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ELECTROCHEMICAL DETECTION OF TRYPTOPHAN METABOLITES FOLLOWING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Electrochemical detection of tryptophan metabolites following separation on a reversed-phase high-performance liquid chromatography column was compared with other means of detection. Of 29 compounds studied, 26 could be detected at a sensitivity comparable to that of fluorescence derivatisation procedures. Response was linear over a wide range of concentrations and selectivity was shown to be superior to ultraviolet detection when analysing urine. Additionally, it was possible to control selectivity so that only those tryptophan metabolites from the tryptophan hydroxylase pathway were detected. This is of particular value in the study of disturbances of serotonin metabolism and is unique to this type of detector.

INTRODUCTION

Abnormal metabolism of tryptophan has been associated with a number of disease states in man, including Hartnup disease[1], phenylketonuria[2], Down's syndrome[3], schizophrenia[4], and depression[5]. The identification and measurement of tryptophan metabolites in biological fluids is therefore an important aid to research into the aetiologies and treatments of these conditions. Chemical methods for the determination of these compounds have tended to be non-specific and this may partially account for some of the conflicting data in the literature[6]. With the introduction of paper chromatography the separation of complex mixtures became more reliable and, in 1962, Sprince *et al.*[7] used this technique to separate the various indoles present in urine. More recently, thin-layer chromatography has been used for this same purpose[8,9]. However, these techniques are not suitable for quantitative work[9], and attention has now turned to high-performance liquid chromatography (HPLC). Graffeo and Karger[10] separated six indoles using a reversed-phase chromatography column, and demonstrated that ultraviolet (UV) detection at 280 nm was not sufficiently selective for the analysis of these compounds in urine. They overcame this problem by measuring the native fluorescence of these indole compounds. Subsequently, other workers enhanced this fluorescence, to improve sensitivity, by forming derivatives with reagents such as *o*-phthalaldehyde, either before or after column

separation[11]. However, these derivatisation procedures require a chemical transformation stage that not only provides another source of error, but also increases analysis time. This is not a problem with electrochemical oxidation which has been successfully applied to the detection of catecholamines[12], proving to be both selective and highly sensitive. As there are structural similarities between tryptophan metabolites and the catecholamines, it was clearly worthwhile to investigate the suitability of electrochemical oxidation for the detection of the former group.

MATERIALS AND METHODS

Instrumentation

The equipment consisted of two Applied Chromatography Systems (Luton, Great Britain) constant-flow pumps (750-02) controlled by a gradient-elution programmer (750-30), a Whatman LIB injection port (Whatman Labsales, Maidstone, Great Britain), a 120 × 5 mm I.D. column packed with ODS-Hypersil (Shandon Southern Products, Runcorn, Great Britain), particle size 5 μm, and an LCA 10 electrochemical detector (EDT Research, London, Great Britain). The cell, adjusted to a volume of 0.5 μl, has a glassy carbon working electrode, the reference potential being supplied by a silver–silver chloride electrode. The detector was used in the d.c. mode throughout, and all operating potentials quoted are measured against the reference electrode potential. The CE 212 variable wavelength UV monitor (Cecil Instruments, Cambridge, Great Britain) was used as an alternative detector. The signal output from either detector was displayed on a Vitatron UR 401 linear recorder (Fisons Scientific Apparatus, Loughborough, Great Britain).

Reagents

Indole-3-carboxylic acid was obtained from Koch-Light Labs. (Colnbrook, Great Britain), and oxindole from Kodak (Liverpool, Great Britain). All other metabolites of tryptophan were purchased from Sigma (Poole, Great Britain). Stock solutions of each substance, at a concentration of 10^{-3} *M*, were prepared by dissolving the calculated quantity of the compound in 0.4 ml of acetonitrile and making up to a final volume of 20 ml with deionised water. These solutions were stored in the dark at 4°.

Three mobile phases were prepared: (a) Potassium nitrate solution (final molarity 0.05), containing 60% methanol. (b) Potassium dihydrogen phosphate buffer, 0.03 *M*, pH 5.2. (c) As (b) but containing 60% methanol.

These solutions were filtered through Whatman GF/F paper, nitrogen was bubbled through each for 5 min, and they were then degassed under vacuum; finally, the dead-space in the solvent reservoir was filled with nitrogen.

