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THE ANALYSIS OF INDOLIC TRYPTOPHAN METABOLITES IN HUMAN URINE

THIN-LAYER CHROMATOGRAPHY AND *IN SITU* QUANTITATION

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SUMMARY

A method is described for the quantitative analysis of urinary indolic tryptophan metabolites. The first step involves the ether extraction of urine under alkaline and acidic conditions, followed by the extraction of the ether-insoluble metabolites from the dried urine residue. The three extracts are worked up by thin-layer chromatography and indolic components are stained with an acidic solution of *p*-dimethylaminobenzaldehyde. The suggested solvent systems permit the optimal separation of the individual indolic components, which is a prerequisite for the subsequent *in situ* quantitation. The assay of the coloured spots is carried out with a thin-layer reflectance photometer. An almost linear relationship between the concentration of the substance in a spot and the peak area was found for amounts up to 5–7 μg per spot.

In normal persons under basal and L-tryptophan loading, indole-3-carboxylic acid, indole-3-acetic acid, 5-hydroxyindole-3-acetic acid, tryptophan and indican were detected and determined. The artefact indole-3-acetamide was found in two instances. The relatively unknown metabolite indole-3-carboxylic acid was isolated and identified by mass spectrometry. The method is particularly suitable for the analysis of known and unknown urinary indolic components in metabolic diseases.

The excretion rates found for indican and indole-3-acetic acid were 10–20 times lower than those published in the literature. The causes for this discrepancy were investigated in a comparison of the present method with two commonly used methods for the determination of indican and an often cited procedure for the determination of indole-3-acetic acid. These investigations suggest that the higher values obtained by other workers resulted from the use of non-specific methods.

INTRODUCTION

Studies in this laboratory of the excretion patterns in inborn errors of metabolism made it desirable to improve on the methods used for the qualitative and quanti-

tative analysis of indolic tryptophan metabolites in urine. In this work, the qualitative and quantitative aspects are equally important, for in metabolic illnesses such as phenylketonuria, Hartnup, etc., it has been observed that the disturbed tryptophan metabolism not only produces the known metabolites in abnormal amounts but also forms new and pathological metabolites. The ability to detect these new products is of extreme importance from the standpoint of pharmacology. Once this is possible, it would seem more likely that direct interrelations between clinical manifestations of these disorders and tryptophan metabolism might be found.

Many of the presently used procedures such as paper chromatography and the simple extraction and photometric methods that are used, for example, to determine indole-3-acetic acid, 5-hydroxyindole-3-acetic acid and indican lack sufficient resolving power and specificity for use in detailed studies

Thin-layer chromatography (TLC) has not yet found extensive use in the study of metabolic diseases. Although the TLC behaviour of pure indolic compounds has often been described, good qualitative and quantitative TLC analysis of body fluids is usually possible only when suitable procedures for the preparation of the sample are available. The *in situ* quantitation method eliminates the "transfer" step, which is normally necessary when going from the chromatographic separation to a quantitation in solution. One of the main prerequisites for a transfer is the ability to locate the required compound and to excise it alone and quantitatively; this cannot always be achieved. The ensuing quantitative extraction of the excised spot presents its own problems. These difficulties are, of course, aggravated when one is dealing with very small amounts. It was the object of this investigation to find the optimal conditions for the qualitative analysis and *in situ* determination, by TLC, of indolic compounds obtained from urine.

METHOD

The method consists of three basic steps: (1) extraction of the urine, (2) work-up of the extracts by TLC and (3) *in situ* quantitation of the separated spots on the TLC plate.

Extraction

Basic and neutral compounds. A 100-ml volume of urine is saturated with 25 g of sodium chloride and then adjusted to pH 10–11 with 25% ammonia solution and centrifuged at *ca.* 3000 rpm for 10 min (Centrifuge: Runne Nachf., Heidelberg, G.F.R.). The supernatant is then extracted three times with 100 ml of diethyl ether–cyclohexane–*tert.*-butanol (80:10:10). The combined extracts, which contain the basic and neutral metabolites, are then evaporated to dryness on a rotary evaporator at 40–50°. The residue is taken up in 1 ml of methanol and kept cold.

