.

Isovalthine was prepared in a similar manner as in ref. 8, by refluxing DL-α-bromoisovaleric acid and L-cysteine in water at pH 7 under nitrogen (the pH being re-adjusted with sodium hydroxide solution) and the reaction mixture was separated on Dowex 50W-X8 using 0.1 *M* pyridinium acetate (pH 3.2) and on Dowex 1-X8 using a gradient of water to 1 *M* acetic acid.

β-(Cystein-S-yl)glutaric acid (βCGA) was prepared in a similar manner[9] from glutaconic acid and L-cysteine.

Isolation

There were two major unknown compounds present in the urine of a woman 1, 2 and 3 days after a methionine load. Both compounds reacted positively to iodoplatinate and one also reacted positively to ninhydrin when stained on a two-dimensional HVE paper chromatogram (Fig. 1). The compounds were isolated from 200 ml of urine (adjusted to pH 2) by ion-exchange chromatography on a 45 × 5 cm I.D. column of Dowex 50-X8 (200–400 mesh) using 0.1 *M* pyridinium acetate buffers of pH 3, 3.2 and 3.8. Fractions containing the compounds, as monitored by HVE at pH 1.9 and 4.0 and staining with iodoplatinate[5,6] and ninhydrin[7], were combined and evaporated to dryness *in vacuo* with repeated addition of water to give fractions 1 (ninhydrin-negative) and 2 (ninhydrin-positive).

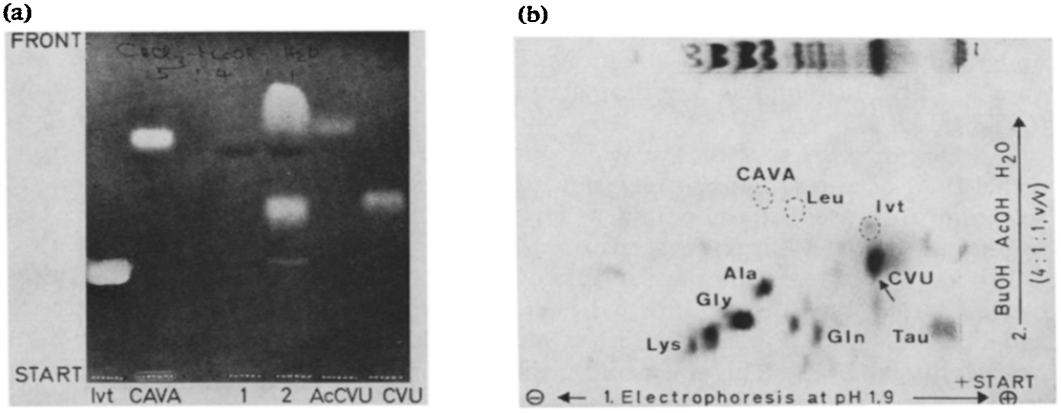

Fig. 1. (a) TLC on cellulose (Merck) of urine samples collected (1) before and (2) 3–6 h after intake of 0.9 g of Bromisoval. Urine volumes equivalent to 30 μg of creatinine were applied after precleaning by adsorption on Porapak Q. Solvent: chloroform–methanol–water (5:4:1, v/v/v); staining for sulphur compounds with iodoplatinate gave white spots on a red background. AcCVU in sample 2 moved faster than the reference because of the high concentration. (b) Two-dimensional HVE paper chromatogram of urine collected 3–6 h after Bromisoval intake; 16 μl, equivalent to 30 μg of creatinine, were applied. Polychromatic staining for amino acids with ninhydrin–copper–collidine according to Moffat and Lytle[7] revealed the following spots of metabolites: a strong grey-brown spot (CVU), a scarcely visible olive green spot (Ivt) and a grey-green spot (CAVA).

Fraction 1 was dissolved in 5% acetic acid, adsorbed on a 5 × 5 cm column of Porapak Q (150–200 mesh), which was rinsed with 250 ml of water, and the compound was then eluted with 250 ml of acetone–water (1:1, v/v). The eluate was evaporated to dryness and the residue dissolved in methanol. An aliquot was esterified

with diazomethane in ether and separated by TLC on cellulose in *n*-hexane–diethyl ether–methanol (4:6:1, v/v/v). The iodoplatinate-positive (white) zone ($R_F \approx 0.1$–0.25) was scraped off and extracted with methanol. The extract was analysed by mass spectrometry (see below).

Fraction 2, a yellow-brown residue, was suspended in 5 ml of methanol and centrifuged and the resulting cream-white powder was dried. Attempts to purify the material by recrystallization failed. However, it could be purified further by adsorption chromatography on the hydrophobic polystyrene resin Porapak Q (ref. 4). A 35-mg amount was dissolved in 6 ml of 0.2 *M* hydrochloric acid and placed immediately on a 21.5 × 1.25 cm I.D. column of Porapak Q (325 mesh), which had previously been washed with acetone and then thoroughly with water, followed by 0.1 *M* hydrochloric acid. After rinsing with water, the compound was eluted with a gradient of water to 10% aqueous ethanol, the absorption being monitored at 254 nm. In the corresponding fractions the compound crystallized spontaneously as long white needles. It was recrystallized from hot water.

