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Assay of S-ethyl-N-acetyl-l-cysteine in urine by high-performance liquid chromatography using post-column reaction detection

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Abstract

The assay of the ethyl chloride metabolite S-ethyl-N-acetyl-L-cysteine in human urine by HPLC is described. The compound is enriched by adsorption on a non-polar adsorbent of graphitized non-porous carbon, and then stripped from positively charged compounds by application onto a strong acid cation-exchanger. Subsequently, an enzymatic deacetylation is carried out and the acylase is removed by centrifugal ultrafiltration. Separation of the sample is performed by cation-exchange chromatography applying an eluent of a very low elution strength (diluted formic acid). In the column effluent S-ethyl-L-cysteine is derivatized by *o*-phthaldialdehyde and the reaction product is detected by fluorescence measurement. In human urine a detection limit in the low ppb range is achieved. © 1997 Elsevier Science B.V.

Keywords: Ethylacetylcysteine; Mercapturic acid

1. Introduction

Ethyl chloride (monochloroethane, ECL) is a colourless substance, industrially produced by photochemical or thermal chlorination of ethene. It shows narcotic effects in humans. Recent in vivo studies demonstrated that the genotoxic potency of ECL is detectable by test systems sensitive to simple alkylating agents. Thus ECL is considered to be a potent carcinogen [1]. ECL conjugation to glutathione (GSH) is catalysed by GSH S-transferases. The glutathione S-transferases plays a physiologically important role in the detoxification and excretion of potential alkylating agents [2]. The S-ethyl glutathione adduct is converted to the mercapturic acid (MA) S-ethyl-N-acetyl-L-cysteine (EMA) and can be detected in urine of mice and rats [3]. It is assumed that the GSH conjugation of ECL is mediating the observed carcinogenic response in the mouse uterus. The evidence available suggests a mechanism for the induction of uterine carcinomas that is species specific [4]. The amount of ECL metabolised via GSH pathway should steadily decrease with decreasing exposure concentrations, while the amounts metabolised via the P450 pathway should remain at the approximately same level [3,4].

The mercapturic acid biosynthesis is generally considered to be an interorgan process based largely on the interorgan distribution of the enzymes. The measurement of urinary MA is becoming an important parameter in the biomonitoring of exposure to electrophilic chemicals [5,6]. One of the pathways for the biotransformation of ECL which leads to the

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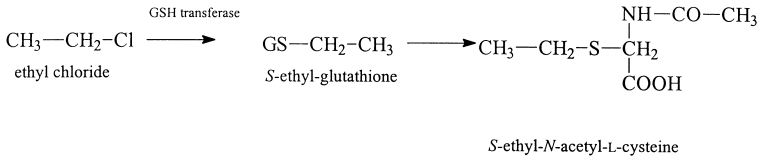

Fig. 1. Metabolic pathway of the formation of S-ethyl-N-acetyl-L-cysteine.

formation of S-ethyl-N-acetyl-L-cysteine is shown in Fig. 1 [1,3].

For the assay of mercapturic acids, various chromatographic techniques such as HPLC in combination with UV [7], electrochemical [8] and fluorescence [9–12] detection, capillary gas chromatography [13,14] and gas chromatography–mass spectrometry [15] have been reported. Generally, UV absorbance detection is limited to a few mercapturic acids with high molar absorptivity. The moderate electrochemical activity of most mercapturic acids facilitates only a rather poor detection by electrochemical methods. The most promising results were obtained by fluorescence measurements after derivatization of the analyte. The determination by GC methods requires derivatization of mercapturic acids in urine in water-free solvents, which is difficult to achieve.