Procedure

In order to determine whether electrochemical detection is suitable for metabolites of tryptophan, 29 of these compounds were tested, in turn. The mobile phase was 0.05 *M* potassium nitrate containing 60% methanol at a flow-rate of 1.0 ml/min. The operating potential was increased step-wise from 0.4 to 1.5 V, and following the injection of 4 μl of each compound, the signal produced by the electrochemical oxidation at each potential was displayed on the chart recorder and the peak height

noted. For each compound the peak height was plotted against the applied potential.

To demonstrate the linearity and sensitivity of the detector, 5-hydroxyindole-3-acetic acid (5-HIAA) was chosen, as this substance is an end-product of tryptophan metabolism commonly found in biological material. Dilutions of the stock 10^{-3} *M* solution of 5-HIAA were prepared in 2% acetonitrile to give a series of concentrations down to $5 \cdot 10^{-7}$ *M*. Each of these solutions (5 μl) was injected onto the column, under the same conditions as in the previous experiment, and the resulting peak heights were measured and expressed in nA. The peak height, in each case, was plotted against the amount of 5-HIIAA injected.

The specificity of the detector was compared with UV detection for the analysis of tryptophan metabolites in biological material. A random sample of urine (20 ml) was saturated with sodium chloride, adjusted to pH 4 with molar hydrochloric acid and extracted into 100 ml of diethyl ether. After evaporation of the ether, the residue was dissolved in 0.4 ml of methanol and stored at 4°. The chromatographic system was programmed for gradient elution using the two potassium phosphate buffers described. The methanol content of the mobile phase was increased from zero to 60% in a three-stage gradient as shown in Fig. 1. At a flow-rate of 0.7 ml/min, the urine extract (2 μl) was injected onto the column and the eluted substances were detected electrochemically at an operating potential of 1.20 V, full scale deflection being given by a current of 1 μA. The procedure was then repeated using UV detection at 280 nm at a sensitivity of 0.1 a.u.

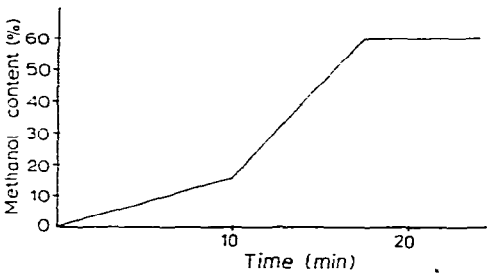

Fig. 1. Gradient-elution programme: percentage of methanol in the mobile phase as a function of time following injection of the sample onto the column.

RESULTS AND DISCUSSION

The relationship between peak height and applied potential for 5-HIAA is shown in Fig. 2. From this polarographic curve the half-wave potential ($E_{\frac{1}{2}}$) for 5-HIAA was estimated to be 0.80 V. The half-wave potentials of the other tryptophan metabolites were estimated in a similar fashion and are shown in Table I.

The electrochemical detector achieves its selectivity by detecting only those compounds which are electrochemically active at the operating potential chosen. This is particularly important in the analysis of biological material as many biological compounds are not electrochemically active and will therefore not interfere. If the compounds of interest can be detected at a lower operating potential than other electrochemically active substances, then the specificity can be improved still further by selecting an operating potential which is too low to detect the interfering compounds, yet high enough to detect the substances under investigation. This point is