Acidic compounds. The aqueous phase from the first extraction is adjusted to pH 4.5–5 with dilute hydrochloric acid and again extracted three times with the same mixture as above. The combined evaporated extracts, which contain the acidic indoles, are taken up in 2 ml of absolute methanol.

Ether-insoluble compounds. After the acidic extraction, the aqueous phase is evaporated to dryness on a rotary evaporator and, after thorough grinding, the salt–urine residue is extracted three times with 100 ml of carbon tetrachloride–ethanol–

25% ammonia solution (60:35:5) shaking the mixture for 30 min each time. After evaporation, the extract, which contains tryptophan and indican, is taken up in 5 ml of methanol–25% ammonia solution (99:1).

Thin-layer chromatography

The chromatography is carried out on 0.25-mm layers of silica gel F_{254} (Merck, Darmstadt, G.F.R.), in a solvent-saturated nitrogen atmosphere: the tanks are lined with filter paper wetted with the solvent, and before use they are left for at least 1 h so as to attain equilibrium. When the chromatograms are developed in successive runs in the same or in two different solvents, they are dried between runs at 45–50° in an incubator equipped with a circulator. Before staining, the completed chromatogram is inspected under ultraviolet light (254 and 360 nm) for unusual patterns and for fluorescent spots. After this examination, the chromatogram is stained by spraying with a 1% solution of 4-dimethylaminobenzaldehyde in ethanol–25% hydrochloric acid (1:1) (Van Urk's reagent). After having been sprayed, the plates are ventilated in a fume-hood for 1–2 h for colour development. If new metabolites are being sought, it is imperative that the stained chromatograms are inspected daily for at least 1 week as some compounds become visible only very slowly.

No attempt was made to list the colours of the individual compounds as colour shades may vary considerably from run to run. (It is recommended that the experimentor accustoms himself to monitoring the colour development of the chromatograms, as many indolic compounds exhibit typical stages before the final colour is reached. This type of experience is very subjective and cannot be formulated without difficulty.)

Extract I: basic and neutral compounds. Aliquots of 60 µl of extract are applied to the plates in such a manner that uniform bands of 3-cm length result. Table I lists the solvent mixtures used and the R_F values of the authentic indolic compounds.

Chromatography in solvent system 1 separates the neutral compounds indole-3-carboxaldehyde (I-3Ald), oxindole (OxI), N-acetyltryptamine (TryAm-N,Ac), indole-3-acetamide (IAAm), tryptophol (I-EtOH), N-acetyl-5-hydroxytryptamine (Sero-N,Ac) indole (I), indole-3-acetonitrile (IAN), skatole (Skat) and 5-hydroxyindole (I-5OH) from the start. Basic compounds such as tryptamine (TryAm) and its derivatives remain at the start. Indole, skatole, 5-hydroxyindole and indole-3-acetonitrile, if present, are not separated from one another in solvent system 1. A good separation, however, is achieved with solvent system 3. Indole-3-carboxaldehyde and oxindole are also not separated in solvent system 1. After staining, oxindole is usually visible as a yellow edge on the lower side of the indole-3-carboxaldehyde spot. These metabolites are separated in solvent system 9.

Solvent system 2 separates the basic compounds 5-methoxytryptamine (TryAm-5OMe), N-methyltryptamine (TryAm-N,Me) bufotenine (Bufo), tryptamine (TryAm) and 5-hydroxytryptamine (Sero).

In thin-layer chromatograms of pathological urines (Fig. 1a) in solvent system 2, tryptamine is usually covered by the urea spot. This results in certain variations in the R_F values and a delay in the staining time which may amount to several days. For this reason, tryptamine in such urines is determined in the form of its N-acetyl derivative. Details have been given elsewhere[1].

