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SEPARATION OF TRYPTOPHAN METABOLITES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC AND FLUORESCENCE DETECTION

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SUMMARY

An isocratic method for the separation of most of the tryptophan metabolites in the oxidative degradation pathway is described The chromatographic analysis utilizes the combined selectivity and sensitivity of amperometric and fluorimetric detection The effect of pH, ionic strength and operating potential on retention times and detector responses are evaluated The use of dual electrochemical cells at two operating potentials together with a programmable fluorescence detector allows for improved selectivity for the detection of metabolites The sensitivity achieved with this method allows for the detection of the metabolites in biological fluids at the picomole level The method has been used to monitor serum samples obtained during a tryptophan load test

INTRODUCTION

The importance of tryptophan and its metabolites in normal and diseased subjects and in many disease states has been emphasized by several workers. These diseases have included cataract formation [1], hepatic porphyrias [2], diabetes [3], carcinoma of the bladder [4], certain haemoblastic disorders [5], some inborn errors of metabolism [6] and alcoholism [7]

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Since our group has an interest in some of these diseases, we required a sensitive method to analyse as many tryptophan metabolites as possible Several methods for the assay of the tryptophan metabolites by high-performance liquid chromatography (HPLC) have been published [7–9], but these have been mainly concerned with only some of the metabolites involved in the oxidative pathway

This article outlines the development of a method which enables the simultaneous quantitation of many of the metabolites of the kynurenine pathway (Fig 1) The method is particularly suitable for following the increase in the concentration of compounds resulting from the Tryptophan Load Test [10] This test, originally introduced to detect patients suffering from pyridoxal deficiency, has also been used for investigating defects in tryptophan metabolism in other disorders [7,8]

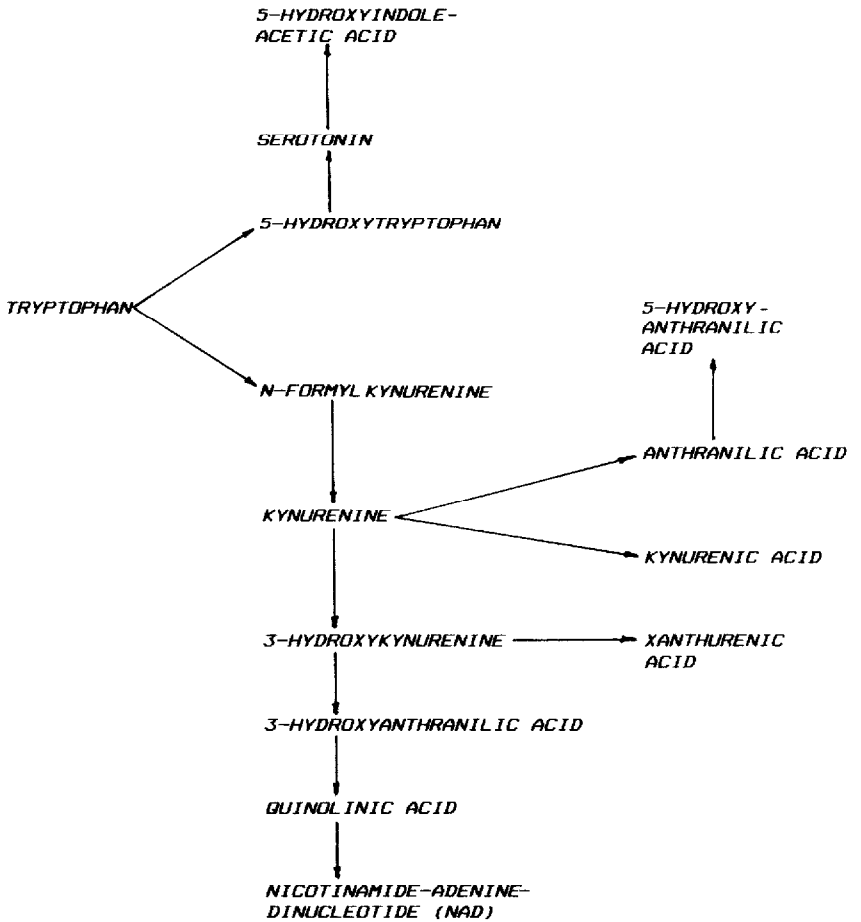

Fig 1 Tryptophan pathway showing the compounds of interest

EXPERIMENTAL

All chemicals used in buffers were of analytical grade Doubly glass-distilled water was passed through a Waters Milli-Q system prior to use

