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THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF INDOLIC TRYPTOPHAN METABOLITES IN HUMAN URINE USING SEP-PAK C_{18} EXTRACTION

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SUMMARY

Tryptophan and some of its indole metabolites were separated by thin-layer chromatography, stained with the Van Urk—Salkowski reagent, and quantitated by scanning densitometry. The application of this technique for the detection of the indoles in urine samples, employing Sep-Pak C_{18} cartridges for extraction, was demonstrated. The proposed method is simple and accurate. The detection limits were 2 µg/ml 5-hydroxytryptophan, 1.75 µg/ml 5-hydroxyindolyl-3-acetic acid, 1.5 µg/ml tryptophan, 0.8 µg/ml indolyl-3-acetic acid, 0.9 µg/ml indolyl-3-butyric acid, 1.75 µg/ml serotonin, and 1.25 µg/ml tryptamine.

INTRODUCTION

An increasing number of investigations involving the determination of indole compounds, especially 5-hydroxyindoles, in biological fluids is being undertaken in several laboratories. Abnormally increased amounts of tryptophan metabolites are associated with many diseases such as malignant carcinoid tumours, schizophrenia, Parkinson's disease, and malignant hyperthermia [1, 2].

Methods of analysis for the determination of tryptophan and its metabolites in biological fluids include thin-layer chromatography (TLC) [3—6], gas—liquid chromatography [7], UV and fluorescence spectrometry [8, 9]. Most of these methods are lengthy and lack sufficient resolving power and specificity. More modern techniques such as gas chromatography—mass spectrometry [10] and radioimmunoassay [11] have been used with varying degrees of success. Recently, reversed-phase high-performance liquid chro-

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matography (HPLC) with fluorescence [12] and electrochemical [13, 14] detection has been successfully employed to determine tryptophan and its metabolites in biological fluids and tissues. TLC has not yet found extensive use in the study of metabolic diseases. Perhaps the main reason is the time-consuming preliminary treatment of the sample necessary to reduce the amount of interfering substances. Although many procedures have been used for analyzing indole compounds in biological fluids by TLC, some are little suited to quantitative determination because of the distorted chromatograms obtained if the samples are not pretreated [15].

This paper describes a convenient TLC method for the assay of tryptophan metabolites in human urine avoiding the need for multiple extractions. The method is based on a rapid extraction procedure from deproteinized urine using Sep-Pak C_{18} cartridges. In particular, we report the separation of tryptophan (TRP), 5-hydroxytryptophan (5-HTRP), tryptamine (T), serotonin (5-HT), indolyl-3-acetic acid (IAA), 5-hydroxyindolyl-3-acetic acid (5-HIAA), and their determination by scanning densitometry using indolyl-3-butyric acid (IBA) as internal standard. Graffeo and Karger [16] developed a rapid and sensitive analytical method for the analysis of the same six indoles in urine. Unfortunately, the selectivity, which is diagnostically important, does not seem to be fully satisfactory for TRP and 5-HIAA (for the latter only at low concentrations) which are masked by the large fluorescence peak in the urine background. Our aim was to develop a simple and rapid assay for tryptophan metabolites which would not offer such a drawback. Briefly, the selectivity arises from three processes: (A) the initial step of sample clean-up, (B) the separation effected by TLC, and (C) the specific reagent used for staining chromatograms.

EXPERIMENTAL

Apparatus

A Camag (Muttenz, Switzerland) TLC/HPTLC 76500 scanner was used for all quantitative measurements, the absorbance of the stained spots being read at 620 nm. In all determinations the instrument was zeroed on a blank area of the thin-layer. A constant drift of the baseline was always observed. The peaks obtained were quantitatively integrated by a Spectra-Physics Minigrator (Darmstadt, G.F.R.). TLC was performed on 0.25-mm precoated layers of silica gel G-60 F_{254} (Merck, Darmstadt, G.F.R.). Aliquots (0.5—1.5 μl) of the solutions to be assayed were spotted quantitatively with a Camag micro-applicator. The plates were developed at room temperature in a twin-trough chamber. Extractions were performed using Sep-Pak C_{18} cartridges, containing octadecylsilane bonded-phase packing (Waters Assoc., Milford, MA, U.S.A.).