Identification

As the crystals from fraction 2 appeared to be pure they were investigated first. Hydrolysis with 6 *M* hydrochloric acid for 16 h gave isovalthine (Fig. 2). In 0.1 *M* sodium hydroxide solution at 60°, the compound was split nearly quantitatively within 2 h to give urea (identified by GC–MS and by enzymic reaction using urease) and an intermediate, which was a ninhydrin-negative but chlorine–tolidine and iodoplatinate-positive weak acid. The intermediate could be hydrolysed further to isovalthine under more drastic hydrolytic conditions. The intermediate was identified by GC–MS on a 1% SE-30 column as 2-isopropyltetrahydro-1,4-thiazin-3-one-5-carboxylic acid (CITO), Fig. 2).

Desulphuration of the isolated compound 2 with active nickel prepared from nickel chloride and sodium borohydride as previously described[10] gave alanine as the sole ninhydrin-positive compound and isovalerylurea, identified by GC–MS. Desulphuration of the thiazinone derivative (see above) gave N-isovalerylalanine.

These results and the mass spectra of the isolated compound as the methyl ester hydrochloride (Fig. 3), acetyl/methyl ester, trifluoroacetyl (TFA), TFA/methyl ester and N,O-per-TMS (Fig. 3) derivatives identified the compound as α-(cystein-S-yl) isovalerylurea (CVU). This was confirmed by chromatographic (Table I), IR (Fig. 4) and mass spectral comparison with the synthetic α- and β-derivatives.

The proton NMR spectrum (100 MHz in trifluoroacetic acid) of CVU was as follows: two doublets at 1.14, 1.18, 1.20, 1.25 (two CH_3 from isopropyl); quartet at 2.24, 2.30, 2.37, 2.43 (CH, isopropyl); doublet at 3.44, 3.50 (CH_2); 3.54, 3.61 (CH); 4.63 broad unresolved (cysteinyl-CH); 7.73 broad (NH_2); and 9.99 (NH, acylurea).

Elemental analyses are given in Table II.

Fraction 1 gave isovalthine after hydrolysis with 6 *N* hydrochloric acid at 110° for 16 h. The mass spectrum of the methyl ester derivative (Fig. 5) indicated the intact compound to be α-(N-acetylcystein-S-yl)isovalerylurea (AcCVU). This was confirmed by mass spectral and chromatographic comparison with the synthetic reference compound. The IR spectrum of AcCVU is shown in Fig. 4.

The two other unknown iodoplatinate- and ninhydrin-positive compounds in the urine (Fig. 1) were identified by chromatographic comparison with the synthetic