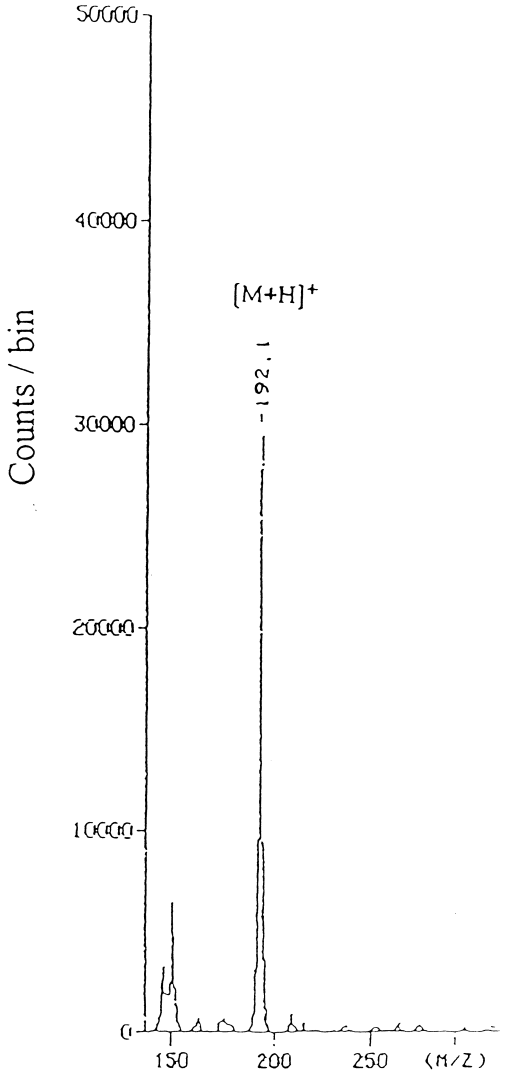

Fig. 2. Plasma desorption mass spectrum of ethyl mercapturic acid (M_r=191).

In this paper, the quantitative determination of ethylmercapturic acid in human urine is described. The high polarity of the compound in conjunction with the complex urine matrix represents a difficult analytical problem. In addition, the absence of chromophores and electrochemical highly reactive groups prevents the sensitive detection of the native analyte. The problem was solved by applying the same separation system for sample preparation as well as for analytical separation. After adsorption of most of the free amino acids and amines on a cation exchanger the analyte was deacetylated. As a result, retention strongly increased and shifted into the chromatographic regions of compounds which have previously been removed. Furthermore, detectability is strongly improved by post-column reaction with *o*-phthaldialdehyde (OPA) and mercaptoethanol due to the formation of highly fluorescent products.

2. Experimental

2.1. Chemicals

S-Ethyl-N-acetyl-L-cysteine was synthesized by a modified method of Boyland and Nery [16]. Analytical grade *o*-phthaldialdehyde and mercap-

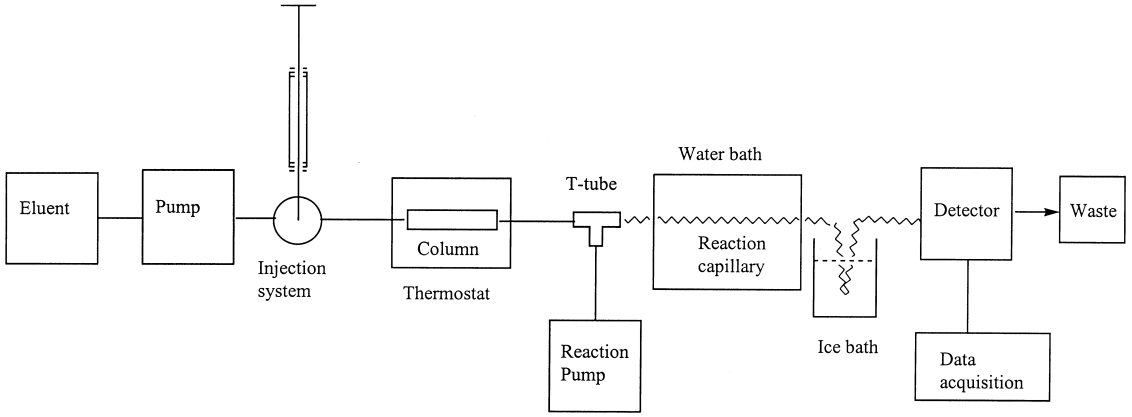

Fig. 3. Configuration of the chromatographic apparatus with post-column reaction detection system.

toethanol were purchased from Sigma (Steinheim, Germany), boric acid, formic acid, potassium hydroxide, sodium dihydrogenphosphate and Li-Chrosolv methanol from Merck (Darmstadt, Germany). Porcine acylase I of grade III was obtained from Sigma.