Fig. 1a shows a typical thin-layer chromatogram of the ammoniacal extract of

TABLE I

R_F VALUES OF INDOLIC METABOLITES OF TRYPTOPHAN ON COMMERCIAL SILICA GEL F_{254} PLATES, 0.25 mm

Solvent systems: (1) dichloromethane–ethanol–ethyl acetate (80:10:10); development, 2 × 10 and 2 × 17 cm (measured from bottom edge of plate). (2) chloroform–methanol–glacial acetic acid (80:15:5); development, 1 × 17 cm. (3) propanol-2–*n*-heptane (25:75); development, 1 × 10 and 1 × 17 cm. (4) chloroform–methanol–glacial acetic acid (75:20:5); development, 2 × 17 cm. (5) butanone-2–*n*-hexane (35:65); silica gel impregnated with 0.05 *M* ammonium formate (pH 4.5); development, 1 × 10 and 2 × 17 cm. (6) propanol-2–water–25% ammonia solution (75:20:5); development, 1 × 17 cm. (7) chloroform–*n*-heptane (65:35); development, 1 × 10 and 1 × 17 cm. (8) butanol-1–ethanol–25% ammonia solution (80:10:10); development, 1 × 17 cm. (9) chloroform–glacial acetic acid (95:5); development, 1 × 17 cm. Abbreviations, see text.

Compound	*R_F in solvent system*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
IAA	74	96	38	93	35	76	0	22	70
I-3CA	84	96	56	85	35	78	0	19	39
IPrA	80	90	47	95	40	83	0	30	68
ILA	6	33	2	40	0	83	0	23	7
IAA-5OH	45	50	17	70	13	76	0	12	10
Try-N,Ac	7	44	2	58	0	83	0	23	8
IAcrA	71	85	45	93	30	81	0	29	57
IPyA	0	24*	0	19*	0	-*	0	19	0
IAAm	78	73	28	90	7	26	0	85	43
I-3AId	88	78	48	96	50	100	6	86	40
OxI	88	95	60	10	37	100	8	86	65
TryAm-N,Ac	84	84	30	96	–	100	9	27	89*
Sero-N,Ac	83	72	23	91	–	100	0	–	18
I-EtOH	92	89	50	96	40	100	0	93	58
IAN	95	97	58	95	73	100	30	95	84
I-5OH	95	88	55	96		100	10	93	42
I	100	100	81	95	95	100	27	100	95
Skat	100	100	87	95	95	100	35	100	95
TryPhanol	2	14	0	28	43**	88	0	56	2
TryAm	0	22	0	30	55**	79	0	61	3
Sero	0	9	0	20	7**	40–65	0	40	0
TryAm-5OMe	0	22	0	33	47**	78	0	58	1
Bufo	0	7	0	13	7**	60–80	0	82	0
TryAm-NMe	0	33	0	36	–	79	0	55	2
Try	0	17	0	25	0	50	0	15	0
Try-5OH	0	2	0	6	0	46	0	2	0
Indican	9	25	0	33	0	90	0	36	0

* Decomposition.
** Ammonia atmosphere.

urine from a phenylketonuric patient after development in solvent system 1 and which, in contrast to normal urines, contains several unidentified indoles.

Extract II: acidic compounds. A 10–20-µl volume of extract is applied in the same manner as above. The standard solvent system 3, which is usually used, separates indole-3-carboxylic acid (I-3CA), indole-3-propionic acid (IPrA), indole-3-acetic acid (IAA), indole-3-acrylic acid (IAcrA) and 5-hydroxyindole-3-acetic acid (IAA-5OH) from the start. Indole-3-lactic acid (ILA) and N-acetyltryptophan (Try-