The reference metabolites investigated were (i) tryptophan, 3-hydroxykynurenine, 3-hydroxyanthranilic acid and kynurenine (as representative of the kynurenine pathway), (ii) xanthurenic acid, 5-hydroxyanthranilic acid and anthranilic acid (as representatives of the metabolic branch pathway), and (iii) 5-hydroxytryptophan (as representative of the serotonin pathway) They were obtained from either Sigma (St Louis, MO, U S A) or from Fluka (Buchs, Switzerland)

Apparatus

The liquid chromatograph used consisted of a Beckman Model 112 pump, an ETP-Kortec Model K65B automated sample injector, a Shimadzu Model L-ECD-6 electrochemical detector and a Hewlett-Packard 1046A programmable fluorescence detector Chromatograms were recorded and peak heights integrated on a Shimadzu CR3A integrator and an Omniscribe recorder

Analytical separations were performed on a 100 mm×4 6 mm I D column packed with 5-μm Microsorb C_8 (Rainin Instrument, Woburn, MA, U S A) A 30 mm long Brownlee RP-8 column was fitted as a guard column

Chromatographic conditions

The buffer was prepared from a mixture of stock solutions of 200 m*M* sodium acetate and 200 m*M* acetic acid to obtain a pH of 4 50 This 200 m*M* buffer was then diluted 1 10 to produce the final mobile phase (20 m*M*), which was then readjusted to pH 4 50 with concentrated hydrochloric acid, filtered and degassed by sonication under vacuum prior to use The solvent flow-rate was 0 6 ml/min and separations were performed at ambient temperature Detection was achieved amperometrically at 480 and 990 mV and also fluorimetrically at excitation and emission wavelengths optimised for each metabolite

Methods

A stock solution of reference metabolites was prepared by dissolving each in 20 m*M* acetate–citrate buffer, pH 5 0 at a concentration of 100 mg/l [7] Aliquots were stored frozen at −30°C Working solutions were diluted as required, usually to 0 5 μg/ml with mobile phase

The parameters listed below were investigated in sequence to optimise the chromatographic separation of the metabolites Individual dilutions of each metabolite were used to measure operating potentials and fluorescence spectra

pH Volumes of 200 m*M* acetic acid and 200 m*M* sodium acetate were varied to produce the desired pH This solution was then diluted 1 19 to obtain the correct acetate concentration

Acetate concentration After establishing the optimal pH, the buffer was prepared with 200 m*M* acetic acid and sodium acetate and this solution was then diluted to achieve the various concentrations If necessary, the pH was adjusted with concentrated hydrochloric acid

Ionic strength In order to test the effect of salt concentration, sodium chloride was added to the 20 m*M* acetate buffer, pH 4 50, to produce a final concentration of chloride ion equivalent to 0 1 and 0 2 *M*.

Applied potential To determine the optimum operating potential, each metabolite was injected (20 μl) on column and the operating potential changed incrementally in a stepwise manner from 300 to 1000 mV

Fluorescence spectra Excitation and emission maxima were determined by stop-flow and manual scanning of each metabolite The detector was then programmed to set each optimised wavelength at a time prior to elution of the appropriate peak

Linearity and sensitivity To determine the linearity and sensitivity of the detectors, increasing amounts of each metabolite were injected onto the column and the detector responses were measured as either peak area (electrochemical detection) or peak height (fluorescence detection)

RESULTS AND DISCUSSION

A C_8 column was chosen as the stationary phase because of the generally reduced k' values and the better peak symmetry reported using this type of packing material [11] The analysis time was longer than that obtained with acetate–citrate buffer [7], however, an improved separation of tryptophan from its closest eluting metabolites was obtained in the current method This is particularly important for samples derived from tryptophan load tests where the tryptophan peak in serum and urine samples is broad