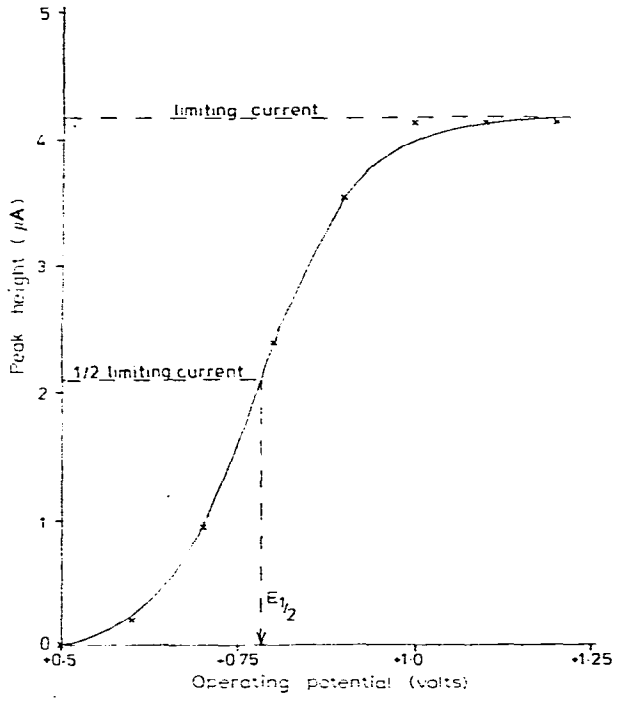

Fig. 2. Relationship between the potential applied to the working electrode (measured against the reference electrode potential) and the height of the peak obtained following the injection of 4 nmoles of 5-HIAA. The half-wave potential ($E_{1/2}$) is determined as that voltage at which the current produced by the oxidation is half of the limiting current.

demonstrated in Table I in which it can be seen that by choosing an operating potential of 1.0 V, it is possible to detect only those tryptophan metabolites with a 5-hydroxy group on the phenol ring, this being the site of oxidation. This allows the selective detection of metabolites in the tryptophan hydroxylase pathway which plays an important role in neurotransmission in the central nervous system. At an operating potential of 1.40 V, twenty of the remaining tryptophan metabolites can be detected, possibly by oxidation of the ring nitrogen to N^+. Three compounds; oxindole, indole-3-carboxaldehyde and indole-3-carboxylic acid could not be oxidised under the conditions used. Of these, only indole-3-carboxylic acid has been reported to be present in biological material[8] and it is possible that its known instability in solution[13] is the reason for the failure to detect it.

In order that HPLC followed by electrochemical detection can be used as a quantitative technique, the detector response must be linearly related to solute concentration as is shown in Fig. 3. The design of the electrochemical cell allows approximately 85% of the solute in the column eluent to react at the glassy carbon surface. However, since this figure will vary each time the detector is set up, standards must be included with each set of experiments.

It can also be seen from Fig. 3 that the limit of detection for 5-HIAA is in the region of 2 pmoles, this limit being taken as twice the level of baseline noise. It is assumed that the detection limits of the other tryptophan metabolites are similar

TABLE I

HALF-WAVE POTENTIALS ($E_{1/2}$) FOR TRYPTOPHAN METABOLITES

Compound	$E_{1/2}$ *(V)*
5-Hydroxy indole	0.80
5-Hydroxy-indole-3-acetic acid	0.80
5-Hydroxy-tryptophol	0.80
N-Acetyl 5-hydroxy-tryptamine	0.80
5-Hydroxy-tryptamine	0.85
5-Hydroxy-tryptophan	0.85
Indoxyl sulphate	1.10
Tryptophol	1.15
5-Methoxy-tryptophol	1.15
3-Methyl indole	1.15
N-Methyl-tryptamine	1.15
Indole-3-acrylic acid	1.15
Indole-3-acetic acid	1.15
Indole-3-pyruvic acid	1.15
Indole-3-propionic acid	1.15
5-Methoxy-indole-3-acetic acid	1.15
Indole-3-lactic acid	1.20
N-Acetyl-tryptophan	1.20
5-Methoxy-tryptamine	1.20
Tryptophan	1.25
Tryptamine	1.25
Indole-3-acetamide	1.25
Indole-3-acetonitrile	1.25
Indole-3-acetaldehyde	1.25
Indoxyl acetate	1.25
Indole	1.30

since the limiting current plateaus, determined as in Fig. 2, varied only slightly in all cases. This shows a thirty-fold increase in sensitivity over measurement of native fluorescence[10], and is comparable to the sensitivity obtained with the derivatisation of tryptophan metabolites with *o*-phthalaldehyde[11].

The selectivity of electrochemical detection when dealing with biological material was mentioned earlier and is demonstrated by the two recorder traces shown

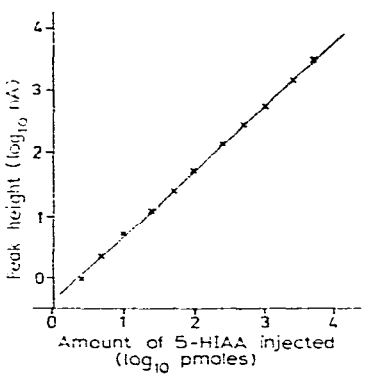

Fig. 3. Proportionality between the amount of 5-HIAA injected and the height of the resulting peak.

