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Note

High-performance liquid chromatography of tryptophan and its irradiation products using UV photometric and voltammetric detection

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Investigation of the radiolysis of amino acids is of great importance, as information is provided on the effect of high-energy radiation on proteins (and thus also on living organisms). Data on radiolysis in solution are necessary in the preparation, storage and purification of amino acids labelled with radionuclides.

Tryptophan belongs among the biologically most important amino acids and its radiolysis has recently been extensively studied (ref. 1 and references therein). Radiolysis of tryptophan in aqueous solution involves complex radiation–chemical reactions in which intermediates and final, stable products are formed due to the interaction of the active species produced by radiolysis of water, *e.g.* OH, H and e_{aq}^-, with tryptophan molecules[2]. Radiation transformation of the indole part of the tryptophan molecule is considered to predominate, but deamination, recombination and other processes also occur.

To study these radiation processes, reliable, selective and sensitive analytical methods are required. So far, tryptophan and its metabolites have been widely analysed in biological fluids and tissues using thin-layer chromatography[3], gas chromatography–mass spectrometry (GC–MS)[5] and especially high-performance liquid chromatography (HPLC) with UV photometric and fluorescence detection[6,7].

Most of the products of tryptophan metabolism or degradation are readily oxidizable (hydroxy derivatives are usually formed) and thus voltammetric HPLC detection of these substances is highly sensitive, with an additional advantage of the selectivity of voltammetry that is useful in analyses of complex mixtures. Hence, numerous papers have described these determinations in biological samples (for a survey see ref. 8, and more recently refs. 9 and 10). In the study of the radiolysis of tryptophan, HPLC with UV photometric detection was used[6,7,11,12].

In view of the high sensitivity and selectivity of voltammetric detection, we studied this method as a means of monitoring radiolysis of aqueous solutions of tryptophan under various experimental conditions. The present paper describes the properties of the analytical method, while the results thus obtained for the radiolysis process are given elsewhere[1].

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EXPERIMENTAL

Apparatus

The HPLC measurements were carried out on a Varian Aerograph 4100 instrument (Varian, U.S.A.) at laboratory temperature, in a reversed-phase system with glass columns (150 cm × 4 mm I.D.) packed with Separon SI C_{18} or Separon SI CN, 5 μm (Laboratorní Přístroje, Czechoslovakia). The separation was obtained using the following mobile phases: I, $1.2 \cdot 10^{-4}$ *M* EDTA (aq.)–20% methanol (pH 5.23); II, 0.0001 *M* KH_2PO_4 (aq.), (pH 5.47); III, 300 ml of 0.1 *M* citric acid + 160 ml of 0.1 *M* sodium acetate (pH 4.5) containing 7% methanol. The samples were injected through a stopped-flow valve using 10- and 25-μl syringes (Hamilton, U.S.A.). Two detectors were connected in series at the column outlet, a variable-wavelength Varian UV photometric detector followed by a carbon fibre voltammetric detector constructed by us and described elsewhere[13]. The electrode potentials are referred to a saturated silver chloride electrode.

Chemicals

All the chemicals employed were of p.a. purity (Lachema, Czechoslovakia) and were not further purified. The standard substances involved L-tryptophan (Koch-Light Labs., U.K.), 5-hydroxy-L-tryptophan, L-kynurenine, 3-indolepropionic acid (Fluka, Switzerland), 3-indoleacetic acid (Lachema, Czechoslovakia) and formyl-L-kynurenine (Calbiochem, U.S.A.). Methanol for UV spectroscopy was used.

Preparation of tryptophan radiolytic products

Tryptophan was dissolved in redistilled water to obtain a concentration of 10^{-2} mol/l and the solution was irradiated in 10-ml ground-glass-stoppered test tubes. The pH was adjusted with sulphuric acid or sodium hydroxide and some solutions were bubbled with nitrogen monoxide. A ^{60}Co gamma-source with an intensity of 2.7 kGy/h was used and the doses varied from 20 to 60 kGy. Samples of the solutions after the irradiation were directly injected into the column.

The substances were identified by comparing their retention data with those of the standard substances. For the determination, peak heights were measured, as they exhibited a better precision than the peak areas; and the calibration curve method was used.

RESULTS AND DISCUSSION

Because of great differences in the character of the tryptophan degradation products (acidic, neutral and weakly basic compounds are involved) it was impossible to carry out a satisfactory isocratic separation on a single column[6]. The problem can be solved either by a step change in the pH of the mobile phase during the separation[6] or by using two different columns. It was found that satisfactory separation of kynurenine, 5-hydroxytryptophan and tryptophan can be achieved using a C_{18} column with mobile phases I–III. 3-Indoleacetic and 3-indolepropionic acids are eluted with the solvent front when using mobile phases I and II. On the other hand, they are eluted after tryptophan with long retention times by mobile phase III. Good results are then obtained when mobile phase III is combined with a CN-stationary phase.