2.1.1. Characterization of S-ethyl-N-acetyl-L-cysteine

The synthesized product, S-ethyl-N-acetyl-L-cysteine was characterized by ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz). The molecular mass (M_r = 191) of the standard (Fig. 2) was confirmed by a Bio-Ion 20 plasma desorption mass spectrometer (Bio-Ion, Uppsala, Sweden) and matrix assisted laser desorption/ionisation mass spectrometry MALDI III (Kratos Analytical, Manchester, UK).

2.2. Sample preparation

Morning urine was collected from ten healthy humans and analysed immediately after collection. A

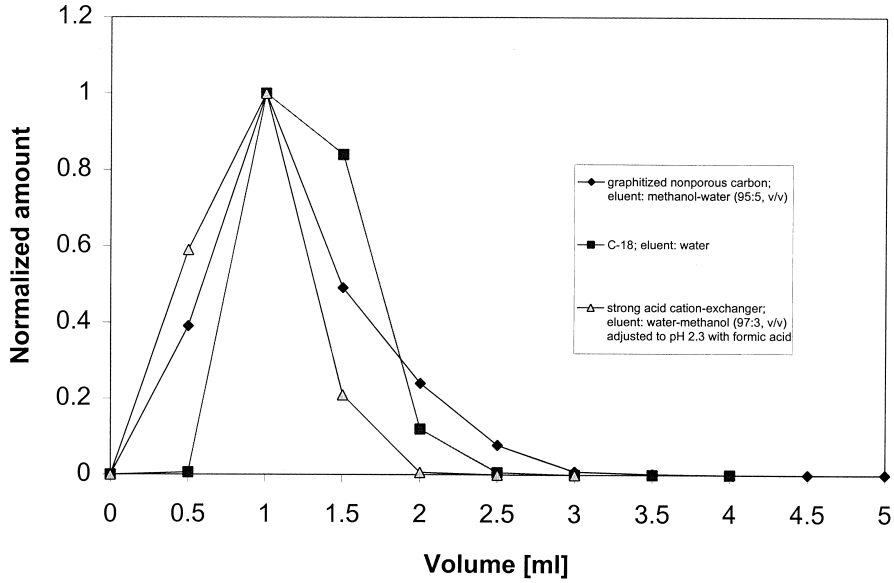

Fig. 4. Retardation of ethyl mercapturic acid on sample preparation cartridges filled with different adsorbents. Conditions: column, 500 mg adsorbent; fraction size: 0.5 ml.

500-mg C_{18} cartridge (Varian, Harbor City, CA, USA) was plugged into a 500-mg Supelclean-ENVI Carb cartridge (Supelco, Bellefonte, PA, USA) and conditioned with 5 ml methanol and 5 ml 0.1 *M* NaH_2PO_4 (pH 7). A 5-ml aliquot of human urine was applied and subsequently washed with 3 ml of water. (EMA was transfered from the C_{18} cartridge onto the Supelclean-ENVI Carb cartridge.) The cartridges were separated, S-ethyl-N-acetyl-L-cysteine was eluted from the Supelclean-ENVI Carb with 4 ml of methanol–water (95:5, v/v) and evaporated to dryness in a vacuum centrifuge. The residue was dissolved in 200 μl of the mobile phase (aqueous formic acid, pH 2.3) and applied to a Chromabond SA cation-exchange cartridge (Macherey-Nagel, Düren, Germany). EMA was not adsorbed and was eluted within the first 3 ml of aqueous formic acid (pH 2.3) which was used as eluent. The sample was evaporated to dryness and the residue reconstituted in 200 μl 0.1 *M* NaH_2PO_4 (pH 7). A 200-μl volume of acylase I solution (3 mg/ml in 0.1 *M* NaH_2PO_4, pH 7) was added and the sample was incubated at 37°C for 6 h. Deproteinization was performed by centrifugal ultrafiltration (120 min at 1000 *g*) through Millex Ultrafree-MC 10000 NMWL filters (Millipore, Eschborn, Germany).

2.3. Chromatography

Analysis was performed by cation-exchange chromatography with a post-column reaction detection system (Fig. 3).