In the procedure of Krstulović et al [7] tryptophan metabolites were separated on a C_{18} column in 40 m*M* acetate–nitrate buffer with 7% acetonitrile Using this mobile phase, 5-hydroxyanthranilic acid, a metabolite of major concern in this study, was found to partially coelute with 3-hydroxykynurenine The organic modifier was reduced but it was found that concentrations of acetonitrile below 1% did not allow elution of the compounds from the column. Resolution was not altered significantly by concentrations below 7% acetonitrile The 10 m*M* acetate buffer at pH 4 84 produced a longer analysis time, but afforded comparatively better resolution on the C_8 column

Changing the pH of the mobile phase in the range 3 0–5 0 was found to affect the retention times of some metabolites, most notably that of anthranilic acid. In general, a mobile phase of low pH produced good separation of the slower eluting compounds, but yielded poor separation of the earlier compounds The highest pH tested (pH 5 0) gave the poorest separation The separation between tryptophan and xanthurenic acid decreased as the pH was raised from

4 5 to 5 0 Since the tryptophan peak in serum samples following a tryptophan load is large it obscures the xanthurenic acid peak if buffers of pH 4 85 or 5 0 are used

Varying the concentration of acetate did not have a major affect on metabolite retention time The best separation was obtained using either 20 or 40 m*M* acetate The effect of the increase in the ionic strength on the retention times of the metabolites was not significant

The limiting currents and the half-wave potentials for each of the compounds are listed in Table I Of the compounds tested only kynurenic acid did not elicit a response from the electrochemical detector up to 1000 mV. This finding agrees with that of others [7]

Non-protein constituents in serum and urine were also found to interfere with electrochemical detection One of these compounds was found to be uric acid, the electrochemical nature of which has been described in earlier reports [12,13] This excretion product elutes as a large broad peak between 5-hydroxyanthranilic acid and 3-hydroxykynurenine. It does not oxidize below 500 mV For this reason we employed dual electrochemical cells connected in series, the first with an operating potential of 480 mV and the second with an operating potential of 990 mV

The chromatogram displayed in Fig 2A shows the electrochemically detected separation of metabolites in 20 m*M* acetate buffer, pH 4 50, each at a concentration of 1 mg/ml or 10 ng on-column per injection, and at an operating potential of 990 mV At this potential all compounds except kynurenine are above or at their limiting current

Since 5-hydroxyanthranilic acid could not be measured in serum samples by either detector because of interference, a third detector was installed. The programmable capability of this fluorescence detector increased the selectivity of the system and also allowed the measurement of kynurenic acid which does not exhibit electrochemical activity

TABLE I

LIMITING CURRENTS AND HALF-WAVE POTENTIALS OF TRYPTOPHAN METABOLITES

Metabolite	Limiting current (mV)	Half-wave potential (mV)
5-Hydroxyanthranilic acid	500	370
3-Hydroxykynurenine	500	430
5-Hydroxytryptophan	600	455
Kynurenine	> 1000	> 1000
3-Hydroxyanthranilic acid	700	635
Kynurenic acid	Non-electroactive	
Tryptophan	1000	810

