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Note

High-performance liquid chromatography of cystathionine, lanthionine and aminoethylcysteine using *o*-phthaldialdehyde precolumn derivatization

M. COSTA, L. PECCI, B. PENSA, M. FONTANA and D. CAVALLINI*

Dipartimento di Scienze Biochimiche, Università di Roma "La Sapienza", and Centro di Biologia Molecolare del Consiglio Nazionale delle Ricerche, 00185 Rome (Italy)

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Cystathionine (CT) has long been known as a key intermediate in the transsulphuration pathway. CT has been measured by ion-exchange chromatography [1], reaction with acidic ninhydrin [2], exchange with labelled cysteine [3] and isotachophoresis [4]. Lanthionine (LT) is known mainly as a degradation product of cystine-containing proteins [5] and is occasionally found as such in biological samples [6–8] and in some antibiotics [9,10]. S-Aminoethylcysteine (AEC), also named thialysine, is chemically produced by reacting cysteine either with bromoethylamine [11] or with ethylenimine [12] and has so far never been identified in vivo.

CT, LT and AEC, together with cystine, have been reported recently to be substrates of L-amino acid oxidase (EC 1.4.3.2) and of glutamine transaminase (EC 2.6.1.15) extracted from bovine tissues [13,14]. The main product of this reaction is a monoketo acid which cyclizes into the respective ketimine forming, together with its unsaturated analogue, a new class of sulphur-containing cyclic imino acids of putative biochemical significance. Some of these cyclic compounds have recently been detected in mammalian samples [15–19].

This paper describes a method for the determination of CT, LT and AEC by high-performance liquid chromatography (HPLC) making use of precolumn derivatization with *o*-phthaldialdehyde (OPA) plus mercaptoethanol according to Jarrett et al. [20]. As a preliminary application of the method, we have

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determined the production of CT, LT and AEC by incubating cystathionine β-synthase (EC 4.2.1.22) with appropriate substrates.

EXPERIMENTAL

Reagents

L-Cysteamine hydrochloride, pyridoxal-5′-phosphate, L-serine and methanol (HPLC grade) were obtained from Merck (Darmstadt, F.R.G.) and L-cysteine hydrochloride, L-homocysteine thiolactone hydrochloride and S-2-aminoethylcysteine from Sigma (St. Louis, MO, U.S.A.). L-LT was prepared as described by Greenstein and Winitz [21]. L-CT, OPA and mercaptoethanol were obtained from Fluka (Buchs, Switzerland). Standard solution of amino acids was obtained from Hamilton (Reno, NV, U.S.A.). DEAE-cellulose (DE-52) was purchased from Whatman (Maidstone, Kent, U.K.). An L-homocysteine solution was prepared immediately before use by dissolving L-homocysteine thiolactone (31 mg) in 1 ml of 0.8 *M* sodium hydroxide solution. After heating at 100°C for 3 min under a stream of nitrogen, the pH was adjusted to 8.5 with 1 *M* hydrochloric acid.

Derivatization procedure

The reagent was prepared by dissolving 54 mg of OPA in 2 ml of methanol, mixing the solution with 10 ml of potassium borate buffer (5.0 g of boric acid and 4.4 g of potassium hydroxide per 100 ml of water) and adding 80 μl of mercaptoethanol. The reagent was kept at 4°C in the dark and 20 μl of mercaptoethanol were added daily to maintain the maximum yield.

A 50-μl volume of CT, LT and AEC (2–40 μ*M*) was mixed with 50 μl of OPA–mercaptoethanol reagent. At a timed 2-min interval after mixing the reactants, a 10-μl aliquot was injected into the HPLC system.

Chromatography

HPLC analyses were carried out with a Waters Assoc. (Milford, MA, U.S.A.) chromatograph equipped with two Model 501 pumps, a Model 680 gradient controller and a U6K sample injector. Eluates from the column were detected by a Perkin-Elmer (Norwalk, CT, U.S.A.) Model LS-1 LC fluorescence detector using a 340-nm filter for excitation with an emission wavelength of 450 nm. The column was a 150 mm×4 mm I.D. Hypersil ODS, 5 μm (Policonsult, Rome, Italy). The mobile phases were (A) 0.05 *M* sodium phosphate buffer (pH 5.5)–methanol (70:30, v/v); (B) 0.05 *M* sodium phosphate buffer (pH 5.5)–methanol (20:80, v/v). The column was preconditioned with solvent A for 15 min before sample loading, followed by a linear gradient from A to 100% B over 30 min. The flow-rate was 1.0 ml/min at room temperature. For the separation of the OPA derivative of LT in the enzymatic incubation mixture, the elution gradient was as follows: a linear increase from 0 to 50% B in 5 min,

then isocratic at 50% B for 15 min followed by 100% in 10 min; the other parameters were the same as above.