2.3.1. Separation

The sample was separated on a 125×4 mm I.D. stainless steel column, packed with Nucleosil 10 SA (Macherey-Nagel) of 10 μm particle size. The column was protected by a 30×4 mm I.D. precolumn packed with octadecylsilica (Varian) of 40–65 μm particle size. Both columns were thermostated at 25°C. The eluent, water–methanol (97:3, v/v) adjusted to pH 2.3 with formic acid, was delivered by a Hewlett-Packard quaternary gradient pump model 1050 (Waldbronn, Germany) at 0.8

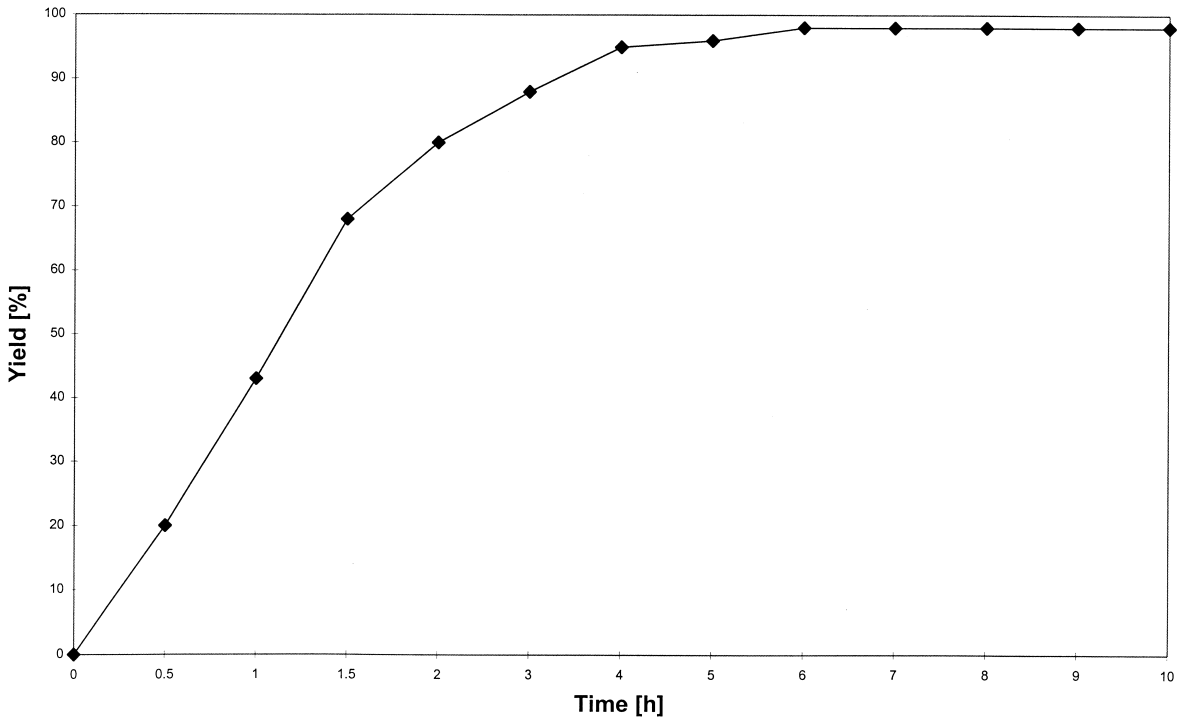

Fig. 5. Yield of the deacetylation of EMA in dependence of time.

ml/min. Samples up to 100 μl were injected by an Hewlett-Packard autosampler model 1050.

2.3.2. Detection

The effluent from the column was derivatized with a solution containing 1 g OPA and 1 ml mercaptoethanol in 1 l of borate buffer (1 *M* boric acid and 1.25 *M* potassium hydroxide, pH 11). Reagent delivery was performed via a T-tube by a Merck isocratic pump model L-6000A at a flow-rate of 0.4 ml/min. A 2.5 m×0.5 mm I.D. PTFE reaction capillary was immersed into a water bath and maintained at 70°C. The last part of the reaction capillary before the detector was cooled in an ice bath to enhance fluorescence intensity. The reaction products were detected by a Hewlett-Packard programmable fluorescence detector model 1046A at an excitation wavelength of 340 nm and measuring the emission at 420 nm. Data acquisition was performed by a Hewlett-Packard CHEMSTATION (Fig. 3).

