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DETERMINATION OF INDICAN AND TRYPTOPHAN IN NORMAL AND URAEMIC PATIENTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A NEW ELECTROCHEMICAL DETECTOR

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SUMMARY

A simple analytical procedure has been developed for the determination of indican and tryptophan in biological fluids by reversed-phase liquid chromatography using a new electrochemical detector consisting of a tubular anode obtained by moulding graphitized carbon black and polyethylene. The hydrodynamic voltammetry of these compounds has been carried out and it has been found that, by operating in isocratic conditions with phosphate buffer (pH 4.0)—methanol (93:7), the reported compounds can be determined directly. The procedure can be applied for the determination of the free compounds on ultrafiltered serum as well as of their total content on serum deproteinized with methanol. Levels of both compounds in normal and uraemic patients have been measured and the relative ratios between free and total content yield a useful marker for patients with renal disease. The limits of quantitation of indican and tryptophan in serum were 5 and 10 ng/ml, respectively. The within-day assay coefficient of variation for total indican and tryptophan ranged from 3.0 to 3.6% and from 3.8 to 4.1%, respectively. The day-to-day assay coefficient of variation for total indican and tryptophan ranged from 3.4 to 3.7% and from 4.6 to 5.0%, respectively.

INTRODUCTION

Patients with chronic renal failure have many metabolic abnormalities, and several analytical procedures [1—10] have been proposed to evaluate specific metabolites either in serum or plasma. In recent years, attention has been paid to naturally fluorescent compounds present in uraemic plasma, which can be separated by high-performance liquid chromatography (HPLC) [8, 10]. Some of them have been identified as indole derivates and related to the metabolism of amino acids. The determination of these species is a rather complicated task

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and difficulty has been experienced when plasma is analysed by HPLC to interpret the chromatograms obtained by using conventional detectors such as ultraviolet and fluorescence.

To achieve adequate detection sensitivity while maintaining the relative simplicity of this assay, a new electrochemical detector has been applied for the concurrent liquid chromatographic analysis of indican and tryptophan in normal and uraemic serum. This investigation has been carried out to find an analytical procedure for fast and reliable determination of these compounds. The developed method has been applied to the determination of free and protein-bound indican and tryptophan in normal and uraemic patients.

EXPERIMENTAL

Apparatus

A Series 10 liquid chromatograph equipped with a Rheodyne Model 7125 injector with a 20-μl loop (all from Perkin-Elmer, Norwalk, CT, U.S.A.) and with an LC-4B electrochemical detector (Bioanalytical Systems) was used with an LC-17 electrochemical cell. The thin-layer cell with glassy carbon electrode was replaced by inserting a tubular electrode as anode, obtained by moulding graphitized carbon black and polyethylene [11]. This recently developed electrode has some advantages over the glassy carbon electrode; it has a higher response and a better signal-to-noise ratio. The cell of the electrochemical detector consisted of a tubular anode, an auxiliary electrode and an Ag/AgCl reference electrode. The electrochemical detector was operated at a potential of +0.9 V vs. the Ag/AgCl reference electrode.

A 25 cm × 4.6 mm I.D. column packed with 10-μm C_{18} reversed-phase (Erbasil, Carlo Erba, Milan, Italy) was used. The analytical column was fitted with a 5 cm × 4.6 mm I.D. precolumn, packed with 30—38 μm diameter Pelliguard from Supelco (Bellefonte, PA, U.S.A.). The mobile phase was methanol—phosphate buffer (7:93), pH 4.0, at a flow-rate of 2 ml/min.

Reagents and standards

All reagents were of ACS-certified grade. Indican (3-indoxyl sulphate) and the other standards investigated were from Sigma (St. Louis, MO, U.S.A.).

Stock indican and tryptophan solutions (1 g/l) were prepared in water and diluted to obtain the working standards. Standard solutions of the other substances were similarly prepared. Chromatographic-grade methanol was obtained from Carlo Erba. Water used for the preparation of the mobile phase was purified by passing it through Norganic cartridges (Millipore, Bedford, MA, U.S.A.). Phosphate buffer (pH 4.0) was prepared by dissolving 2.72 g of potassium dihydrogen phosphate in 1 l of fresh distilled water and adjusting the pH to 4.0 with phosphoric acid.