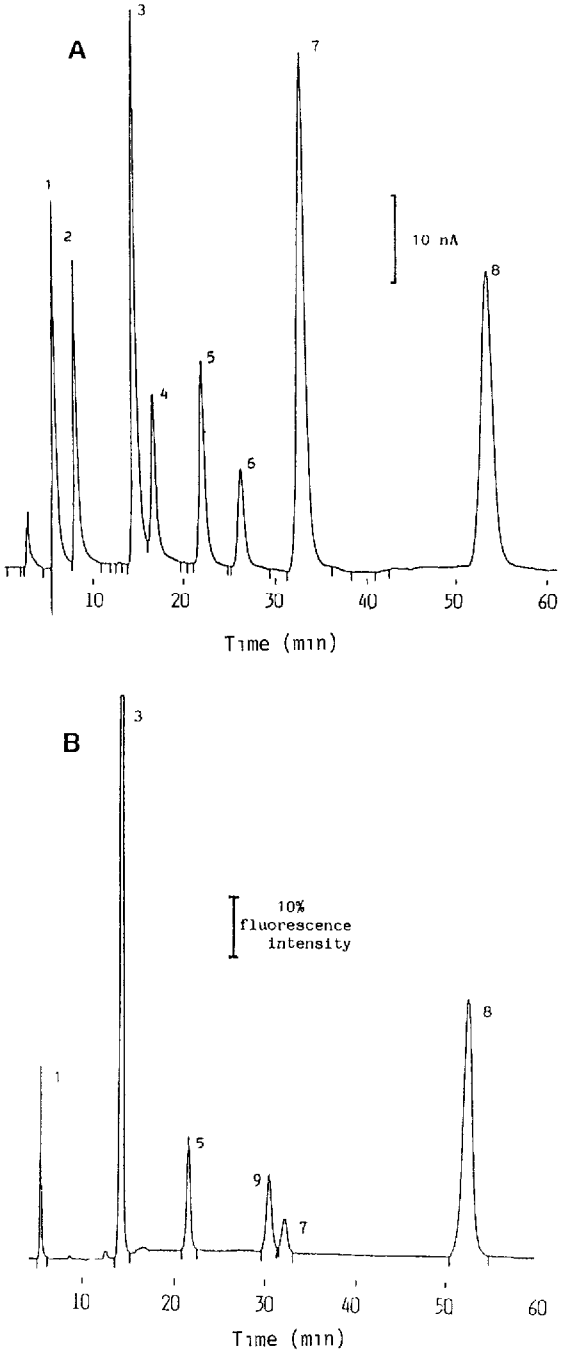

Fig 2 Chromatogram illustrating the separation of reference compounds (30 μl injected) detected (A) electrochemically at 990 mV and (B) by programmable fluorescence detection Mobile phase is 20 m*M* acetate buffer, pH 4 5 and flow-rate 0 6 ml/min Peaks 1 = 5-hydroxyanthranilic acid, 2 = 3-hydroxykynurenine, 3 = 5-hydroxytryptophan, 4 = kynurenine, 5 = 3-hydroxyanthranilic acid, 6 = xanthurenic acid, 7 = tryptophan, 8 = anthranilic acid, 9 = kynurenic acid

Of all the tryptophan metabolites investigated, only three compounds (3-hydroxykynurenine, kynurenine and xanthurenic acid) did not show any native fluorescence at the operating pH of 4 50 Fluorescence data reported elsewhere [14] indicate that these compounds do fluoresce, and also that kynurenic acid has a higher maximum than that found in the current study However, as indicated in the instrument's operating manual, the design of the fluorescence detector makes exact comparisons of spectral data, obtained on other equipment, difficult The fluorescence spectra of the metabolites at pH 4 50 are listed in Table II and the chromatogram of metabolites fluorimetrically detected in 20 m*M* acetate buffer is shown in Fig 2B

Table III lists the sensitivity and linearity of each of the metabolites expressed in picomoles injected on-column The limit of detection was taken as a signal-to-noise ratio of 2 1 The detector response was found to be linear for

TABLE II

FLUORESCENCE SPECTRA OF TRYPTOPHAN METABOLITES

Metabolite	Excitation wavelength (nm)	Emission wavelength (nm)
5-Hydroxyanthranilic acid	237	453
3-Hydroxykynurenine	Non-fluorescent at pH 4 50	
5-Hydroxytryptophan	230	340
Kynurenine	Non-fluorescent at pH 4 50	
3-Hydroxyanthranilic acid	235	450
Kynurenic acid	238	375
Xanthurenic acid	Non-fluorescent at pH 4 50	
Tryptophan	227	356
Anthranilic acid	244	403

TABLE III

PERFORMANCE CHARACTERISTICS

Compound	Detector (mV)	Sensitivity (pmol)	Linearity (pmol)	Recovery (%)	Signal-to-noise ratio
5-Hydroxyanthranilic acid	Fluorescence	6	1300	99	14
3-Hydroxykynurenine	480	9	890	50	–
5-Hydroxytryptophan	990	4	910	76	45
Kynurenine	990	96	192	–	20
3-Hydroxyanthranilic acid	990	6	1300	98	20
Kynurenic acid	Fluorescence	5	105	76	7
Xanthurenic acid	990	7	970	–	–
Tryptophan	990	2	980	–	10
Anthranilic acid	990	7	290	92	10