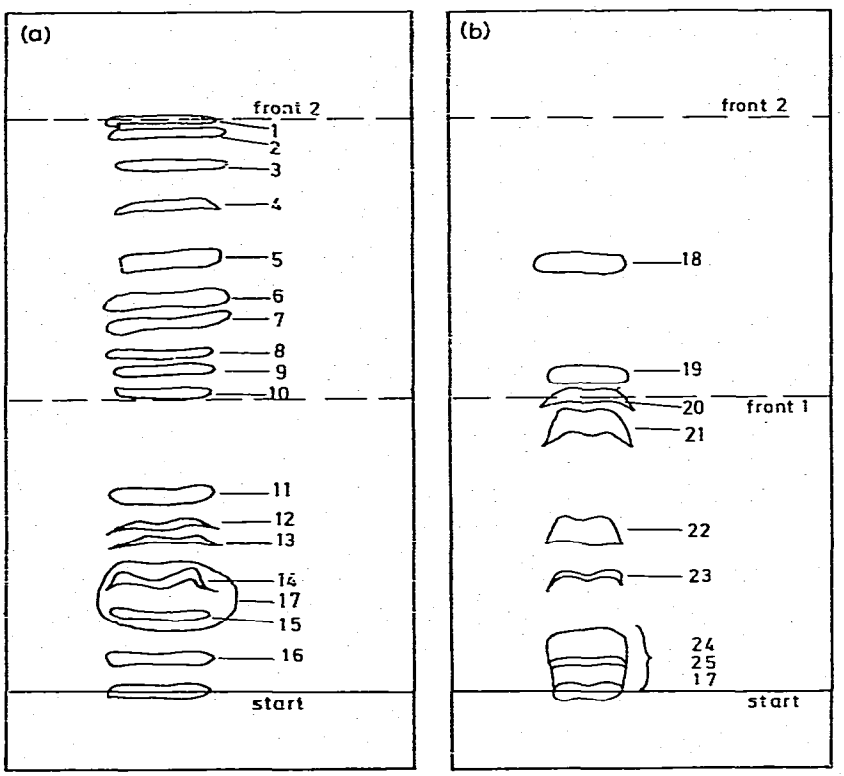


Fig. 1. Thin-layer chromatogram of (a) an alkaline and (b) an acidic urine extract from a patient with untreated phenylketonuria in solvent systems 1 and 3, respectively. Staining with Van Urk's reagent. 1 = IAN; 2 = BN31; 3 = I-3Ald; 4 = BN26; 5 = TryAm-N,Ac; 6 = IAAm; 7 = BN27; 8 = BN28; 9 = BN39; 10 = unknown (yellow); 11 = BN29; 12 = unknown (yellow); 13 = unknown (yellow); 14 = BN32; 15 = TryAm; 16 = BN30; 17 = urea; 18 = S6; 19 = I-3CA; 20 = IPrA; 21 = IAA; 22 = IAA-5OH; 23 = S17; 24 = Try-N,Ac; 25 = ILA. The symbols BN and S refer to unidentified metabolites positive to Van Urk's reagent found in the alkaline and acidic extracts from patients with phenylketonuria (for details, see ref. 1).

N,Ac), which remain at the start, are separated together with 5-hydroxyindole-3-acetic acid in solvent system 2.

The developed chromatograms are stained with Van Urk's reagent and treated as described above. Fig. 1b shows, as an example, the thin-layer chromatogram of the acidic extract of urine from an untreated phenylketonuric patient.

Extract III: ether-insoluble compounds. Aliquots of 10–20 μl of extract III are chromatographed in solvent systems 4 and 6. Solvent system 4 is used for the *in situ* determination of tryptophan (Try). Although solvent system 6 proved to be excellent for the qualitative analysis of extract III, it is not recommended for the determination of tryptophan because of the crescent-like shape of the spot. On the other hand, indican shows well separated and regular spots, which are easily scanned. Solvent system 8 is used for the separation of tryptophan from kynurenine when the latter is present in excessive amounts, as in some pathological urines.