Preparation of serum samples

The serum was taken from normal subjects and patients with chronic renal disease. Aliquots of serum were divided into two portions, one was used to measure free indican and tryptophan and the other to determine their total content. For the determination of the former, 500 μl of serum were ultra-

filtered through an Amicon membrane (Centriflo CF-50) and centrifuged at 2000 *g* for 10 min; for the latter, 200 μl of methanol were added to 100 μl of serum in a screw-capped vial. After vortex-mixing, the tubes were centrifuged at 6000 *g* for 10 min and the resulting supernatant was analysed. In both cases, a 10—20-μl sample was injected.

Quantitation

The assignment of peak identities was based on retention times and co-chromatographing with the reference compounds. Quantitative determination has been carried out by comparing the peak height of indican and tryptophan in the sample with that of a standard solution chromatographed under the same operating conditions.

RESULTS AND DISCUSSION

Electrochemical behaviour

In order to apply an electrochemical detection system to indican and tryptophan in a flowing system, their electrochemical behaviour has been studied by anodic and cyclic voltammetry.

Cyclic voltammetry was performed at a sweep-rate of 20 mV/s in the reported phosphate buffer at pH 4.0; anodic voltammetry was investigated

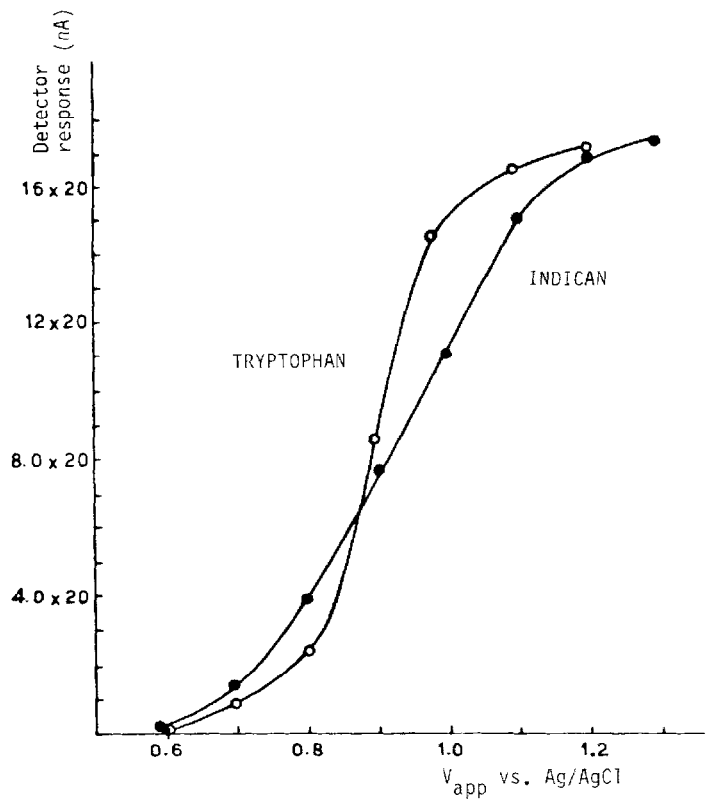

Fig. 1. Response of the graphitized carbon black electrochemical detector for indican and tryptophan at various applied potentials.

in various solutions in the pH range 2—7. The behaviour by cyclic voltammetry is similar to that observed with glassy carbon; by anodic voltammetry, indican and tryptophan yield well defined waves as shown in Fig. 1. The diffusion current has been found proportional to concentration in the investigated range $1.98 \cdot 10^{-6}$—$6 \cdot 10^{-6}$ *M*.

The information acquired indicated the feasibility of this electrochemical sensor for the determination of indican and tryptophan by liquid chromatography; it was used at a potential of +0.9 V vs. the Ag/AgCl reference.

Experiments have been carried out to optimize the analytical variables relative to their determination by HPLC.