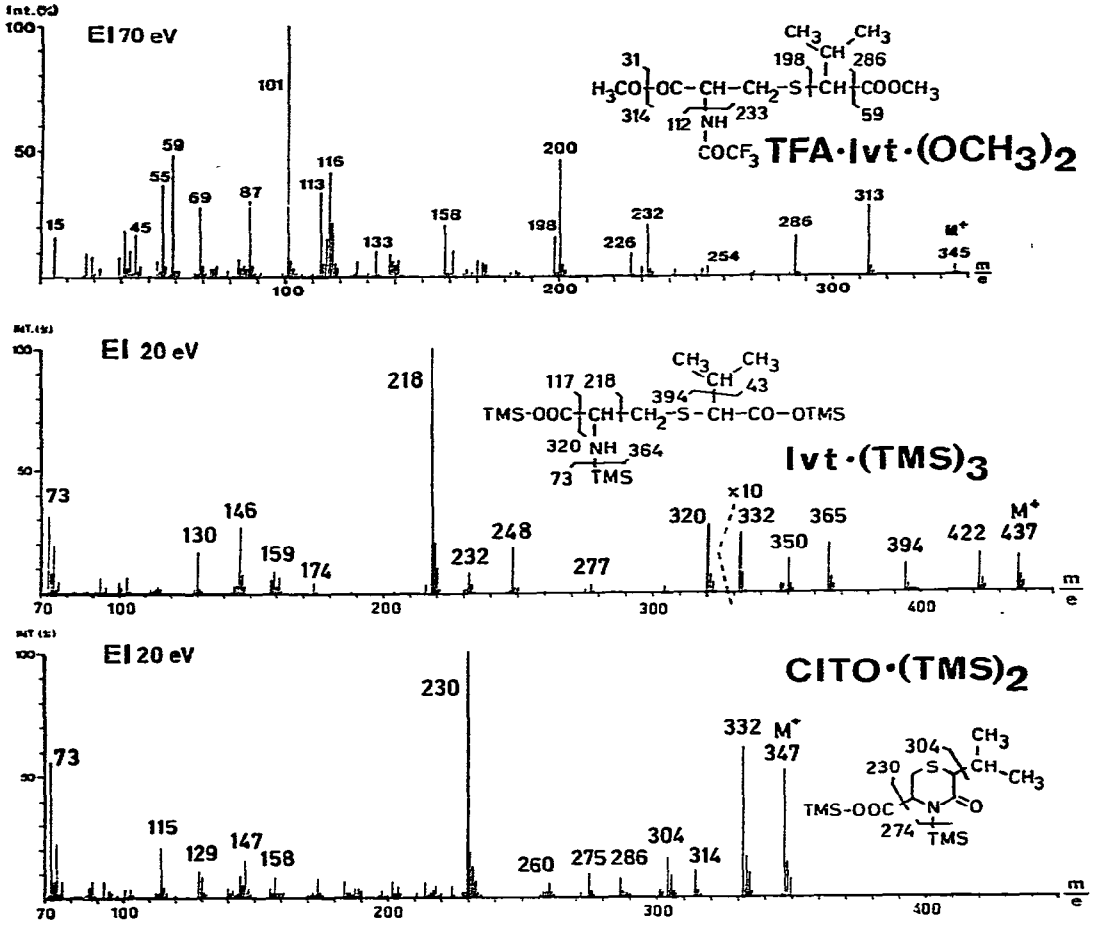

Fig. 2. Electron impact mass spectra of isovalthine and a thiazine derivative (below), compounds resulting from total and partial hydrolysis of CVU, respectively. TFA-isovalthine $(OCH_3)_2$ at 70 eV (GC–MS on LKB 9000); 345 (M), 313 (M−CH_3OH), 286 (M−$COOCH_3$), 254 (M−$COOCH_3$−CH_3OH), 242 (M−$COOCH_3$−propene), 232 (M−CF_3CONH_2), 200 (M−CF_3CONH_2−CH_3OH), 158 (200−propene), 158 (M−$COOCH_3$−OCH_3−$COCF_3$), 116 (158−propene), 113 (CF_3CONH_2), 101 (M−184−60). $(TMS)_3$-isovalthine at 20 eV (GC–MS on VG Micromass F-16): 437 (M^+), 422 (M−15), 394 (M−propyl), 365 (M−72), 350 (365−15), 332 (M−15−urea-TMS), 320 (M−COOTMS), 227 (320−propyl), 248 (320−72), 146 (218−72), 130 (M−218−OTMS). 2-Isopropyl-tetrahydro-1,4-thiazin-3-one-5-carboxylic acid (CITO), bis-TMS derivative, at 20 eV (GC–MS on VG Micromass F-16): 347 (M^+), 332 (M−15), 314 (M−15−18), 304 (M−propyl), 286 (M−propyl−18), 275 (M−72), 260 (M−15−72), 230 (M−COOTMS).

reference compounds isovalthine (Ivt) and α-(cysteamin-S-yl)isovaleric acid (CAVA). They also gave the same stains (olive-green and grey-green, respectively) with the Moffat–Lytle ninhydrin reagent[7].

Synthesis of reference compounds

α-(L-Cystein-S-yl)isovalerylurea (CVU). DL-α-Bromoisovalerylurea (2 g, 8 mmole) (obtained from DL-α-bromoisovalerylchloride and urea by boiling with

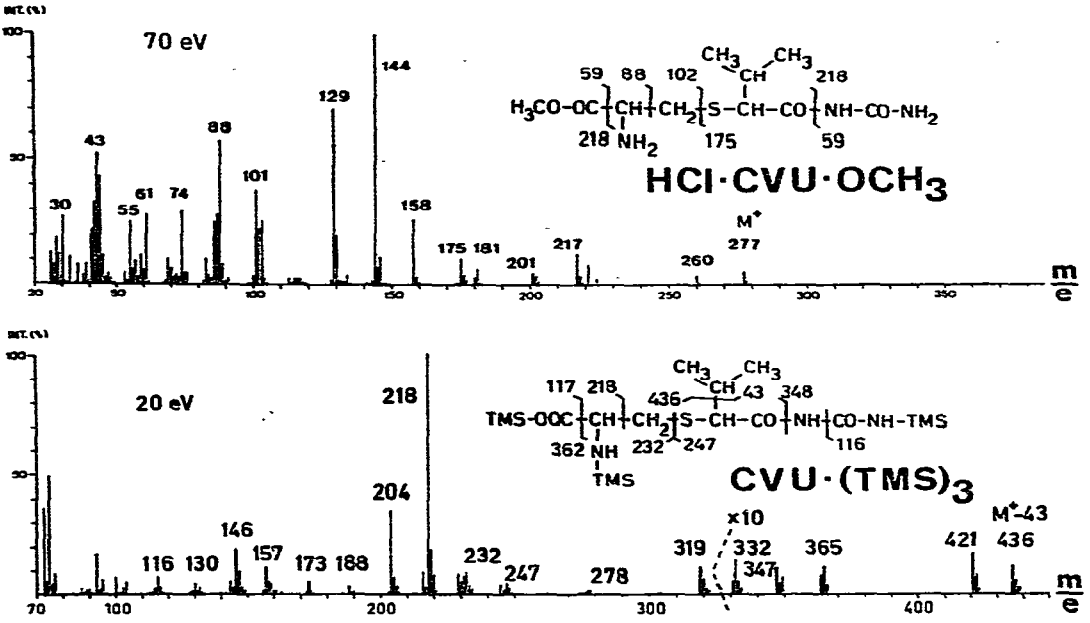

Fig. 3. Electron impact mass spectra of CVU as methyl ester hydrochloride (above) and as the per-trimethylsilyl (TMS) derivative. Methyl ester hydrochloride at 20 eV (LKB 9000, solid inlet): 277 (M^+), 260 ($M-NH_3$), 245 ($M-NH_3-15$), 217 (M—urea), 201 ($M-NH_3-59$), 158 (M—urea—$COOCH_3$), 144 ($M-88-CO-NH_3$), 129 (M—88—urea), 101 (M—88—urea—CO). Methylation with methanol-d_0:d_3 (1:1) gave doublets at *m/e* 277, 260, 245, 231, 217, 103, 102, 101 and 88. Tris-(trimethylsilyl) derivative (GC–MS on VG Micromass F-16): 479 (M^+, not visible), 436 (M—propyl), 421 (436—15), 365 (M—42—72), 347 (M—15—COOTMS), 332 (M—15—urea—TMS), 319 (M—propyl—COOTMS), 204 (247—propyl), 146 (218—72).

