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DETERMINATION OF N-ACETYL-S-(N-ALKYLTHIOCARBAMOYL)-L-CYSTEINE, A PRINCIPAL METABOLITE OF ALKYL ISOTHIOCYANATES, IN RAT URINE★

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SUMMARY

A simple and rapid analytical procedure is described for N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteine (alkyl = benzyl, allyl, methyl, ethyl or *n*-butyl), a mercapturic acid with an unstable dithiocarbamic acid ester structure, which is found in rat urine as the principal metabolite of the corresponding alkyl isothiocyanate. Because such mercapturic acids decompose at pH values greater than 5 to N-acetylcysteine and alkyl isothiocyanate, the free isothiocyanate is converted with *n*-butylamine into the corresponding disubstituted thiourea and, after extraction, measured by high-performance liquid chromatography using an ultraviolet detector. The recovery is ca. 100% and the precision is very good. The lower limit of detection is ca. 0.5 μg of thiourea. The 24-h renal excretion of these mercapturic acids was determined in rats after administration of benzyl, allyl, methyl, ethyl or *n*-butyl isothiocyanate.

INTRODUCTION

Alkyl isothiocyanates with the structures RCH_2NCS and $RR'CHNCS$ occur in nature in bound form as glucosinolates in various plants [1]. The isothiocyanates are rapidly released by the enzyme myrosinase (EC 3.2.3.1) after injury to such plants [1]. When garden cress or horseradish are consumed, isothiocyanates, namely benzyl and allyl isothiocyanate, are ingested by humans as constituents of food. Mustard also contains isothiocyanates.

Since in vitro investigations showed that benzyl isothiocyanate, in particular, possessed effective antibiotic properties [2–5], it was introduced for treatment of respiratory and urinary tract infections under the trademark Tromacaps® (Dr. Madaus GmbH, Cologne, F.R.G.) [6–9].

★This paper is dedicated to Dr. Rolf Madaus on the occasion of his 65th birthday.

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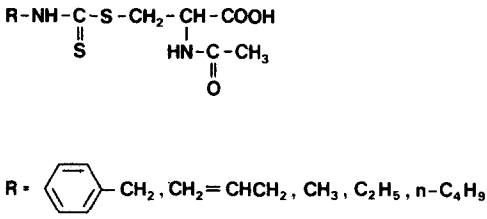

Fig. 1. Structural formula of N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteine (alkyl=benzyl, allyl, methyl, ethyl or *n*-butyl).

N-Acetyl-S-(N-benzylthiocarbamoyl)-L-cysteine was found to be the principal metabolite of benzyl isothiocyanate in urine of rats and humans [10,11] (Fig. 1). N-Acetyl-S-(N-benzylthiocarbamoyl)-L-cysteine is a mercapturic acid with an unstable dithiocarbamic acid ester structure, which converts until equilibrium is achieved into benzyl isothiocyanate and N-acetylcysteine at pH values exceeding 5 [10]. On the basis of these in vitro investigations, it appears that the benzyl isothiocyanate liberated at higher urinary pH values is the active agent in urinary tract infections. Allyl, methyl, ethyl and *n*-butyl isothiocyanates, but not aryl isothiocyanates, are also excreted in the form of these labile dithiocarbamic acid ester derivatives, at least with regard to the rat [12].

A satisfactory method of analysis for these labile mercapturic acids has not yet been described. Since different alkyl isothiocyanates may occur as constituents of food and since benzyl isothiocyanate is also used therapeutically, a method of analysis with general applicability was developed for their unstable principal metabolite. In this method, the alkyl isothiocyanate released at high pH is converted with *n*-butylamine into the corresponding disubstituted thiourea which is then quantified by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals

Benzyl, allyl, methyl, ethyl and *n*-butyl isothiocyanates were obtained from Fluka (Buchs, Switzerland). The solvents and other commercial chemicals used were analytical grade supplied by Merck (Darmstadt, F.R.G.). The dicyclohexylamine salt of N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteine (alkyl=benzyl, allyl, methyl, ethyl or *n*-butyl) was synthesized according to Brüsewitz et al. [10] and Mennicke et al. [12]. The purity was checked by thin-layer chromatography and elemental analysis.