In situ quantitation

The direct *in situ* quantitative determination is carried out with the Zeiss thin-layer spectral photometer (Zeiss, Oberkochen, G.F.R.). Metabolites that are stained blue or violet with Van Urk's reagent are assayed at 500 nm and those stained red or rose (I-3CA and I-3Ald) at 600 nm. The peaks that result from absorption by the spots are cut out, weighed on an analytical balance and compared with those from a dilution series of the authentic compound. The relationship between the concentration of a compound in a spot and the peak weight is non-linear at concentrations above *ca.* 7 μg per spot. In the range 0.5–5 μg per spot, the relationship is, however, linear for all purposes. A typical curve from a dilution series is shown in Fig. 2.

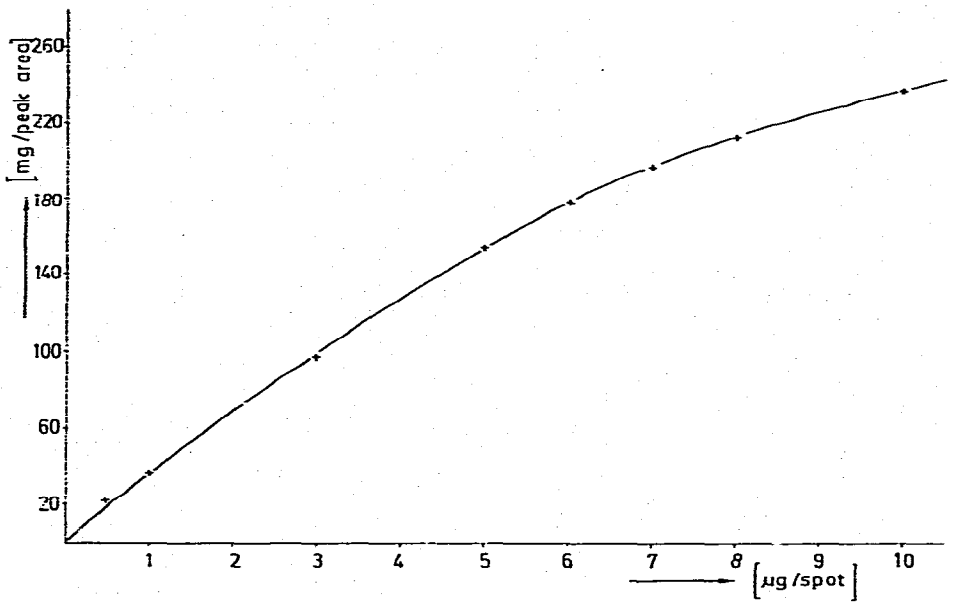


Fig. 2. Calibration graph for a stained indolic compound (indole-3-acetonitrile) on thin-layer chromatogram (reflectance photometry with a thin-layer spectrophotometer, 500 nm).

RESULTS

Recovery

In order to test the reliability of the extraction method, several recovery experiments were performed.

(1) Recovery of the test compounds from aqueous salt solution. A 200-μg amount of each of IAN, I-EtOH, IAAm, I-5OH, IAA, I-3CA, IPrA, IAcrA, IAA-5OH, ILA, Try-N.Ac, TryAm, TryAm-5OMe, Sero (hydrogen oxalate), TryPhanol, Try and indican was dissolved in 50 ml of water saturated with 12 g of sodium chloride and worked up as described above.

(2) Recovery of added test compounds from urine. A 200-μg amount of the above test compounds was added to 50 ml of urine saturated with 12 g of sodium chloride and worked up as described above (the urine had been quantitatively analyzed).