benzene; recrystallized from ethanol–water to give crystals with the correct elemental analysis) was added to a solution of 3 g (19 mmole) of L-cysteine hydrochloride adjusted to pH 7.5 with sodium hydroxide solution. The mixture was refluxed under nitrogen. After 20 min the pH was re-adjusted to 7.5 by the addition of sodium hydrogen carbonate and refluxing was continued for 50 min. After being cooled in ice for 1 h, the crystals were filtered off, washed thoroughly with ice-cold water, absolute ethanol and diethyl ether and recrystallized from 300 ml of ethanol–water (1:5, v/v). The yield was 1.4 g (5.3 mmole, 59%) of pure, very voluminous crystal fibres, m.p. 205°. For the elemental analysis, see Table II.

β-(L-Cystein-S-yl)isovalerylurea (βCVU). A few millilitres of ethanol and 2.4 g (15 mmole) of 3,3-dimethylacrylurea (obtained from 3,3-dimethylacrylchloride and urea by refluxing with benzene for 2.5 h; twice recrystallized from ethanol–water, 4:1, v/v; yield 70%; correct elemental analysis) were added to about 30 ml of an aqueous solution containing 2.36 g (15 mmole) of L-cysteine hydrochloride, adjusted to pH 7 with sodium hydroxide solution. The mixture was refluxed for 1.5 h under nitrogen, whereby the urea derivative dissolved completely. Thereafter, white crystals began to precipitate. After being cooled, the crystals were washed thoroughly with ice-cold water, ethanol and diethyl ether, and recrystallized from ethanol–water.

TABLE I

$R_F \times 100$ VALUES AND RELATIVE ELECTROPHORETIC MOBILITIES OF BROMISOVAL METABOLITES

Thin-layer chromatography on cellulose plates (Merck) in solvent 1 (1-butanol–acetic acid–water, 5:4:1, v/v/v), solvent 2 (chloroform–methanol–water, 5:4:1, v/v/v) and solvent 3 (*n*-hexane–diethyl ether–methanol, 4:6:1, v/v/v). High-voltage electrophoresis at 100 V/cm in 0.05 *M* pyridinium acetate (pH 4.0) and in acetic acid–formic acid–water (120:26:850, v/v/v) (pH 1.9).

Compound	*TLC*			*HVE*	
	Solvent 1	*Solvent 2*	*Solvent 3*	*pH 4.0*	*pH 1.9*
AcCVU	79	69		− 92	− 4
AcIvt	88	76		−148	− 4
CVU	38	47	0	0	+ 26
βCVU	30	40	0	0	+ 28
Ivt	50	42		−100	+ 30
CAVA	64	57		+ 13	+ 64
AcCVU-OCH_3	98		21t*		
AcIvt-OCH_3	97		81		
CAVA-OCH_3			0t*		
Methionine	44	45			
Glutamic acid	19	15			+ 45
Lysine	5	2			+100

* t = Tailing.

The yield was 2.8 g (10.7 mmole ≈ 71%) of pure crystals of m.p. 207°. For the elemental analysis ,see Table II.

α-(N-Acetylcystein-S-yl)isovalerylurea (AcCVU). CVU (158 mg, 0.6 mmole) was suspended in 3 ml of water, dissolved by addition of 500 μl 1 *N* sodium hydroxide solution to give a pH of 10.5 and then immediately reacted with 50 μl of acetic anhydride for 2 min. Addition of sodium hydroxide solution and acetic anhydride was repeated several times until a total of 3.5 ml of 1 *N* sodium hydroxide solution and 0.25 ml of acetic anhydride had been added. Then 3.5 ml of 1 *N* hydrochloric acid were added and the mixture was evaporated to dryness *in vacuo*. The residue was extracted with absolute ethanol, the extract evaporated to dryness, the residue dissolved in warm water and the solution rinsed through a 5 × 0.9 cm column of Dowex 50-X8 (H^+) with 50 ml of warm water. The eluate was evaporated to dryness *in vacuo* with repeated addition of water and the residue was recrystallised from about 3 ml of water. The yield was 127 mg (44 mmole, 67%) of AcCVU · $\frac{1}{2}H_2O$, m.p. 128–131°, elemental analysis 42.24% C (42.03% calc.), 6.65% H (6.41%), 13.37% N (13.37% calc.).

Trideutero-AcCVU was prepared in a similar manner by using hexadeuteroacetic anhydride, with a similar yield and the correct elemental analysis for the semihydrate.

α-(Cysteamin-S-yl)isovaleric acid (CAVA). Equimolar amounts of DL-α-bromoisovaleric acid and cysteamine hydrochloride were dissolved in water by adjusting the pH to 8 with sodium hydroxide solution and the solution was refluxed under nitrogen for 30 min. The pH was re-adjusted to 6 by addition of sodium hydrogen carbonate and refluxing was continued for a further 60 min. The main product was isolated by ion-exchange chromatography on Dowex 50W-X8 with 0.2 *M* pyridinium acetate (pH 3.3). It moved similarly to alanine in HVE at pH 1.9. The residue of