For the preparation of the N-alkyl-N′-*n*-butylthiourea (alkyl=benzyl, allyl, methyl, ethyl or *n*-butyl), 5 g of isothiocyanate were dissolved in 30 ml of ethanol and treated with 7.5 ml of *n*-butylamine. After a reaction time of 1 h at 60 °C, the solvent was evaporated in vacuo. The residue was dissolved in 50 ml of ethyl acetate and extracted twice with 10 ml of 1 *M* sulphuric acid to remove residual amine. Then the organic phase was washed with water until neutral and dried. The solvent was then evaporated in vacuo.

N-Benzyl-N′-*n*-butylthiourea (m.p. 48.5°C), $C_{12}H_{18}N_2S$, requires: C=64.8%, H=8.2%, N=12.6%, S=14.4%. The experimental values were: C=64.7%, H=8.1%, N=12.7%, S=14.6%; R_F=0.61 (silica gel, toluene–ethyl acetate, 70:30).

N-Allyl-N′-*n*-butylthiourea (m.p. 39°C), $C_8H_{16}N_2S$, requires: C=55.8%, H=9.4%, N=16.3%, S=18.6%. The experimental values were: C=55.3%, H=9.4%, N=16.1%, S=18.4%; R_F=0.22.

N-Ethyl-N′-*n*-butylthiourea (m.p. 31°C), $C_7H_{16}N_2S$, requires: C=52.5%, H=10.1%, N=17.5%, S=20.0%. The experimental values were: C=52.0%, H=9.9%, N=17.4%, S=20.0%; R_F=0.35.

N-Methyl-N′-*n*-butylthiourea and N,N′-di-*n*-butylthiourea were prepared as described in the literature [1,3,14].

Processing of the urine samples

A 1.0-ml urine sample was mixed with 50 µl of *n*-butylamine and heated in glass culture tubes (100×12 mm O.D.) (Duran®, Schott, Mainz, F.R.G.) with PTFE-lined screw caps to 60°C for 30 min. After adding 300 µl of 25% sulphuric acid, it was extracted with 5.0 ml of diethyl ether. Following centrifugation (10 min at 1100 *g*) and removal of the aqueous layer, the organic phase was washed consecutively with 1.0 ml of 0.1 *M* sodium hydroxide and 1.0 ml of water, and centrifuged after each washing step. The aqueous phases were discarded and 4.0 ml of the organic phase were then rapidly evaporated to dryness (in vacuo) at 0°C since losses of thiourea otherwise occurred. The residue was dissolved in 1.0 ml of mobile phase and 50 µl were injected into the chromatograph.

HPLC conditions

The HPLC system consisted of a Series 3B liquid chromatograph, an LC 75 spectrophotometric detector, an ISS-100 sampling system (all supplied by Perkin-Elmer) and a column (250×4 mm I.D.) filled with Nucleosil® 10 C_8, particle size 10 µm (Macherey & Nagel, Düren, F.R.G.). Methanol–water (1:1, v/v) was used as the mobile phase. For N-benzyl-N′-*n*-butylthiourea and N,N′-di-*n*-butylthiourea the flow-rate was 1.5 ml/min, otherwise it was 1.0 ml/min. Detection was made at 240 nm. The chromatograms were recorded with an LCI-100 laboratory computing integrator (Perkin-Elmer).

Calibration curves and recovery

To prepare the calibration curves, peak areas were plotted against the concentration of N-alkyl-N′-*n*-butylthiourea over the range 1–100 µg/ml.

Blank urines (1.0 ml) were mixed with various amounts of N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteine (as the dicyclohexylamine salt, dissolved in 200 µl of methanol), which were equimolar to 1–100 µg of the corresponding alkylbutylthiourea, and then processed in the same way as the urine samples. To determine the recovery, alkylbutylthioureas (dissolved in 50 µl of methanol) were injected directly. Their peak areas were compared with those of corresponding amounts of thiourea that had been obtained from equimolar amounts of N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteine after addition to blank urines and subsequent reaction with *n*-butylamine.