Reagents

Analytical reagent grade chemicals were used without further purification. All solutions were made up with deionized and distilled water. All reference compounds were purchased from Sigma (St. Louis, MO, U.S.A.). Stock standard solutions in methanol—citrate buffer (0.1 *M*, pH 4) (1:1, v/v) were prepared containing 510 μg/ml TRP, 550 μg/ml 5—HTRP, 400 μg/ml T, 700

μg/ml 5-HT, 175 μg/ml IAA, 475 μg/ml 5-HIAA, and 200 μg/ml IBA. All solutions were kept refrigerated in tinfoil-wrapped amber bottles, and prepared fresh every four weeks. Working standard solutions were made by dilution to appropriate concentrations with methanol, and discarded after a single use.

Chromatographic procedure

Bright light was avoided throughout the procedure. After spotting, the plate was air-dried and placed in the empty trough of a twin-trough chamber, the second trough containing *n*-hexane. After 15 min of preconditioning, the layer was developed with the solvent system acetone—33% aqueous dimethylamine solution—isopropanol (1.8:2.5:5.6), the run being 9 cm. Then the plate was dried until all traces of solvent had evaporated (10 min). Before staining, the chromatogram was examined under UV light for fluorescing and/or quenching spots. After this examination, the plate was dipped into a solution of the Van Urk—Salkowski reagent [17] for 10 sec, removed and blotted with a dry paper towel. The plate was then heated for 5 min in a 100°C oven connected with a vacuum pump, removed and immersed three times in distilled water to be washed. After the last wash, the plate was dried at 45°C and the stained spots were quantitated by scanning densitometry. The plate was also sprayed with the chromogenic reagent, but the calibration graphs so obtained showed lower correlation coefficients in comparison with those obtained by the dipping procedure, though the slopes were the same. As a consequence, the dipping method was preferred for staining indole compounds.

Extraction procedure

All manipulations were carried out in the dark allowing only slight indirect bulb light. To 50-ml tubes containing 10 ml of urine 1 ml of aqueous zinc sulphate solution (100 g/l) and 1 ml of 1 *M* aqueous sodium hydroxide solution were added. The sample was vortexed for 30 sec to ensure good protein precipitation; 12 ml of 0.2 *M* phosphate buffer (pH 6) were then added and the tubes were centrifuged. The clear supernatant was made up to 25 ml with phosphate buffer; 5-ml aliquots were extracted by passing the supernatant through a Sep-Pak C_{18} cartridge fitted to a Luer-Lok glass syringe. The Sep-Pak C_{18} cartridge was prepared by flushing with 5 ml of methanol followed by 5 ml of aqueous phosphate buffer (0.2 *M*, pH 6). After the deproteinized sample was passed through the cartridge, it was washed with 1.8 ml of redistilled water and 2 ml of methanol. The latter solvent eluted the indole compounds from the cartridge. After washing with a further 5-ml of methanol the cartridge was ready for re-use. The 2-ml methanol fraction was evaporated to dryness in a rotary vacuum evaporator at 40°C. The residue was redissolved in 100 μl of methanol and taken for chromatography.

Preparation of standard curves

The standard curves were prepared by spiking urine samples with varying amounts of TRP, 5-HTRP, T, 5-HT, IAA, 5-HIAA, and with 10 μg/ml IBA as internal standard. The supplemented samples were assayed by the extrac-

tion procedure described above. Results for the standard curves were calculated using the internal standard method. A control urine extract containing only the internal standard was used to estimate the background level associated with each compound. These values were used as corrections for the data obtained before any statistical analysis was carried out. The relative weight response values for each of the tested compounds were determined by at least five independent analyses of calibration standards.