Detector response

The electrochemical detector response, measured in terms of the peak height, is not affected by ionic strength; appreciable differences have been observed by operating with solutions at various pH. The higher response has been observed in the pH range 4–5 (Fig. 2). A similar effect has been observed by Molnar and Horvath [12] and it seems to be related to the dependence upon oxidation of the catechol ring, which has been shown to be enhanced by increased pH.

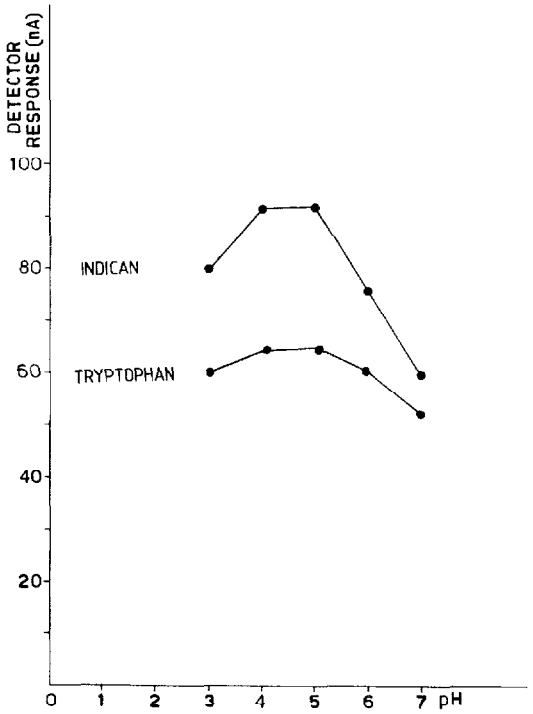

Fig. 2. Detector response of the graphitized carbon black detector for indican and tryptophan at various pH values.

Chromatographic behaviour

The chromatographic behaviour of indican and tryptophan has been investigated in various media and it has been found that phosphate media are

TABLE I

RETENTION TIMES OF COMPOUNDS OF INTEREST IN URAEMIC PATIENTS: DEPENDENCE OF RETENTION TIMES UPON THE MOLARITY OF THE PHOSPHATE BUFFER (pH 4.0) USED

Molarity phosphate buffer	Retention time (min)		
	5-Hydroxytryptophan	Indican	Tryptophan
0.01	4.4	5.1	9.5
0.015	4.3	5.5	9.2
0.02	4.3	6.6	9.2
0.05	4.3	8.0	9.2
0.1	4.3	9.2	9.2

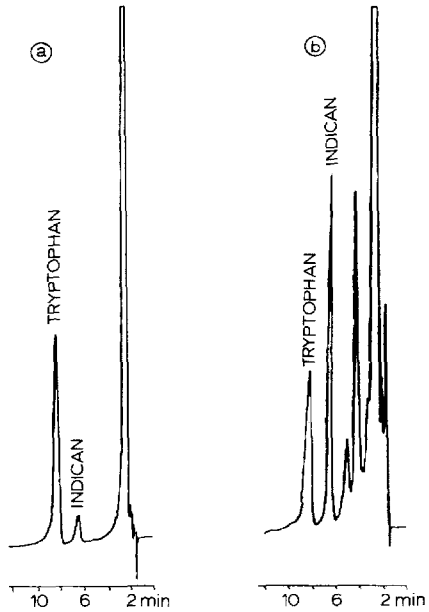

Fig. 3. Representative chromatograms of (a) normal patient containing 0.13 μg/ml indican and 1.41 μg/ml tryptophan (range 10 nA) and (b) uraemic patient containing 3.22 μg/ml indican and 2.01 μg/ml tryptophan (range 20 nA).

the more suitable eluents; however, both pH and ionic strength affect the chromatographic behaviour, as shown in Table I, where the retention times of indican, tryptophan and 5-hydroxytryptophan are collected; they have been obtained by using eluents of the same pH but with increasing phosphate concentration. It has been observed, however, that at high phosphate concentration the separation of indican and tryptophan is not feasible, so that the medium described above has been selected.

The response of the electrochemical detector is linearly related to the mass of indican and tryptophan in the range 0.1—105 ng. Typical chromatograms of normal and uraemic serum under the described operating conditions are shown in Fig. 3a and b.