In selecting the mobile phase, the requirement of a sufficient electrical conductivity had to be satisfied if voltammetric detection was to be used.

The effect of the mobile phase flow-rate was followed from 0.1 to 1.0 ml/min, from the point of view of optimization of voltammetric detection. The signal of the detector was virtually independent of the flow-rate within this interval (*cf.* also ref. 13) and thus a value of 0.3 ml/min was selected, which was optimal from the point of view of the pressure in the system and the time of analysis.

To find the optimal detection conditions, the UV absorption curves and the voltammograms of the substances were obtained directly in the detector cell in the appropriate mobile phases. The UV absorption maxima lie at wavelengths of 220 and 250–290 nm. As the mobile phase itself absorbs strongly at 220 nm, a wavelength of 256 nm was selected for the determination. Typical hydrodynamic voltammograms are given in Fig. 1; on their basis, an electrode potential of +1.4 V was selected as the value at which the highest signal-to-noise ratio is obtained, in agreement with the literature[14].

The very high sensitivity of voltammetric detection, compared with UV photometric detection, and its suitability for monitoring tryptophan radiolysis products are demonstrated in Fig. 2a and b. The figure shows that the radiolysis products include kynurenine, 5-hydroxytryptophan, 3-indoleacetic and 3-indolepropionic acid. Formylkynurenine was not found among the products. The other substances that appeared in the chromatogram could not be identified because of a lack of the appropriate standards.

The calibration data for the UV photometric and voltammetric detection are given in Table I. The table shows that the voltammetric detector exhibits good lin-

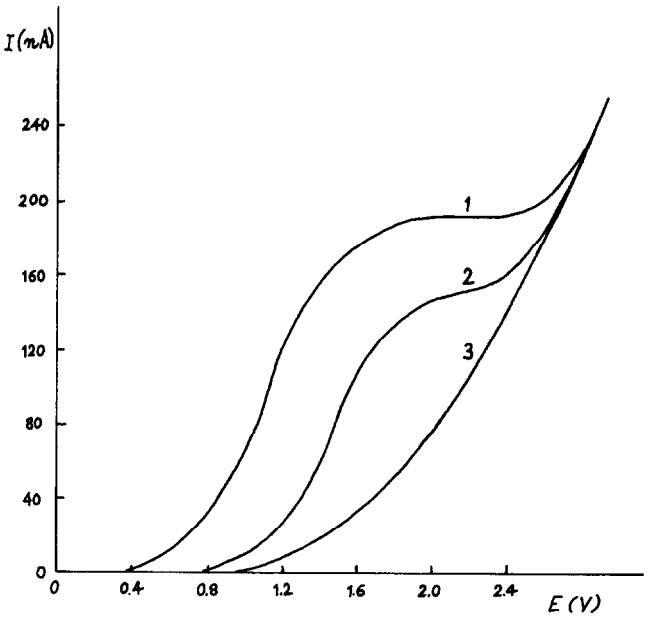

Fig. 1. Hydrodynamic voltammograms of 5-hydroxytryptophan (1), tryptophan (2) and the mobile phase (3). Mobile phase, 0.001 *M* KH_2PO_4; flow-rate, 0.3 ml/min.