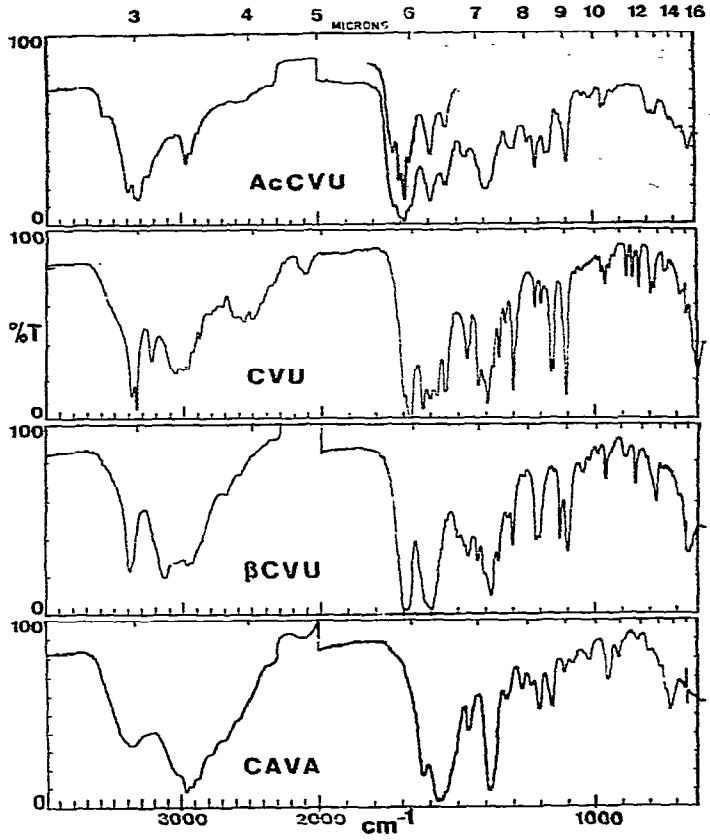

Fig. 4. Infrared spectra in KBr of α-(cystein-S-yl)isovaleryl-urea (CVU, above, isolated from urine, spectrum identical with that of synthetic CVU), of the isomeric β-(cystein-S-yl)isovalerylurea (βCVU, synth.), of α-(N-acetylcystein-S-yl)isovalerylurea (AcCVU, synth.) and of α-(cysteamin-S-yl)isovaleric acid (CAVA, synth.).

the combined evaporated fractions was dissolved in hot absolute ethanol and precipitated with diethyl ether. Colourless crystals, m.p. 197°, were obtained.

Quantitation of AcCVU and CVU

The sum of the urinary mercapturic acids AcCVU and acetylisovalthine was measured quantitatively by selected ion monitoring (mass fragmentography, iso-

TABLE II

RESULTS OF ELEMENTAL ANALYSES

Compound	*Element (%)*			
	C	*H*	*N*	*S*
$C_9H_{17}N_3O_4S$ (mol. wt. 263.3), theoretical	41.05	6.51	15.96	12.18
Isolated material, fraction 2	41.12	6.55	16.05	11.83
αCVU, synthetic, found	41.30	6.71	15.83	12.27
βCVU, synthetic, found	41.04	6.76	15.65	11.89

butane chemical ionization) after their conversion into N-acetylalanine methyl ester by desulphuration with active nickel and esterification with diazomethane. Trideutero-AcCVU was added to the urine samples as an internal standard and the samples were pre-cleaned by adsorption on Porapak Q. Details of this procedure, which seems to be generally applicable to mercapturic acid measurements, will be published elsewhere.