TABLE II

RETENTION TIMES OF POTENTIAL INTERFERENCE

Compound	Retention time (min)
L-Norepinephrine	2.2
Epinephrine	2.6
L-Tyramine	2.8
Dopamine	3.8
L-5-Hydroxytryptophan	4.3
Indican	6.6
L-Tryptophan	9.2
L-5-Hydroxytryptamine	10.0
3,4-Dihydroxyphenylethyl alcohol	10.5
5-Hydroxyindole-3-acetic acid	17.2
Vanilmandelic acid	19.4
Homovanillic acid	26.2
Tryptamine	36.0
5-Methoxytryptamine	60.0
3-Methoxytyramine	60.0

Specificity

In order to evaluate the possibility of using this procedure for the determination of indican and tryptophan in biological fluids and to evaluate possible interference, a number of compounds that might be present in biological fluids have been investigated under the same operating conditions. The retention times of the investigated compounds are reported in Table II; none of these substances interfere with the analysis of indican and tryptophan.

Treatment of the sample

As described, the determination can be carried out for the evaluation of free and protein-bound indican and tryptophan. Deproteinization, which is required for the determination of the total compounds, can be carried out with methanol, acetonitrile or trichloroacetic acid. Deproteinization with methanol is preferred because the use of acid does not allow the samples to be stored for a long period of time, as degradation may take place.

Recovery and precision

The recovery of indican and tryptophan and the precision of the analytical procedure has been assessed by analysing aliquots of serum of normal and chronic renal failure subjects. The mean analytical recovery of total indican was 98.8% (range 97.9–100.1%) and for tryptophan it was 98.5% (range 98.1–99.6%); these values were calculated by comparing results for aqueous solutions. In Table III, the results for within-day and day-to-day assays are summarized.

Sensitivity

The limits of sensitivity (signal-to-noise ratio = 3) for indican and tryptophan were 5 and 10 ng/ml, respectively. At such concentrations, the coefficient of variation for indican was 9.7%, while the corresponding value for tryptophan was 10.5%.

TABLE III

PRECISION OF THE ASSAY FOR TOTAL INDICAN AND TRYPTOPHAN

Compound	Concentration (mean ± S.D., n = 6) (μg/ml)	Coefficient of variation (%)
Within-day precision		
Indican	0.56 ± 0.02	3.6
	33.1 ± 0.99	3.0
Tryptophan	1.33 ± 0.05	3.8
	4.15 ± 0.17	4.1
Day-to-day precision		
Indican	0.54 ± 0.02	3.7
	33.8 ± 1.15	3.4
Tryptophan	1.31 ± 0.06	4.6
	4.16 ± 0.21	5.0

Applications

The free and total levels of indican and tryptophan, measured in normal subjects and chronic renal failure patients, are reported in Tables IV and V. According to the analytical results, it has been found that "free" indican and tryptophan in the serum of normal subjects ranged from 0.05 to 0.13 μg/ml and from 0.80 to 2.00 μg/ml, respectively, whereas "total" indican and tryptophan ranged from 0.53 to 1.17 μg/ml and from 10.89 to 17.61 μg/ml, respectively.

In the serum of patients with chronic renal failure, there is a noticeable increase of free and total indican and tryptophan; the former ranged from 1.80 to 6.98 μg/ml (free indican) and from 1.17 to 2.88 μg/ml (free tryptophan), while the total indican ranged from 10.27 to 34.61 μg/ml and total tryptophan

TABLE IV

SERUM LEVELS OF INDICAN AND TRYPTOPHAN IN NORMAL PATIENTS

Subject No.	Serum level (μg/ml)			
	Indican		Tryptophan	
	Free	Total	Free	Total
1	0.09	0.55	1.35	12.65
2	0.10	0.75	1.21	13.76
3	0.07	0.53	1.49	14.41
4	0.11	1.17	1.81	14.51
5	0.10	0.83	1.12	15.12
6	0.11	0.65	2.00	17.61
7	0.13	1.12	1.41	15.87
8	0.13	0.91	1.04	10.89
9	0.05	0.53	0.80	15.94
10	0.09	0.62	1.70	13.39
Mean	0.10	0.77	1.39	14.41