RESULTS

Calibration curves

The calibration curves for N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteine, measured as N-alkyl-N′-*n*-butylthiourea, were linear over the range investigated. The regression equations were: for benzyl, $y = -0.071 + 0.937x$; for allyl, $y = 0.125 + 1.692x$; for methyl, $y = 0.281 + 0.940x$; for ethyl, $y = 0.159 + 1.327x$; for butyl, $y = -0.062 + 0.956x$. The correlation coefficients were greater than 0.998.

Accuracy and precision

To determine the accuracy of the method, blank rat urine samples were mixed with known amounts of N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteine, equivalent to 2–50 μg of the corresponding thiourea, converted with *n*-butylamine and measured. The accuracy within the series and also from day to day was determined for the benzyl and allyl derivatives, but for the methyl, ethyl and *n*-butyl derivatives it was only determined from day to day. The precision of the method, expressed as the coefficient of variation, is reported in Table I.

Recovery

The recovery for the N-alkyl-N′-*n*-butylthioureas, obtained from equimolar amounts of N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteine, over the concentration range 2–50 μg/ml was 99.8 ± 1.4% for the benzyl derivative, 100.0 ± 1.4% for the allyl derivative, 101.2 ± 2.1% for the methyl derivative, 99.8 ± 0.8% for the ethyl derivative and 100.7 ± 1.5% for the *n*-butyl derivative.

Lower detection limit and selectivity

The lower detection limit was 0.5 μg of thiourea per ml of urine. Fig. 2 shows typical chromatograms of an extract of blank rat urine, of blank rat urine with N-acetyl-S-(N-benzylthiocarbamoyl)-L-cysteine added and of urine after oral administration of benzyl isothiocyanate, each after reaction with *n*-butylamine.

Animal experiments

Groups, each of five Wistar rats (250–350 g body weight), received orally 10 mg of isothiocyanate per animal in 1 ml of olive oil. The controls received 1 ml of olive oil. Urine was collected for 24 h. Food and water were freely available during the experiment. Renal excretion of the metabolite N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteine (alkyl = benzyl, allyl, methyl, ethyl or *n*-butyl) as a percentage of the dose administered was 40 ± 10% after giving benzyl isothiocyanate, 37 ± 11% after allyl isothiocyanate, 47 ± 10% after methyl isothiocyanate, 30 ± 10% after ethyl isothiocyanate and 24 ± 15% after *n*-butyl isothiocyanate. Ioannou et al. [15] found ca. 80% of the dose of ^{14}C-labelled allyl isothiocyanate in the urine as the mercapturic acid after administration to Fischer 344 rats. This high excretion as mercapturic acid may be attributable to the fact that the isothiocyanate was administered in the presence of an emulsifier.

TABLE I

ACCURACY AND PRECISION OF THE DETERMINATION OF N-ACETYL-S-(N-ALKYL-THIOCARBAMOYL)-L-CYSTEINE IN URINE, MEASURED AS N-ALKYL-N'-*n*-BUTYL-THIOUREA ($n=5$)

Concentration of added mercapturic acid★ (μg/ml)	Equimolar concentration of thiourea (μg/ml)	Intra-assay		Inter-assay	
		Concentration of thiourea found (μg/ml)	C.V.★★ (%)	Concentration of thiourea found (μg/ml)	C.V.★★ (%)
Alkyl=benzyl					
111.0	50	49.3±0.6	1.22	50.0±0.7	1.40
66.6	30	30.3±0.5	1.65	30.2±0.3	0.99
22.2	10	9.8±0.3	3.06	10.0±0.1	1.00
11.1	5	4.8±0.3	6.25	4.9±0.1	2.04
4.4	2	2.0±0.1	5.00	2.0±0.1	5.00
Alkyl=allyl					
128.8	50	50.1±0.6	1.20	50.3±1.1	2.19
77.3	30	30.2±0.3	0.99	30.6±1.1	3.59
25.8	10	9.9±0.1	1.01	10.1±0.3	2.97
12.9	5	5.1±0.1	1.96	5.0±0.1	2.00
5.2	2	2.0±0.1	5.00	2.0±0.1	5.00
Alkyl=methyl					
143.8	50	Not determined		50.1±0.1	0.20
28.8	10			10.1±0.2	1.98
5.7	2			2.0±0.1	5.00
Alkyl=ethyl					
134.7	50	Not determined		50.1±0.2	0.40
26.9	10			9.7±0.2	2.06
5.4	2			2.0±0.1	5.00
Alkyl=butyl					
122.0	50	Not determined		50.2±0.4	0.80
24.4	10			9.9±0.3	3.03
4.9	2			2.0±0.1	5.00