Urinary CVU was measured on a Biochrom amino acid analyser after pre-

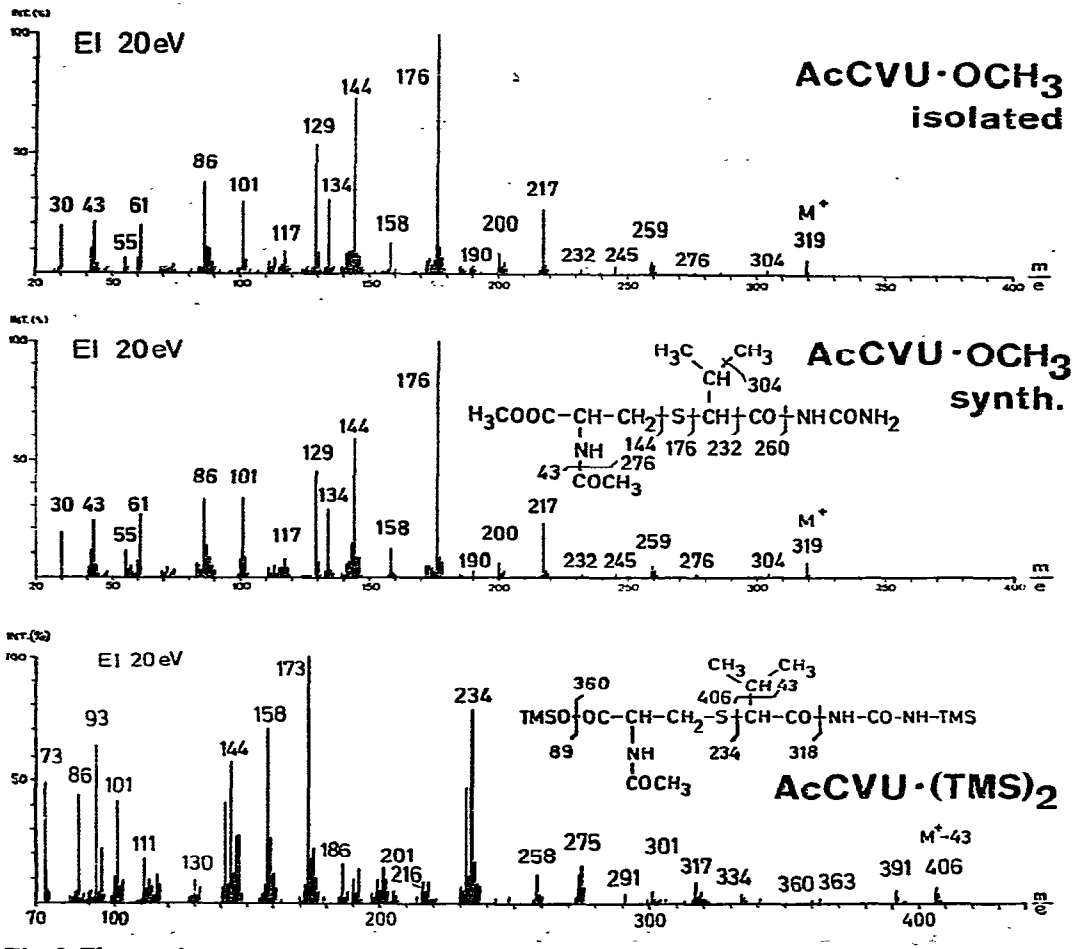

Fig. 5. Electron impact mass spectra at 20 eV of isolated and synthetic AcCVU as the methyl ester and bis-TMS derivative, respectively. AcCVU·OCH_3 (VG Micromass F-16, solid inlet): 319 (M^+), 304 (M−15), 276 (M−acetyl), 259 (M−urea), 245 (M−15−$COOCH_3$), 232 (M−$CONHCONH_2$), 200 (259−$COOCH_3$), 176 (M−isovalerylurea radical). For 217, 158, 144, 129 and 101, see Fig. 3. Methylation with methanol-d_0:d_3 (1:1) gave doublets at *m/e* 319, 304, 276, 259, 232 and 176. AcCVU·$(TMS)_2$ (GC–MS on 15 cm × 2 mm 1% Dexsil on Chromosorb AW DMCS at 180°; VG Micromass F-16). 449 (M^+, not visible), 406 (M−acetyl), 391 (406−15), 363 (M−isopropyl−acetyl), 360 (M−OTMS), 334 (M−TMS−isopropene), 317 (M−15−COOTMS and M−urea-TMS), 275 (317−isopropene), 258 (317−CH_3CONH_2), 234 (M−isovaleryl residue), 201 (M−NHCONHTMS−COOTMS), 173 (M−CONHCONHTMS−COOTMS). For 158, 144 and 101, see Fig. 3.

cleaning the sample by HVE at pH 1.9. An appropriate amount (as estimated by preliminary HVE) of β-cysteinylglutaric acid (βCGA) as an internal standard was added to 0.1–5 ml of urine and the mixture was evaporated to dryness *in vacuo*. The residue was dissolved in 0.1–5 ml of 5% acetic acid and separated by HVE at pH 1.9. Strips along both margins of the paper were stained with ninhydrin and zones corresponding to βCGA (these were of an appropriate width to ensure that all CVU and βCGA was collected) on the non-stained paper were cut out, eluted with 5% acetic acid, and CVU and βCGA were measured on the amino acid analyser.

Loading tests with Bromisoval

Two normal adults were given 0.9 g (4.03 mmole) of Bromisoval in a single dose orally and urine was collected before loading and 0–3, 3–6, 6–12 and 12–24 h after loading. These control subjects experienced no sedative effect. Urinary AcCVU + AcIvt and CVU were measured as described above.

Of the ingested drug, the two controls excreted 64 and 43 mole-%, respectively, in the form of the mercapturic acids AcCVU + AcIvt, and 4.6 and 5.9 mole-% as CVU within 24 h. The excretion rate of these compounds was maximal between 4.2 and 5.5 h, as estimated graphically (Fig. 6). In the urine portions collected 3–6 h after loading, concentrations up to 12.5 mmole/l of AcCVU + AcIvt (3.8 g/l of AcCVU) and 1.1 mmole/l (0.29 g/l) of CVU were observed. From the relative intensities of the chromatographic spots, the proportion of AcIvt was estimated to be less than 5% of that of AcCVU. Thus, the figures given for AcCVU + AcIvt represent principally AcCVU.

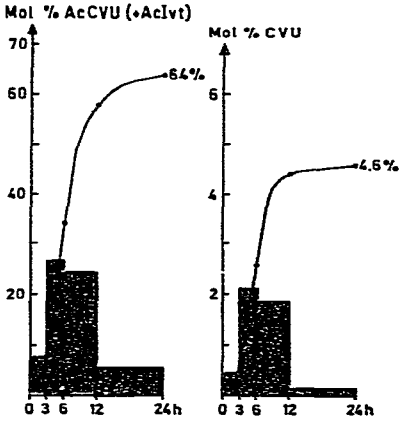

Fig. 6. Urinary excretion of AcCVU and CVU after a single dose of 0.9 g (4.03 mmole) of Bromisoval. Loading of the second control subject gave very similar curves, but with excretion of 43 mole-% as AcCVU + AcIvt and 5.9 mole-% as CVU within 24 h.

Normal 24-h urines (without drugs) gave low values (about 0.1 mmole/l) of mercapturic acids. The values mentioned above were corrected for the corresponding blank values.