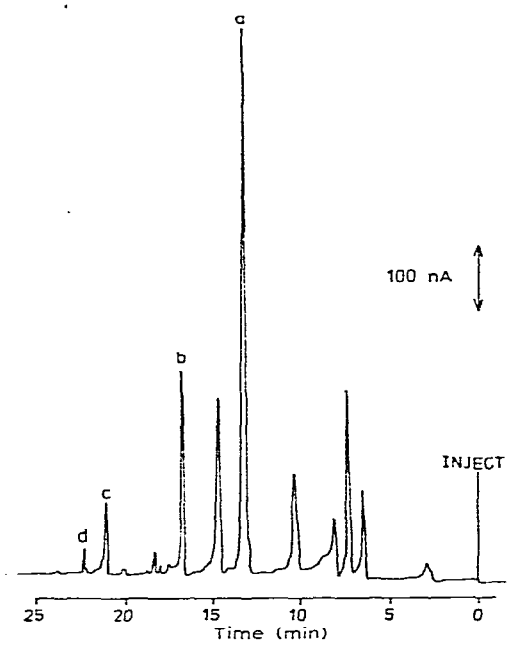

Fig. 4. Chromatogram of an ether extract of acidified urine with electrochemical detection. Column: ODS-Hypersil, mobile phase: gradient elution, potassium phosphate buffer (0.03 *M*, pH 5.2) with increasing concentration of methanol as shown in Fig. 1, flow-rate 0.7 ml/min, detector operating potential 1.20 V, sensitivity 1 μA. Peaks: a = 5-HIAA; b = indole-3-lactic acid; c = indole-3-acetic acid; d = N-acetyl tryptophan.

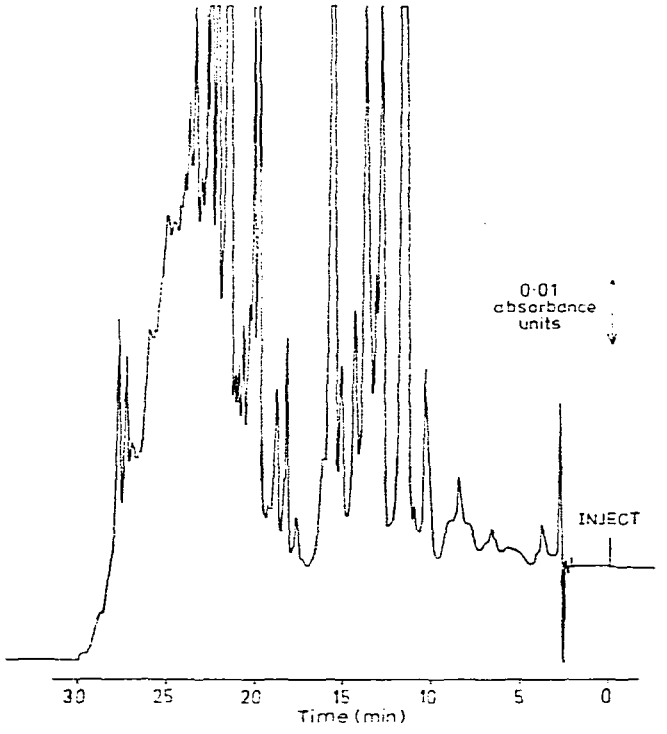

Fig. 5. As Fig. 4 but with UV detection at 280 nm, sensitivity 0.1 a.u.

in Figs. 4 and 5. Using UV detection following the column separation of an ether extract of urine (Fig. 5), very little information could be obtained owing to the large number of peaks present and to the drifting baseline caused by the refractive index effects of the methanol gradient. With electrochemical detection, even at a relatively high, and therefore non-selective potential, the number of peaks obtained is far fewer, and as shown in Fig. 4, several of these peaks have been identified as tryptophan metabolites by standard addition techniques (unpublished results). The baseline also remains constant provided that the conducting solute concentration is not varied throughout the gradient.

In conclusion, electrochemical detection has been shown to be a useful alternative to fluorescence detection for the analysis of tryptophan metabolites. It is as sensitive as fluorescence derivatisation procedures, linear over a wide range of concentration, and highly selective, particularly for the important metabolites from the tryptophan hydroxylase pathway.

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