3. Results and discussion

3.1. Sample preparation

EMA is a polar organic compound soluble in polar solvents such as water and methanol, slightly soluble in aprotic solvents such as acetonitrile and non-soluble in organic solvents such as *n*-hexane or ethyl acetate. The substance was only slightly retained on octadecylsilica but strongly on graphitized non-porous carbon. The octadecylsilica cartridge, which was plugged into the carbon cartridge, reduced the amount of non-polar compounds which would otherwise be eluted from graphitized carbon and, in addition, increased sample capacity. From octadecylsilica EMA was transferred onto the carbon cartridge with 3.0 ml water and completely eluted with 3.5 ml of methanol–water (95:5, v/v) (Fig. 4). For desorption, different mixtures of acetonitrile, 2-propanol or methanol with water were used. The best recoveries were found with methanol.

In the second step most of the positively charged compounds (amino acids and amines) were removed by a strong acid cation-exchanger. The best results were achieved with diluted formic acid, representing an eluent of a very low pH and elution strength. The low retardation of the polar and uncharged EMA may be explained by a slight interaction with the non-polar phenyl-spacer of the cation-exchange material (Fig. 4).

To achieve a better retention on the same separation system, EMA was deacetylated and converted into a cation. Time and yield of the deacetylation depended on the particular mercapturic acid [10,12,17,18]. For EMA we found that deacetylation was completed after 6 h (Fig. 5).

3.2. Chromatography

3.2.1. Separation

Cation-exchange chromatography with a post-col-

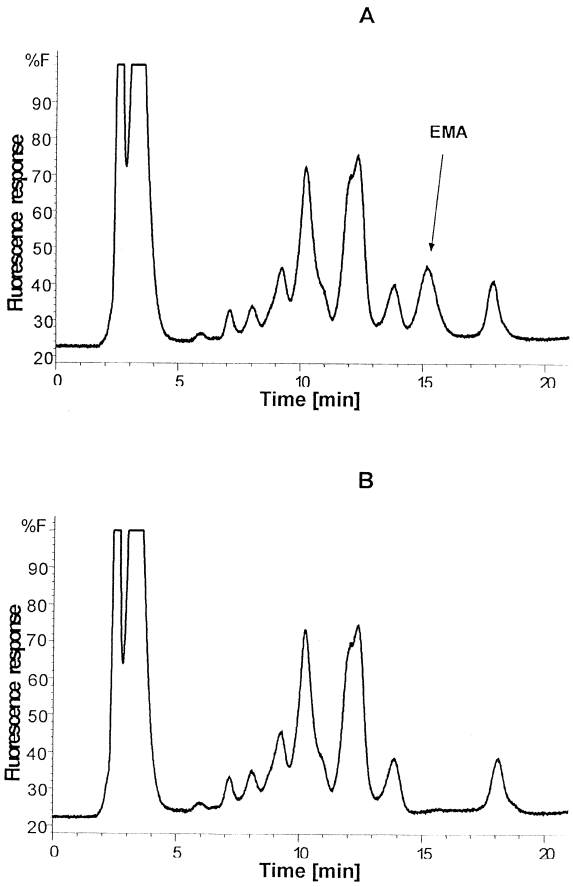

Fig. 6. Chromatogram of an spiked (A) and unspiked (B) urine sample.

umn reaction detection system was used for the determination of EMA. After the enzymatic deacetylation of EMA, a positively charged ion was formed in acidic solution. The retention was strongly increased and it was completely separated from urine components. Fig. 6 shows the chromatograms of spiked and unspiked urine sample. The retention and separation from the matrix components is influenced by temperature and concentration of the organic modifiers in the eluent. A good compromise was obtained at a column temperature of 25°C and 3% (v/v) methanol in the eluent.