In two 24-h urines of the woman mentioned in the Introduction, we found 4.8 and 3.8 mmole of AcCVU + AcIvt and 0.95 and 1.17 mmole (250 and 308 mg) of

CVU. The origin of the drug could not be evaluated in this instance. Several urine specimens collected at a later date lacked these compounds.

The trace amount of 2-hydroxyisovaleric acid present in normal urine was not increased after administration of Bromisoval. Hence, this acid is not a metabolite of Bromisoval under the conditions used in our work.

A proposed test for the Bromisoval metabolite AcCVU

A 5-ml volume of urine, adjusted to pH 2 with hydrochloric acid, is applied to a 5 × 1.5 cm column of Porapak Q (150–200 mesh), which has previously been rinsed thoroughly with acetone followed by copious amounts of distilled water[4]. The column is rinsed with 20 ml of water (rinsing rejected) and the metabolites are eluted with 25 ml of acetone–water (1:1, v/v). The eluate is evaporated to dryness *in vacuo*, the residue dissolved in 1 ml of methanol and the clear solution, which may contain AcCVU, is transferred into a small vessel. The remaining solids may contain CVU and can be stored dry.

Of the methanolic solution, 0.15 ml is applied along 15-cm starting lines of two cellulose thin-layer plates (Merck). From a similarly treated control urine, 10 μl of the methanolic solution are applied along a 1-cm starting line at the margin of the plate. After separation in chloroform–methanol–water (5:4:1, v/v/v, chamber saturation), the chromatogram is dried and the margin of the plate with the control urine and a 2-cm wide zone of the sample are stained with iodoplatinate. If a white spot appears on the sample chromatogram (R_F 0.7–0.9), the corresponding zone on the unstained part of the plate is scraped off, extracted with methanol, esterified with diazomethane, separated by TLC on cellulose in *n*-hexane–diethyl ether–methanol (4:6:1, v/v/v) and extracted with methanol from the R_F zone 0.1–0.3. AcCVU can be identified in this extract by mass spectrometry (solid inlet) or by IR spectroscopy.

DISCUSSION

Bromisoval is used as a sedative and hypnotic bromourea. The pharmacology of bromo compounds has been reviewed recently[11]. Bromisoval is thought to be harmless[12] if not abused and has been superseded by the barbiturate hypnotics. Nevertheless, an increasing use and misuse of bromoureas, probably due to their free availability, has been noted[13].

The metabolism of the monoureide Bromisoval has not yet been established[11]. 2-Bromoisovaleric acid has been described as a minor metabolite[14]. However, Bromisoval is debrominated quickly, with a half-life of only 1.2–3 h in rats[14], and the resulting inorganic bromide is excreted very slowly. Only speculations about the mechanism of debromination exist. Isovalerylurea (3-methylbutyrylurea), identified in Bromisoval poisoning by several investigators, was shown later to be an impurity in the pharmaceutical preparations[15]. In retrospect, the most important study made was that of Haruna[16], who isolated isovalthine in the hydrolysates of an "N-covered" and an "N-free" urine fraction after Bromisoval intake. Despite strong indications for a mercapturic acid pathway, obviously no one has studied the unhydrolysed native sulphur-containing Bromisoval metabolites.

It seems to be characteristic of the present situation that important metabolites of older, freely available drugs are not investigated systematically but have to be de-

tected by chance. This was the case in the present study (see Introduction). We identified unequivocally AcCVU and CVU in human urine after intake of Bromisoval. They constitute an "N-covered" and an "N-free" compound, respectively, and are probably the compounds that Haruna[16] hydrolysed in the urine fractions from which he isolated isovalthine.

In our study, 50% and even 70% of the Bromisoval metabolites were found in urine as cysteine derivatives. Hence the dominating metabolic pathway of Bromisoval is catalysed by glutathione-S-transferases. This group of enzymes has been recognized to function in the detoxification of many drugs and other xenobiotics[17–19]. Accordingto present knowledge[18], we postulate the pathway depicted in Fig. 7. Bromisoval is debrominated enzymically by the attack of a thiolate anion; corresponding non-enzymic reactions have been used by us for the synthesis of CVU, CAVA and Ivt. The first metabolic step (Fig. 7) is thought to occur in the liver and needs glutathione. Therefore, administration of bromisoval theoretically should decrease the concentration of glutathione in the liver. The conjugates of glutathione, cysteinylglycine, cysteine and N-acetylcysteine of several xenobiotics have been shown to be excreted in the bile. After re-absorption in the intestine (enterohepatic circulation) usually only the cysteine- and/or N-acetylcysteine conjugate (mercapturic acid) is excreted in the urine. In our work we found about 10 times more AcCVU than CVU. AcCVU is more soluble at physiological pH than CVU, and this could be one of the biological reasons for the N-acetylation.