RESULTS AND DISCUSSION

Chromatographic separation

Prior to the analysis of urine samples the chromatographic conditions were optimized for the separation of the six indolic compounds and the internal standard. The solvent system used allowed the effective separation of all the indoles examined as can be seen from the R_F values reported in Table I. Under the conditions described the R_F values were quite reproducible, as demonstrated by the coefficient of variation which was less than 2%. In contrast to other researchers [18] we found no loss of indolic compounds during chromatography.

TABLE I

CHROMATOGRAPHIC PROPERTIES OF INDOLE METABOLITES OF TRYPTOPHAN

Compound	$R_F \times 100$	RRF*	Limit of detection (ng)
5-HTRP	33	0.11	40.0
5-HIAA	42	0.16	35.0
TRP	50	0.37	30.0
IAA	60	0.70	25.0
IBA	67	1.00	20.0
5-HT	80	0.71	35.0
T	93	0.41	25.0

*Relative response factor, derived from the mole ratio, compared to the internal standard IBA. Each value is an average of five independent analyses (C.V. = 6%).

Calibration

Calibration curves for the determination of the tested compounds by TLC were prepared by spotting different amounts of the seven indoles and plotting peak areas against concentration. Altogether almost identical linear calibration curves, passing through the origin by extrapolation, were obtained by diluting the stock standard solutions. Linearity was observed up to 1.5 nmol for IAA and IBA, 3.75 nmol for TRP, 5-HTRP, 5-HIAA and T, and 5 nmol for 5-HT. The wide linear range is more than adequate for tryptophan metabolites and for the clinical investigation of urinary concentrations of indole substances. Allowing a signal-to-noise ratio of 2 the detection limits were estimated and the results are reported in Table I. In practice twice these amounts can be easily quantitated. The precision of the proposed method was deter-

mined by spotting the same amount of each of the seven indoles, varying from 100 to 700 ng, and performing five independent runs. The coefficients of variation averaged less than 8%.

Sample clean-up for TLC analysis

A very important prerequisite for TLC analysis of urine is the preliminary treatment of the sample in order to eliminate the bulk of interfering substances. This is of special importance in relation to the assay of tryptophan metabolites which are difficult to analyze because of their overall lability, as well as their exceedingly small quantities in biological fluids. To minimize losses of indole metabolites (amines and acids) throughout the isolation from urine and to simplify sample clean-up, we have investigated the use of the Sep-Pak C_{18} cartridges for urine extractions. Details of the extraction efficiency of this method are recorded in Table II. The use of the Sep-Pak C_{18} cartridges for urine extraction had several advantages over the solvent extraction methods. It provided chromatographically cleaner extracts because of the partial separation which it produced and effected a significant time saving for extraction because it was a single operation. Moreover, the extraction efficiency was at least equal to that of the solvent extraction methods, the recovery of all indoles being approximately 100%. In agreement with Allan et al. [19], we found that a Sep-Pak C_{18} cartridge could be re-used many times without any loss of performance.

TABLE II

RECOVERIES OF THE TESTED COMPOUND FROM SEP-PAK C_{18} EXTRACTION

Each result represents the average of five assays.

Compound	Theoretical content (μg)	Recovery	
		μg (mean ± S.D.)	%
5-HTRP	55	52.7 ± 1.2	95.8
5-HIAA	47.5	48.2 ± 1.1	101.4
TRP	51.0	50.2 ± 0.8	98.4
IAA	17.5	17.1 ± 0.6	97.7
IBA	20.0	20.3 ± 0.8	101.5
5-HT	70.0	68.2 ± 1.7	97.4
T	40.0	39.4 ± 1.2	98.5

Urinary indole analysis

The chromatogram for a mixture of pure standards is shown in Fig. 1a. This separation was achieved with standard substances and thus represents the best performance of the system. To explore the analysis of the indoles in urine, a 10-ml urine sample was supplemented with 0.5–4 μmol of each indole and 100 μg of the internal standard to simulate pathological conditions and processed as described under Experimental. The fact that indoles

are readily lost during manipulation and evaporation steps [8] showed the convenience of reducing them to a minimum and keeping the evaporation temperature below 40°C. The chromatogram of a urine sample to which tryptophan metabolites were added before analysis is shown in Fig. 1b. As can be seen, the indoles are well separated and free from interference from other naturally occurring constituents of urine except for a small peak which appears between the IAA and IBA peaks. The R_F values of the seven indoles after extraction from urine samples show a slight difference in comparison with the standard solutions, but reproducibility is still good.