TABLE V

SERUM LEVELS OF INDICAN AND TRYPTOPHAN IN URAEMIC PATIENTS

Subject No.	Serum level (μg/ml)			
	Indican		Tryptophan	
	Free	Total	Free	Total
1	5.29	33.08	2.20	4.10
2	4.76	32.83	2.12	4.18
3	2.18	19.05	1.17	4.00
4	3.99	25.12	2.35	4.56
5	3.22	16.12	2.01	4.35
6	6.52	31.50	1.94	3.18
7	2.93	17.59	2.76	5.32
8	2.98	19.35	2.54	5.28
9	1.90	13.31	1.90	3.80
10	3.62	23.14	1.57	3.18
11	4.58	30.22	2.26	3.85
12	3.81	20.21	2.86	6.85
13	2.32	13.47	1.81	3.08
14	6.93	32.84	2.28	3.78
15	2.64	16.77	2.71	5.35
16	3.69	21.65	2.31	4.66
17	4.86	26.76	2.07	3.60
18	2.16	14.25	2.36	4.36
19	2.20	12.14	1.61	3.21
20	3.71	18.54	2.26	3.85
21	3.13	25.34	2.69	4.04
22	3.86	23.57	2.05	3.91
23	4.87	29.25	1.84	3.41
24	5.57	27.70	2.07	3.61
25	2.17	11.94	1.81	3.70
26	2.00	11.38	2.88	5.62
27	1.80	11.75	2.83	6.22
28	3.32	10.27	2.17	4.68
29	5.09	34.61	2.80	5.50
30	4.17	28.76	1.78	3.91
Mean	3.68	21.75	2.20	4.30

TABLE VI

SERUM LEVEL RATIOS BETWEEN INDICAN AND TRYPTOPHAN IN NORMAL SUBJECTS AND URAEMIC PATIENTS

	Serum level ratio	
	Normal	Uraemic
Free indican/total indican	0.13	0.17
Free tryptophan/total tryptophan	0.10	0.51
Free indican/free tryptophan	0.70	1.67
Total indican/total tryptophan	0.05	5.12

from 3.80 to 6.85 μg/ml. In Table VI, serum level ratios of the mean value obtained for indican and tryptophan for normal subjects and uraemic patients are presented. These ratios indicate that more than the absolute value of either indican or tryptophan determined in a specific subject, the ratios of free indican to free tryptophan and total indican to total tryptophan might be used to evaluate the conditions of a patient.

REFERENCES

1 E.J. Knudson, Y.C. Lau, H. Veening and D.A. Dayton, Clin. Chem., 24 (1978) 686.
2 A.O. Vladutiu, E.E. Cunningham and J. Walshe, Clin. Chem., 24 (1978) 1084.
3 H.A. Schwertner, S.T. Weintraub and S.B. Hawthorne, Clin. Chem., 26 (1980) 1927.
4 A. Saito, T. Niwa, K. Maeda, K. Kobayashi, Y. Yamamoto and K. Ohta, Am. J. Clin. Nutr., 33 (1980) 1402.
5 A.O. Vladutiu, Clin. Chem., 27 (1981) 1139.
6 A.C. Schoots, F.E.P. Mikkers, H.A. Claessen, R. De Smet, N. Van Landschool and S.M.G. Ringoir, Clin. Chem., 28 (1982) 45.
7 H.A. Schwertner, Nephron, 31 (1982) 209.
8 P. Faguer, N.K. Man, G. Cueille, S. Di Giulio and J.L. Funck-Brentano, Clin. Chem., 29 (1983) 703.
9 J.S. Swan, E.Y. Kragten and H. Veening, Clin. Chem., 29 (1983) 1082.
10 A.L. Barnett and H. Veening, Clin. Chem., 31 (1985) 127.
11 A. Liberti, M. Mascini and C. Morgia, Anal. Chim. Acta, 173 (1985) 157.
12 I. Molnar and Cs. Horvath, Clin. Chem., 22 (1976) 1497.