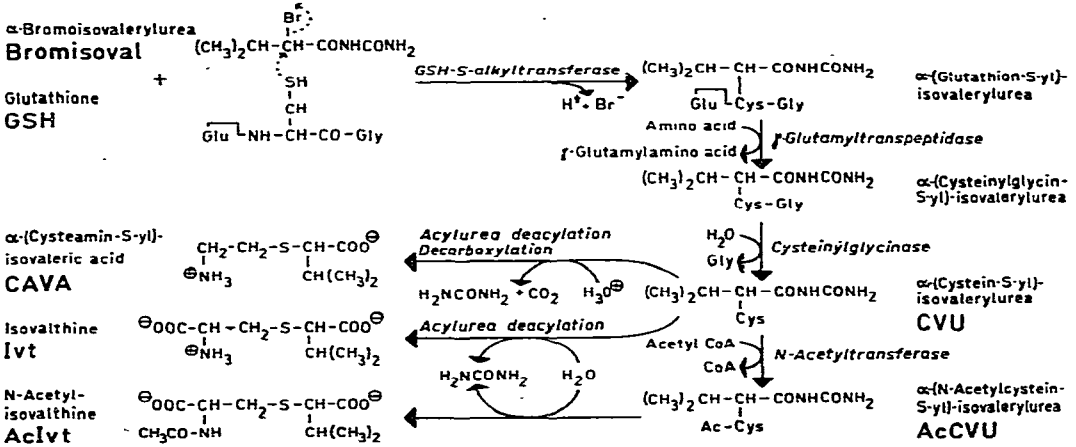

Fig. 7. Postulated metabolic pathway of Bromisoval in humans. The conjugates are excreted into bile, re-absorbed in the intestine and excreted in urine, mainly as AcCVU (mercapturic acid), to a lower extent as CVU, and trace amounts as N-acetylisovalthine (AcIvt), isovalthine (Ivt) and α-(cysteamin-S-yl)isovaleric acid (CAVA).

We did not search for the glutathione and cysteinylglycine conjugates in the urine; if present at all they can only be minor constituents. However, it can be assumed that these are the main metabolites in human bile.

CAVA, Ivt and AcIvt have been found in only trace amounts in urine. Their formation affords the splitting off of urea and, with CAVA, a decarboxylation. Probably the latter will occur by interaction with the intestinal flora. Fresh urine

fractions containing CAVA were checked and found to be almost free of bacteria. Hence, the formation of CAVA by urinary bacteria can be excluded. Possibly the appearance of CAVA in urine may be an indication for the excretion of part of the cysteine-containing Bromisoval metabolites in faeces (not investigated).

The pharmacological effects of Bromisoval is not very strong. However, it is an open question whether the observed pharmacological effect is due to Bromisoval itself or one of its metabolites. The list of possible candidates includes not only the metabolites described above but also the corresponding cysteaminyl derivatives. We did not measure the concentrations of these metabolites in blood or cerebrospinal fluid.

Isovalthine was found in hydrolysates of urine from hypercholesterolemic patients[8,20] and normal cats[8]. Experimental isovalthinuria could also be induced in other animals by administration of isovaleric acid, leucine and a number of other compounds known to be related to some extent with cholesterol metabolism. However, it has been reported that administration of even "the most strong inducers", such as isovaleric acid, bile acids or glucocorticoids occasionally does not induce isovalthinuria[21]. The exact condition necessary for isovalthine induction and the mechanism of induction are still ambiguous[21]. The sulphur atom of Ivt has been found to originate from cysteine or methionine[22]. However, all experiments to find the precursor of the isovaleric acid residue of Ivt failed and [2-^{14}C] acetic acid, [U-^{14}C]valine and [U-^{14}C] leucine have been excluded[21].

Questions still remain concerning the conditions of these studies. One must consider the discrepancies and the fact that α-bromisovalerylurea is a proved precursor of Ivt, together with the possibility that this freely available sedative may be present in mixtures that are not designated as being drugs. It seems likely that diets and other experimental conditions were not controlled strictly enough to exclude any exogeneous α-bromoisovaleryl compounds. Except in the first isolation[8], Ivt has obviously been identified only by chromatographic means, and only in hydrolysates. In our opinion, these studies should be reassessed under carefully controlled conditions and Ivt, if present at all, together with its native (unhydrolysed) molecular species should be identified unequivocally by GC–MS. As long as this has not been verified, the above isovalthine observations must be treated with caution.

The GC of AcCVU is possible as its trimethylsilyl derivative (Fig. 5). However, poor reproducibility was observed with this compound from day to day and often no peak was obtained. For identification, mass spectrometry in the solid inlet or IR spectroscopy after chromatographic purification is recommended. Because AcCVU was difficult to analyse by GC, we decided to analyse the desulphurated product acetylalanine. This technique is not specific for AcCVU alone and should also be applicable to the analysis of many other mercapturic acids. As an alternative, HPLC on a reversed phase could also be used.

Several possibilities exist for the quantitation of CVU. The simplest is by automatic amino acid analysis. However, on our amino acid analyser, CVU was eluted near methionine. Low concentrations could be measured only after pre-cleaning the urine samples by HVE. Another possibility is to measure alanine resulting from desulphuration of CVU in samples that have previously been purified on Porapak Q. In this way specificity is lost because other cysteine conjugates would also give alanine. Specificity could be retained by hydrolysing CVU at pH 10–11 to give the

thiazinone intermediate (see *Identification)*. This compound or its desulphurated product, N-isovalerylalanine, could be measured by GC or by mass fragmentography.

The sum of all cysteinyl derivatives of Bromisoval can be quantitated as Ivt after total hydrolysis of the sample with 6 *N* hydrochloric acid, using chemical ionization selected ion monitoring of the trifluoroacetyl methyl ester. Depending on the GC conditions, two peaks of the Ivt diastereoisomers may be obtained.

Carbromal [Adalin, (2-bromo-2-ethylbutyryl)urea] may possibly be metabolized similarly to Bromisoval (Fig. 7). However, a metabolite of Adalin is 2-hydroxy-2-ethylbutyric acid[23]; the analogous acid, 2-hydroxyisovaleric acid, was not excreted in urine after Bromisoval intake.

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