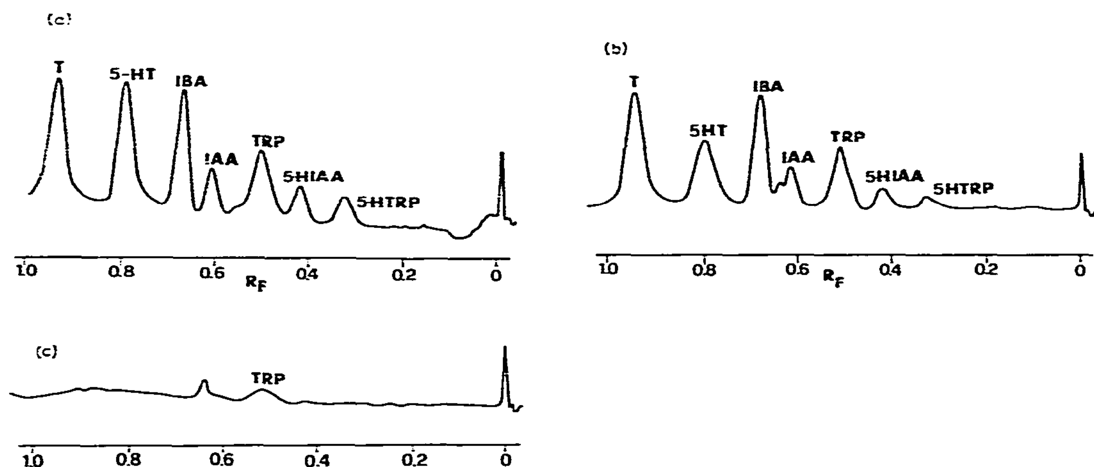

Fig. 1. Chromatograms of (a) a mixture of pure indole standards, (b) urine sample supplemented with pure indole standards, and (c) urine blank.

Analytical recovery of indole compounds added to urine samples

The mean recoveries of the seven indoles added to urine samples are reported in Table III. Since they were not all extracted equally, it was necessary to prepare an extracted standard curve as described under Experimental. The low recoveries obtained for 5-HTRP, 5-HIAA, and 5—HT after extraction from supplemented urine samples indicate that the loss of these indoles occurs during deproteinization. In fact, such a loss cannot be ascribed to the dipping process or to extraction on the Sep-Pak C_{18} cartridge since a standard solution chromatographed directly gives the same response as a standard solution extracted on Sep-Pak C_{18}. Although the recoveries achieved with the extraction procedure described above are sufficient to allow the determination of endogenous levels of tryptophan metabolites, we are presently working to improve the overall extraction procedure further by the use of a Pellicon membrane (Millipore) for deproteinizing urine samples. In conclusion, the proposed technique for the assay of indole tryptophan metabolites in urine, though less sensitive, offers several advantages over other currently used methods. It is a simple, selective and reliable method which can easily be put to routine use for diagnostic application.

TABLE III

RECOVERIES OF THE TESTED COMPOUNDS AFTER THE COMPLETE EXTRACTION PROCEDURE

Each result represents the average of five assays.

Compound	Amount added to 1 ml or urine (μg)	Recovery	
		μg (mean ± S.D.)	%
5-HTRP	27.5	14.3 ± 1.0	52
5-HIAA	23.5	16.2 ± 0.9	69
TRP	25.5	21.2 ± 1.3	83
IAA	8.5	8.2 ± 0.6	97
IBA	10.0	9.8 ± 0.6	98
5-HT	35.0	19.3 ± 1.1	55
T	20.0	17.6 ± 1.2	88

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