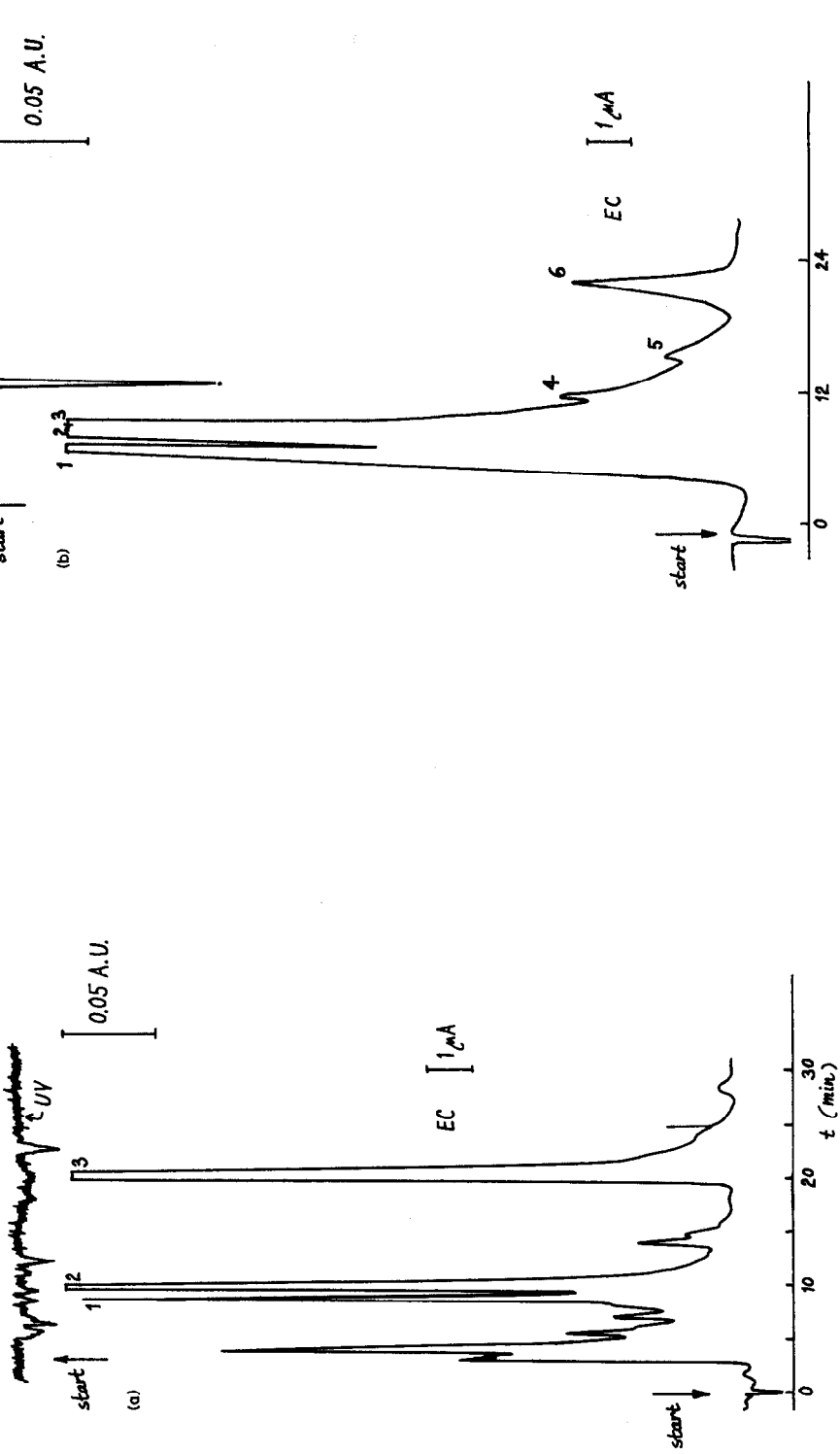

Fig. 2. Separation of the tryptophan radiolytic products on a C_{18} column (a) and on a CN column (b) with voltammetric (EC) and UV photometric (UV) detection. Mobile phase III, flow-rate 0.3 ml/min. (a) 1 = Kynurenine, 2 = 5-hydroxytryptophan, 3 = tryptophan. (b) 1 = Hydroxytryptophan, 2 = kynurenine, 3 = tryptophan, 4 = 3-indoleacetic acid, 5 = unidentified, 6 = 3-indolepropionic acid.

TABLE I

CALIBRATION DATA FOR THE UV PHOTOMETRIC AND VOLTAMMETRIC DETECTOR

For experimental conditions, see the text. The detection limit equals twice the peak-to-peak noise.

Substance	*UV photometric detector*			*Voltammetric detector*		
	Corr. coeff.	*Slope of regression dependence (A.U./ng)*	*Detection limit (ng)*	*Corr. coeff.*	*Slope of regression dependence (μA/ng)*	*Detection limit (ng)*
3-Indolepropionic acid	0.999	$7.59 \cdot 10^{-3}$	3.9	0.999	$3.75 \cdot 10^{-2}$	0.7
3-Indoleacetic acid	0.998	$6.63 \cdot 10^{-3}$	4.8	0.995	$1.77 \cdot 10^{-2}$	1.8
Kynurenine	0.997	$6.92 \cdot 10^{-3}$	4.3	0.999	$3.72 \cdot 10^{-2}$	0.9
5-Hydroxytryptophan	0.999	$5.58 \cdot 10^{-3}$	6.7	0.999	$6.30 \cdot 10^{-2}$	0.5
Tryptophan	0.993	$6.98 \cdot 10^{-3}$	4.1	0.998	$6.10 \cdot 10^{-2}$	0.8
Formylkynurenine	0.999	$9.38 \cdot 10^{-3}$	1.3	Electro-inactive		

earity, is more sensitive than the UV photometric detector and yields substantially lower detection limits for the test substances, with the exception of formylkynurenine which is electro-inactive. The connection of a UV photometric and a voltammetric detector in series is especially advantageous, as comparison of the detector responses facilitates identification of solutes in complex mixtures.

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