★As the dicyclohexylamine salt.
★★C.V. = coefficient of variation.

DISCUSSION

N-Acetyl-S-(N-alkylthiocarbamoyl)-L-cysteine, a mercapturic acid, was found as the principal metabolite of alkyl isothiocyanates with the formula RCH_2NCS [10,12]. Since these polar and unstable mercapturic acids are difficult to extract from urine, use was made of the fact, for assay, that they partly decompose at higher pH values into N-acetylcysteine and isothiocyanate. The isothiocyanate released can be removed from the equilibrium by reaction with an amine.

When ammonia was used as the simplest amine base, rather polar extraction solvents had to be used to extract the monosubstituted thiourea. Despite this, the recovery was not satisfactory. In addition, other components of the urine were co-extracted and distorted the determination, especially at low concentrations.

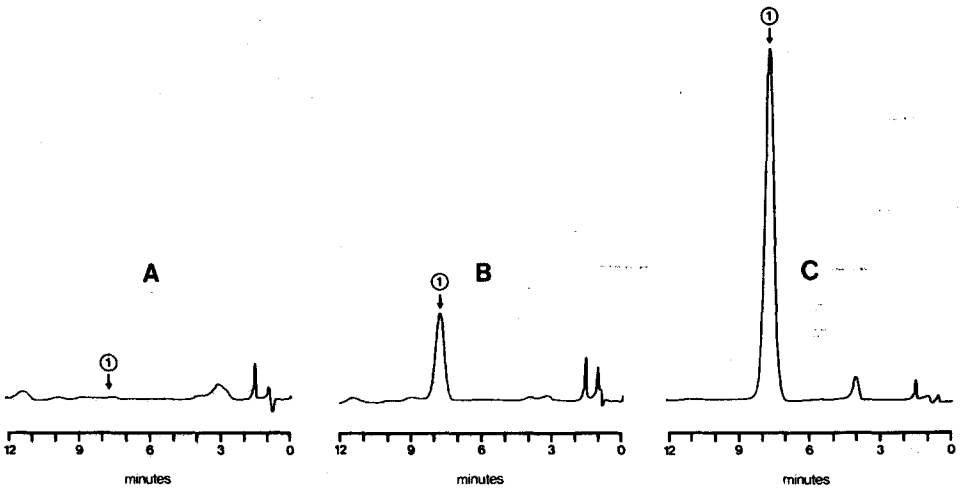

Fig. 2. Chromatograms of an extract of (A) blank rat urine, (B) blank rat urine after addition of 11.1 μg of mercapturic acid (as the dicyclohexylamine salt, equivalent to 5.0 μg of N-benzyl-N′-*n*-butylthiourea) and (C) urine after oral administration of benzyl isothiocyanate, each after reaction with *n*-butylamine. UV detection at 240 nm; (A) and (B) 0.08 a.u.f.s., (C) 0.16 a.u.f.s. Peak 1 = N-benzyl-N′-*n*-butylthiourea.

Therefore, the relatively non-volatile *n*-butylamine was selected as the reactant since it was quite soluble in water and also gave easily extractable thioureas, even with methyl and ethyl isothiocyanates, with a recovery of ca. 100%.

A disadvantage of the disubstituted thioureas is their volatility when the solvent is evaporated at room temperature. This behaviour was also observed with the monosubstituted thioureas. However, if the disubstituted thioureas were extracted with diethyl ether and the solvent was evaporated rapidly in vacuo at 0°C, no loss of thiourea was observed. Measurable losses occurred at 0°C if higher boiling extraction solvents were used, because of longer evaporation time.

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