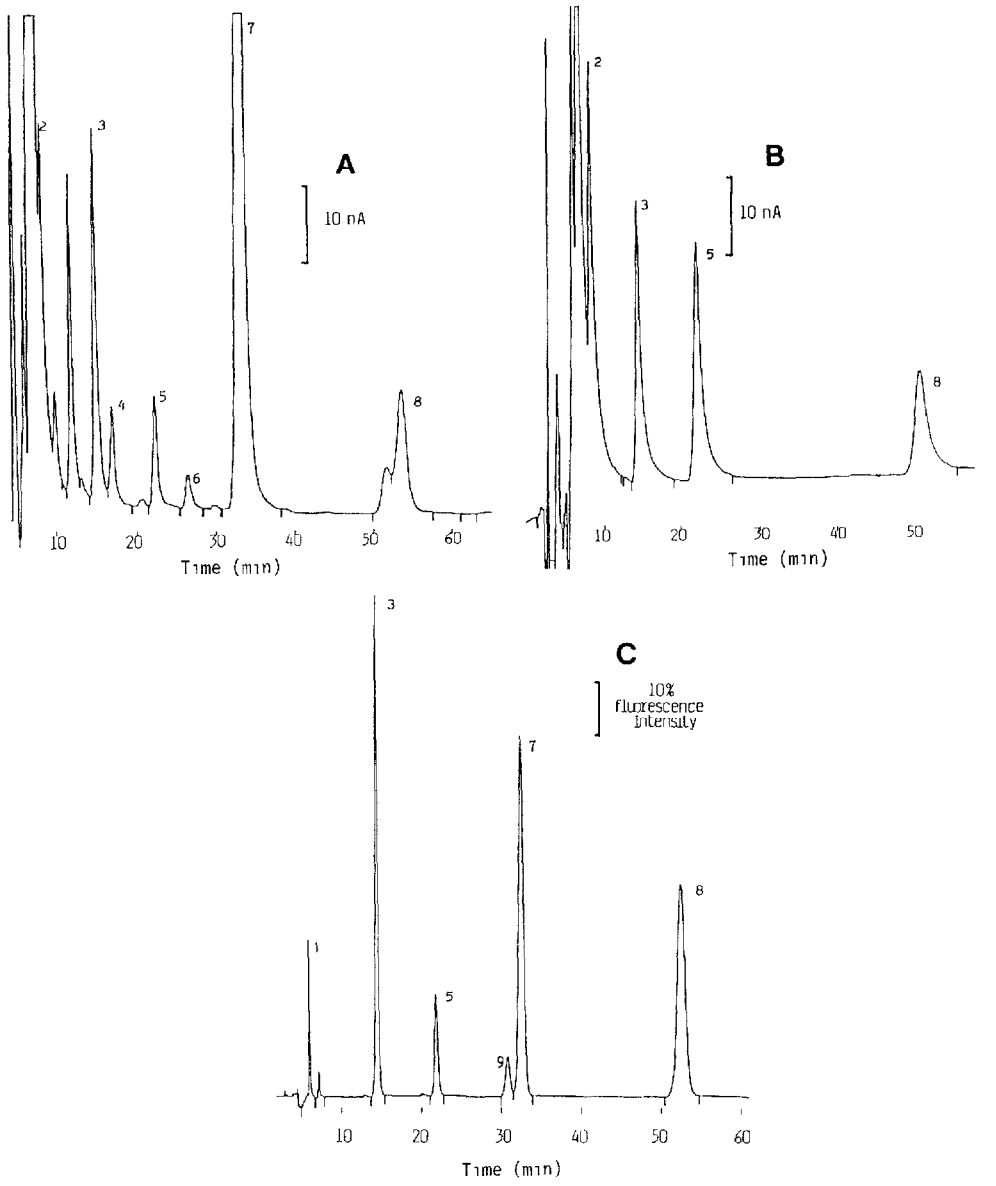

Fig 3 Chromatogram illustrating the separation of a spiked serum sample (prepared as specified in the text) as detected by the three detectors (A) electrochemical detection at 990 mV, (B) electrochemical detection at 480 mV, (C) programmable fluorescence detection Chromatographic conditions as in Fig 2

