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Note

Investigation of the synthesis of tryptathionine using high-performance liquid chromatography

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The amino acid tryptathionine, which occurs in phalloidin and related toxic peptides of *Amanita phalloides*, provides a crosslink between a tryptophan and a cysteine residue[1]. The most successful synthetic route to this amino acid, the Savige–Fontana reaction[2,3] involves the reaction of cysteine (or other thiols in the case of related amino acids) with 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo-[2,3-*b*]indole-2-carboxylic acid[4].

During a study of the kinetics of the reaction the need arose for a rapid method for the separation and estimation of a variety of tryptophan oxidation products. Existing methods used in this laboratory[3,5] although satisfactory with regard to separation, proved to be too slow for our purposes. In this communication we describe the results obtained using a high-performance liquid chromatography (HPLC) system in conjunction with a photodiode-array detector.

EXPERIMENTAL

Materials

Samples of a 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid (Hpi)[4], dioxindolyl-3-alanine[6], and tryptathionine and some of its analogues[3], were prepared according to published methods. All other materials were obtained from normal commercial sources.

Method and instrumentation

HPLC analysis was performed using a Perkin-Elmer Series 4 solvent delivery system and a LCI-100 laboratory computing integrator. Sample injection was performed using a Rheodyne 7125-075 syringe-loading sample injector with a 6-μl loop.

Elutions were monitored at 289 nm using a Hewlett-Packard 1040A photodiode-array detector controlled by an 85B microcomputer. Chromatography was performed on a Vydac 218 TP (250 × 4.6 mm I.D.) 5 μm, C_{18} reversed-phase column at 60°C.

All samples were run at 1.0 ml/min with solvent A (1 g/l aqueous ammonium acetate) and solvent B (acetonitrile). The column was eluted for 4 min with solvent A and then for 11 min following a linear gradient to 60% B.

A mixture of L-cysteine hydrochloride monohydrate (18.5 mg, 0.105 mmole)

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and Hpi (29.9 mg, 0.126 mmole) in 25% trifluoroacetic acid (1.00 ml) was kept at room temperature. Aliquots (50 μl) were taken at recorded hourly intervals and added to water (950 μl). The diluted samples (2 μl) were then analysed by HPLC using tryptathionine and Hpi as controls.

RESULTS AND DISCUSSION

Table I summarizes the chromatography of tryptophan and the separation of the reactants and possible products of the Savige–Fontana reaction. These conditions were also found to be suitable for rapid quantitative assays for products resulting from various oxidative treatments of tryptophan, even though some derivatives were found to be eluted close together. The use of the photodiode-array detector enabled simultaneous confirmation of the identity of peaks by comparison of their ultraviolet absorption spectra with those of known standards. This feature made the procedure particularly suitable for materials with distinctive UV-absorbing characteristics, such as the tryptophan oxidation products.

TABLE I

SEPARATION OBTAINED BY HPLC OF TRYPTOPHAN, REACTANTS AND POSSIBLE PRODUCTS OF TRYPTATHIONINE SYNTHESIS, AND TRYPTOPHAN OXIDATION

Compound	*Retention time (min)*	*Colour factor 289 nm (Trp = 1)*
L-Tryptophan	8.80	1.00
Reactants		
L-Cysteine	2.68	–*
L-Hpi	4.28	0.37
Products		
L-Tryptathionine	6.02	2.20
L-Cystine	2.61	–*
Dioxindolyl-3-alanine	4.12, 4.48	0.23**
L-Kynurenine	5.70	0.06
Formyl-DL-kynurenine	7.00	–*
L-Oxindolyl-3-alanine	7.38	0.24
Tryptamine	10.29	0.83

* 289 nm is unsuitable for detection of these compounds. Their retention times were established by detection at a wavelength suitable for their absorption maximum.