RESULTS

Chromatography

The elution pattern of the fluorimetrically measured OPA derivatives of CT, LT and AEC in the presence of OPA derivatives of a standard amino acid mixture is shown in Fig. 1. LT-OPA eluted in an intermediate position, well separated from the other amino acids (before the methionine-OPA derivative). The CT-OPA derivative co-eluted with that of valine. The AEC-OPA peak eluted well before the OPA derivative of lysine.

Dilution series gave reliable linear peak heights down to 20 pmol per injection. Lower concentrations were also studied and the detection limit was found to be 2 pmol per injection.

As the amino acid OPA derivatives degraded fairly rapidly with time, accurate timing of the reaction and injection was maintained and analysis was carried out exactly 2 min after derivatization.

Applications

The procedure described above was applied to measure the amounts of CT, LT and AEC as products of the cystathionine β-synthase activity. Chicken and rat liver are good sources of cystathionine synthase. We also tested the enzy-

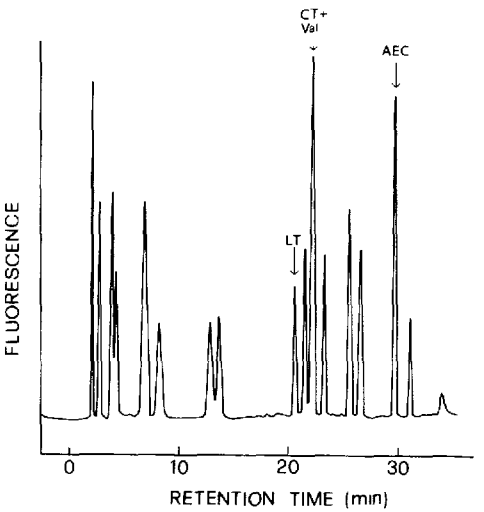

Fig. 1. Elution pattern of LT-OPA, CT-OPA and AEC-OPA (100 pmol each) plus OPA derivatives of a standard mixture of seventeen amino acids (25 pmol of each amino acid). Conditions as under Experimental.

matic activity in the bovine brain stimulated by the finding that the cyclic derivatives of CT and LT are present in this organ [15,16,19]. The enzyme was partially purified according to Boresok and Abeles [22] up to the DEAE-cellulose chromatography step. Typical HPLC elution profiles of the enzymatic incubation mixtures for CT, LT and AEC formation are shown in Fig. 2. Quantitation of CT, LT and AEC was based on duplicate assays of samples spiked with a known amount of amino acid similar to that found in the sample injection and by measuring the increase in the peak height. Suitable blanks without enzyme or substrates, processed and analysed in parallel, did not show any peak corresponding to CT-OPA and AEC-OPA. The determination of LT

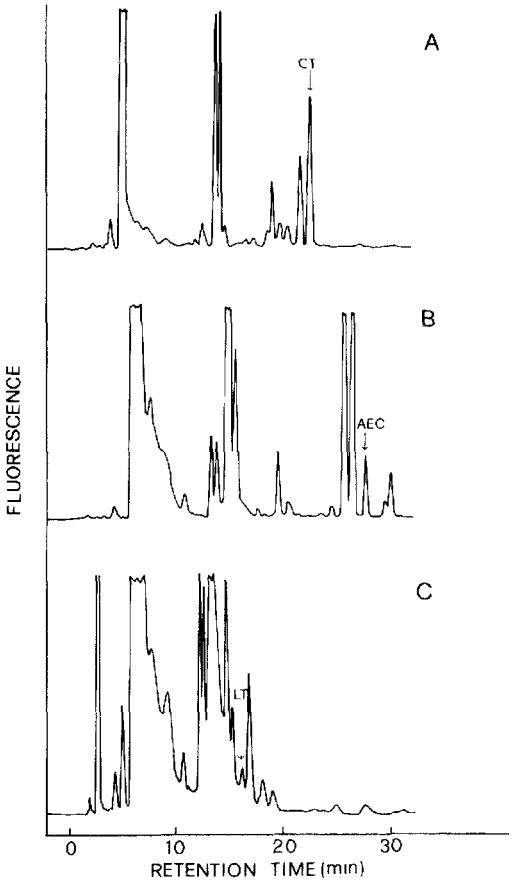

Fig. 2. Elution pattern of the enzymatic incubation mixtures for the detection of (A) CT-OPA, (B) AEC-OPA and (C) LT-OPA. The enzyme (1 mg), from chicken liver, was incubated with appropriate substrates as reported in Table I. Aliquots (10 μl) of the incubation mixtures were reacted with 100 μl of OPA–mercaptoethanol reagent and different amounts (5–20 μl) were analysed.

proved to be more difficult because of the existence of a component that had a retention time similar to that of the LT-OPA peak. However, a good separation was obtained by adjusting the elution gradient as reported under Experimental.