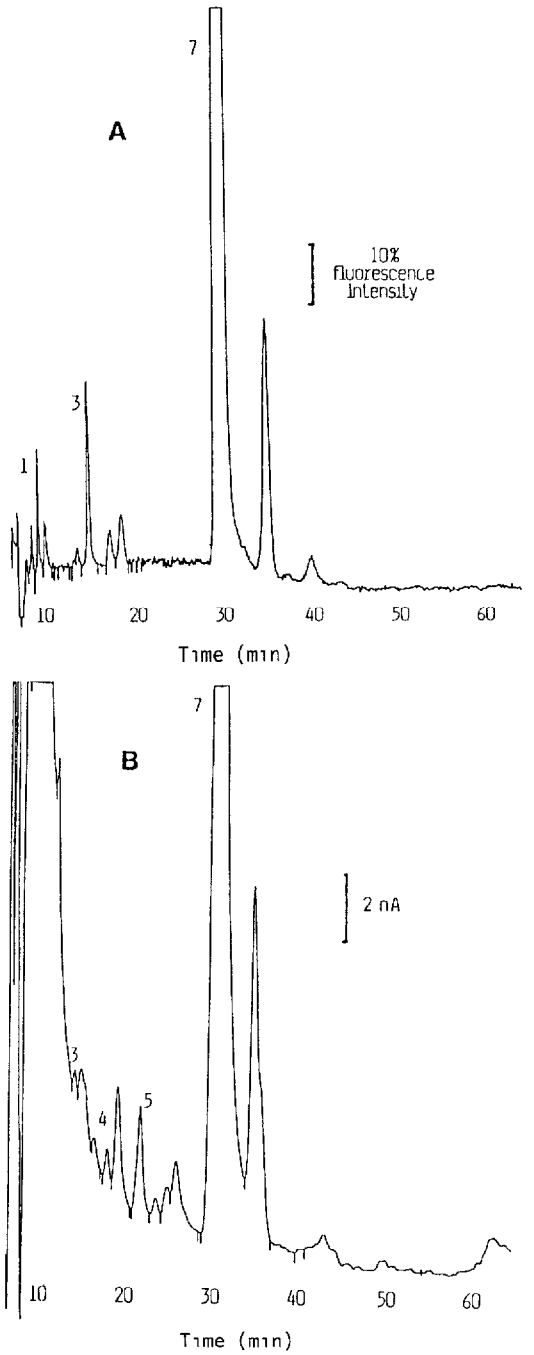

Fig 4 (A) Fluorescence chromatogram of a serum sample taken 6 h after an oral dose of 50 mg/kg tryptophan (B) Simultaneously recorded chromatogram from 990 mV electrochemical detector of the same serum sample Chromatographic conditions and sample preparation as in Figs 2 and 3

the concentration range likely to be observed in our samples, but for some metabolites larger amounts were injected and the response was still found to be linear

Fig 3 shows the chromatograms obtained from each of the three detectors, of a serum sample (1 ml) spiked with 0 5 μg of each metabolite, and precipitated with 0 67 ml of cold 10% trichloroacetic acid A 30-μl volume of supernatant was injected on-column Some large peaks were found to be present in serum which interfered with the quantitation of 5-hydroxyanthranilic acid and 3-hydroxykynurenine at 990 mV This interference was reduced by performing the oxidation at 480 mV Although 5-hydroxyanthranilic acid was completely masked at this potential, 3-hydroxykynurenine could be quantified Levels of 5-hydroxyanthranilic acid can be obtained by fluorescence detection without any interference

The method described provides a sensitive and selective assay for the major metabolites of the tryptophan–kynurenine degradation pathway and for tryptophan metabolites arising from branch pathways Using the three detectors in series, the system has been used successfully to monitor samples obtained from tryptophan loading tests, an example of which is given in Fig 4

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