(3) Recovery of test compounds from a saturated sodium chloride solution through continuous extraction with diethyl ether under reflux (perforation). (a) For acidic indoles, 200 μg each of IPrA, IAA, IAcrA, IAA-5OH and I-3CA were added to 50 ml of saturated sodium chloride solution. The solution was perforated with diethyl ether for 8 h after adjustment to pH 4.5 with dilute sulphuric acid. The ether extract was worked up as described above. (b) For neutral indoles, 200 μg each of IAN, I-5OH, indole, I-EtOH and IAAm were subjected at pH 7 to the same procedure as in (a). (c) For basic indoles, 200 μg each of TryAm, TryAm-5OMe, TryPhanol and Sero were perforated as in (a) at pH 10.5 (adjusted with dilute sodium hydroxide solution).

(4) Recovery of added test compounds from urine through perforation. A 200-μg amount of each of the compounds listed in the first two recovery experiments, with the exception of Try and indican, was added to 50 ml of urine saturated with sodium chloride and the pH was adjusted to 10.5. The mixture was perforated for 8 h with 150 ml of diethyl ether, after which the ether extract (extract I) was worked up. The remaining aqueous phase of the urine was adjusted to pH 4.5 with dilute sulphuric acid and perforated again with 150 ml of diethyl ether. The ensuing procedure was carried out as above.

TABLE II

RECOVERY OF TEST COMPOUNDS FROM (I) SATURATED SODIUM CHLORIDE SOLUTION, (II) URINE, (III) SATURATED SODIUM CHLORIDE SOLUTION (PERFORATION) AND (IV) URINE (PERFORATION)

Mean values from five assays.

	Recovery (%)			
Compound	*I*	*II*	*III*	*IV*
IAA	107.3	111.2	108.0	107.6
I-3CA	103.6	99.8	112.0	77.8
IPrA	98.8	107.4	99.3	103.7
IAcrA	83.7	75.2	84.9	84.9
IAA-5OH	96.3	104.0	80.4	102.8
Try-N,Ac	105.7	97.4	—	98.7
ILA	97.3	98.5	—	103.5
IAN	99.9	96.0	107.9	97.1
I-5OH	87.9	94.5	105.8	93.5
I	78.7	34.4	74.7	90.1
I-EtOH	107.5	94.4	84.5	96.7
IAAm	117.5	97.9	82.0	112.1
TryAm	101.1	87.1	102.7	112.4
Sero	46.6	73.8	57.1	68.7
TryAm-5OMe	100.4	112.8	89.8	106.8
TryPhanol	94.5	89.4	108.0	86.2
Try	87.9	91.4	—	—
Indican	75.3	81.6	—	—

The average recoveries from five assays each are presented in Table II. It can be seen that, with the exception of serotonin and indole, the recovery is quantitative.

The precision of the *in situ* quantitation was assessed by the standard deviation (S) found when the same amount of a urine extract was spotted several times. From

Table III, it can be seen that S varies from 3.74 to 12.59% and tends to increase with the concentration of the compound in the spot. The mean of the standard deviation is 6.57%. This value is dependent on the staining technique (spraying, method of colour development, time between spraying and assaying, etc.) and the experience of

TABLE III

DETERMINATION OF CONCENTRATIONS OF I-3CA, IAA AND IAA-5OH IN AN ACIDIC URINE EXTRACT

Comparison of results from repeated measurements on two different spot concentrations (μg per μl of extract). $\bar{X}(A)$ = mean value, plate A; $\bar{X}(B)$ = mean value, plate B; $\bar{X}(AB)$ = mean values, plates A + B; $S(AB)$ = standard deviation over plates A + B. $S = [\Sigma (\bar{X} - X)^2/(n-1)]^{\frac{1}{2}}$.