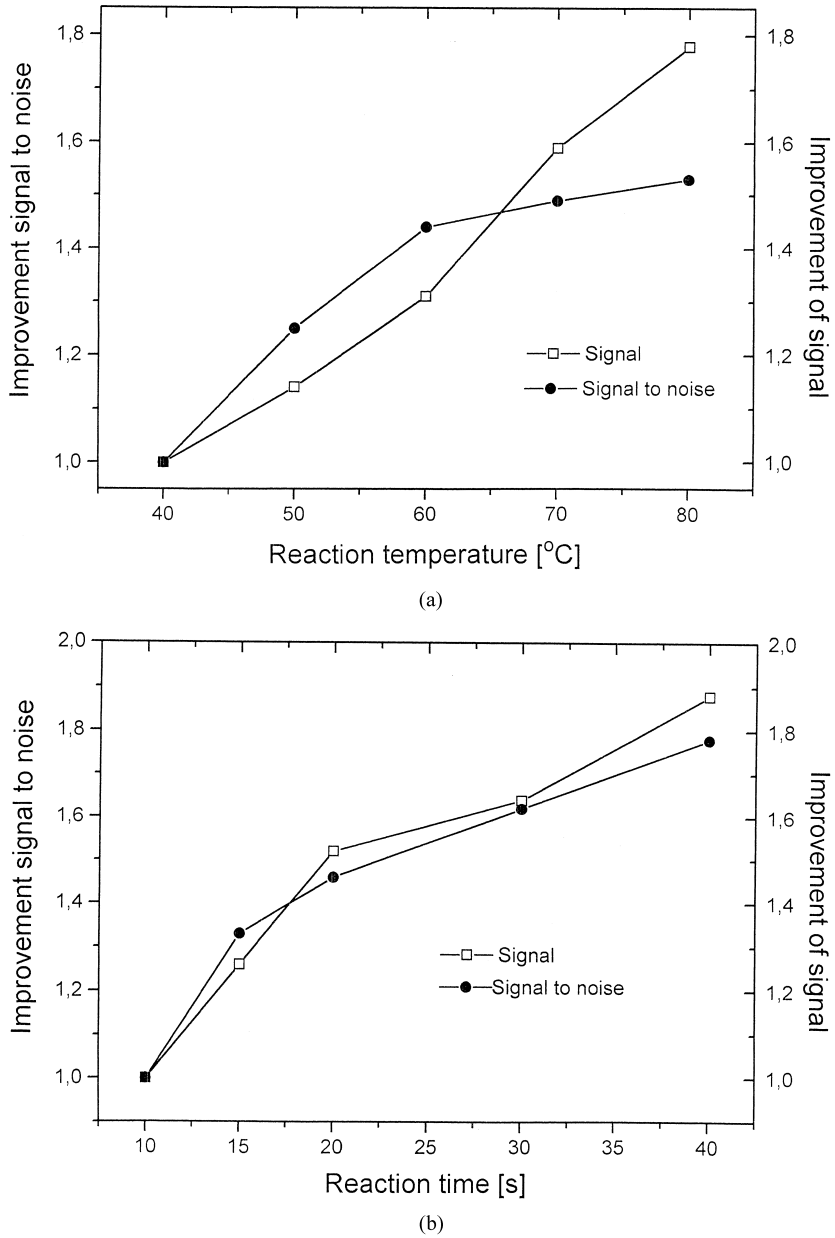

Fig. 7. (a) Influence of the reaction temperature on signal response; (b) influence of the reaction time on signal response.

3.2.2. Detection

The column effluent was derivatized with a buffered solution of OPA and mercaptoethanol. At a flow-rate of 0.8 ml/min of the separation system, the flow-rate of the reagent pump should be at least 0.3 ml/min to achieve a pH>9 which is necessary for high reaction yield. Fig. 7a shows that the reaction yield and the signal-to-noise ratio improved with increasing temperature. Above 60°C an overproportional increase of noise was observed in conjunction with a high baseline signal. The use of longer capillaries increased the reaction time but also resulted in a linear increase of the signal and signal-to-noise ratio together with additional peak broadening and decrease of resolution (Fig. 7b). Under the experimental conditions, optimal resolution was achieved at a reaction time of 20 s.

The precision of the method was determined from repeated analyses of blank urine samples spiked with different amounts of mercapturic acid. The urine samples were obtained from three different non-exposed healthy humans who were on a diet for 7 days. The received calibration function showed good linearity in the range between 7 and 260 μg/l (r= 0.9973). Recoveries of the method from urine samples spiked with 20 μg/l, 50 μg/l, 100 μg/l and 260 μg/ml of EMA were 94,2% (±2.8%, n=5). The within day precision of the method was better than 6% and day-to-day variations were lower than 10%. A detection limit at a signal-to-noise ratio of 3 of about 7 μg/l was estimated.

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