** Summation of two peaks.

When the technique was applied to the products obtained in the tryptathionine synthesis, separation of the reactants, tryptathionine and the byproducts was clearly obtained (Fig. 1). The method of Savige and Fontana[3] had specified a 48-h reaction time, but from our study (Fig. 2) any increase in time beyond 8 h is not necessary. The yield does increase slightly with longer times but so do the amounts of secondary products. A small quantity of oxindolyl-3-alanine was identified as a byproduct of the reaction, also reported by Savige and Fontana[3], but the major byproducts could not be identified. They did not correspond to any of a number of possible oxidation

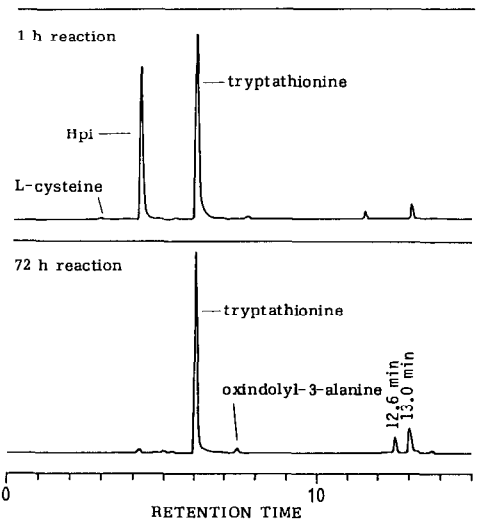


Fig. 1. HPLC trace of samples taken from the tryptathione reaction mixture after 1 h and 72 h at 289 nm. L-Cysteine does not absorb maximally at this wavelength.

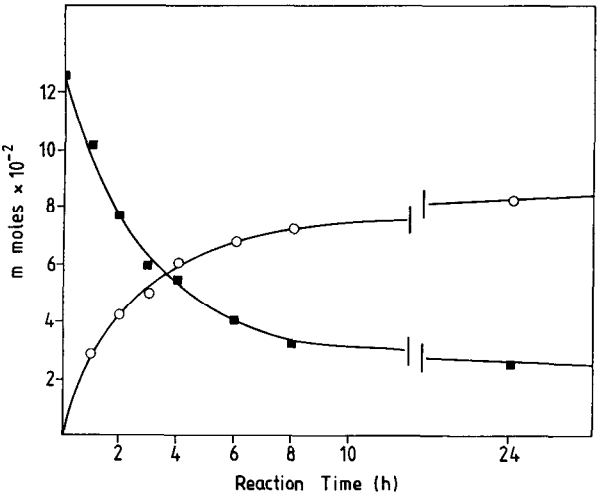


Fig. 2. The formation of tryptathionine (○) and the consumption of Hpi (■) plotted against time of reaction.

products of tryptophan reported in Table I and the UV-absorption spectrum of the major peak most closely resembled that of a tryptophan dimer (Fig. 3)[7].

The HPLC procedure described also proved to be suitable for the detection and analysis of a range of tryptathionine analogues (Table II), without modification to the chromatography. These compounds were eluted on the reversed-phase column in the manner consistent with their polarity and structure.

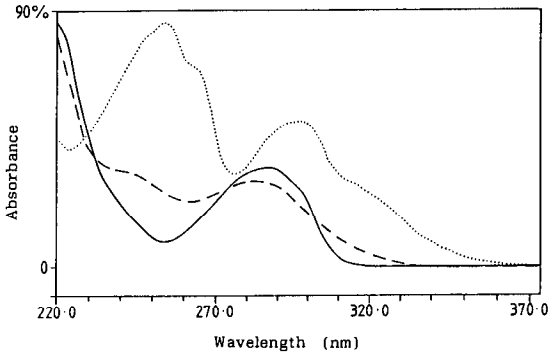
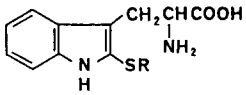


Fig. 3. The UV-absorption spectra of the byproducts (shown in Fig. 1) compared with that of tryptathionine obtained by the photodiode-array detector. ——, Tryptathionine; ...., compound eluted at 12.6 min; – – –, compound eluted at 13.0 min.

TABLE II

SEPARATION OBTAINED BY HPLC OF TRYPTATHIONINE AND ITS ANALOGUES

Basic formula	*R*	*Retention time (min)*
	$CH_2CH(NH_2)COOH$ (tryptathionine)	6.02
	CH_2COOH	8.65
	CH_2CH_2COOH	10.65
	CH_2CH_2OH	11.99
	CH_3	12.43
	CH_2CH_3	13.25

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