Fig. 3 shows a linear relationship between the amounts of LT, CT and AEC formed by the chicken liver enzyme and incubation time. Moreover, the enzyme activity was proportional to the protein concentration in the range 1–4

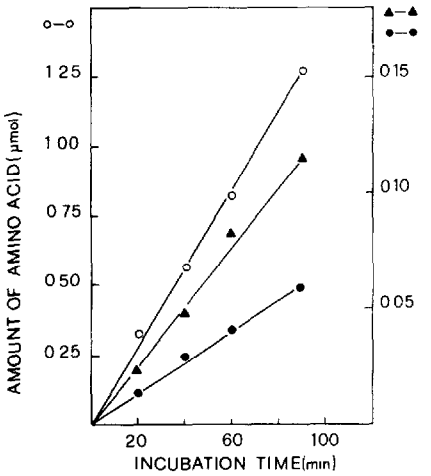

Fig. 3. Time course of the production of (○) CT, (●) LT and (▲) AEC by cystathionine synthase from chicken liver.

TABLE I

COMPARATIVE PRODUCTION OF CYSTATHIONINE (CT), AMINOETHYLCYSTEINE (AEC) AND LANTHIONINE (LT) BY CYSTATHIONINE β-SYNTHASE

The final concentrations of the assay components in 1 ml of incubation mixture were 100 m*M* potassium phosphate buffer (pH 7.8), 5 m*M* pyridoxal-5′-phosphate, 5 m*M* EDTA, 50 m*M* serine, 20 m*M* homocysteine and enzyme. For AEC and LT production the homocysteine was replaced with cysteamine and cysteine, respectively. The mixtures were incubated at 37°C for 60 min and the proteins were precipitated by perchloric acid. After neutralization with 10 *M* potassium hydroxide solution, 10-μl aliquots were allowed to react with 100 μl of OPA–mercaptoethanol reagent and analysed by HPLC. Activity expressed as a percentage of the cystathionine produced is given in parentheses. The values are means of three single determinations.

Source	Production (nmol/mg·h)		
	CT	AEC	LT
Chicken liver	875 (100)	90 (11.5)	31.5 (4 7)
Rat liver	240 (100)	30 (12.5)	6.5 (2.7)
Bovine brain	12 (100)	2.8 (23.3)	[a]

[a]The activity of the brain enzyme was too low for the precise determination of LT

mg. Table I shows the comparative production of CT, LT and AEC by cystathionine synthase from the three different sources.

DISCUSSION

The HPLC technique described here is sufficiently simple and sensitive for the determination of CT, LT and AEC in biological samples, as illustrated by the quantitation of cystathionine β-synthase products. With minor modifications the procedure could also be used for the determination of these sulphur amino acids in tissues and biological samples. The partial resolution of the CT-OPA derivative and valine did not affect the quantitation of CT produced by the synthase in this work owing to the absence of valine in our enzymatic incubation mixtures. When valine is assumed to be present, pretreatment of the sample with performic acid, as adopted previously [23], is the simplest way to remove CT and to measure the contribution of valine to the CT peak. This method can be made more specific and versatile by employing electrochemical detection as for the determination of ornithine [24]. One result, particularly relevant to our interest in investigating the biochemical generation of cyclic derivatives of CT, LT and AEC, is the confirmation of the involvement of cystathionine β-synthase in the production of LT and AEC. This enzymatic reaction has been reported earlier by Braunstein et al. [25] and by Porter et al. [26]. At that time, the enzymatic products were detected by paper and thin-layer chromatography and the significance of this reaction was not further investigated.

Cystathionine β-synthase, possibly identical with serine sulphydrase, is a ubiquitous enzyme of wide substrate specificity able to replace, with different efficiency, serine-OH and cysteine-SH with a number of thiol-containing compounds [27]. The confirmation of the production of LT and AEC by this enzyme and the development of a sensitive analytical procedure for their determination will certainly be of help in understanding the overall enzymatic mechanism for the production of the cyclic derivatives of CT, LT and AEC found in mammals.

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