Spot No.	*I-3CA (10 μl)*	*IAA (10 μl)*	*IAA-5OH (10 μl)*	*I-3CA (20 μl)*	*IAA (20 μl)*	*IAA-5OH (20 μl)*
1	0.122	0.193	0.150	0.130	0.229	0.120
2	0.124	0.192	0.157	0.119	0.191	0.128
3	0.124	0.192	0.151	0.130	0.174	0.148 plate A
4	0.135	0.198	0.156	0.120	0.189	0.128
5	0.131	0.199	0.156	0.110	0.201	0.141
6	0.128	0.200	0.147	0.126	0.199	0.139
7	0.136	0.204	0.151	0.124	0.223	0.148
8	0.128	0.210	0.149	0.126	0.212	0.147 plate B
9	0.125	0.217	0.165	0.120	0.205	0.106
10	0.133	0.175	0.169	0.122	0.183	0.106
$\bar{X}(A)$	0.127	0.195	0.154	0.122	0.197	0.133
$\bar{X}(B)$	0.130	0.201	0.156	0.124	0.204	0.129
$\bar{X}(AB)$	0.129	0.198	0.155	0.123	0.201	0.131
$S(AB)$ (%)	± 3.90	± 5.79	± 3.74	± 4.73	± 8.68	± 12.59

the operator. A typical example of the irregularities that arise in staining is exhibited by the 20 μl-assay on IAA-5OH: although the average values on plates A and B are nearly equal, the standard deviation is 12.59%. The differences between $\bar{X}(A)$ and $\bar{X}(B)$ are small for both concentrations, indicating that different chromatograms from the same run are comparable.

Analysis of urine from healthy persons

The 24-h basal urine from six healthy persons was collected; a 2.0-g L-tryptophan load was administered orally in fruit juice; the urine excreted during the next 48 h was collected in two 24-h portions. No preservative was added to the urine, which was frozen within a few hours after collection. All persons were on a normal diet. Table IV presents a review of the persons taking part in the experiment.

A 100-ml volume of each of the urines was worked up according to the extraction procedure described above. Table V presents the results of the quantitative analysis. The basal urines contained the following compounds: indole-3-carboxylic acid, indole-3-acetic acid, 5-hydroxyindole-3-acetic acid, tryptophan, indican and, in two instances, indole-3-acetamide. The most interesting finding is that almost all of the normal urines (and all of the pathological urines[1]) contain considerable amounts of indole-3-carboxylic acid. Because this metabolite has not been well documentated in

TABLE IV

REVIEW OF CONTROL SUBJECTS

b = basal urine; l_1 = urine from first 24 h after oral loading with 2.0 g of L-tryptophan (20–27 mg/kg body wt.); l_2 = urine from second 24 h post-tryptophan.

No.	*Subject*	*Sex*	*Age (yrs)*	*Weight (kg)*	*Urine volume per 24 h (ml)*	*Urine pH*
1	E.A.	F	8	25	b: 280	5.5
2	K.K.	M	12	74	b: 400	5.5
					l_1: 475	6.0
					l_2: 500	6.4
3	S.M.	F	13	74	b: 590	5.2
					l_1: 640	6.1
					l_2: 485	5.5
4	K.J.	F	13	86	b: 1060	5.7
					l_1: 390	7.0
					l_2: 1500	5.9
5	T.F.	M	26	58	b: 840	5.8
					l_1: 870	5.9
					l_2: 950	6.5
6	K.W.	M	41	99	b: 1175	5.4
					l_1: 975	5.3
					l_2: 975	5.4

the literature as a urinary component, it was isolated and examined by combined gas chromatography–mass spectrometry (GC–MS). An acidic urine extract was evaporated to dryness and the residue taken up in 10 ml of 99% aqueous methanol and treated with ethereal diazomethane at 10 . After evaporation to dryness, the residue was mixed with 15 ml of 10% sodium hydrogen carbonate solution and extracted twice with 5 ml of diethyl ether. The residue from this ether extract was taken up in 500 μl of methanol and investigated by GC–MS (see Fig. 3).

The mass spectrum of the methyl ester shows a molecular ion peak at *m/e* 175. Loss of the methoxy group and of carbon monoxide leads to the fragments at *m/e* 144 and 116. The indoleyl cation fragments in the usual manner. The biogenetic derivation of indole-3-carboxylic acid from tryptophan has been demonstrated in an experiment with D,L-tryptophan–[^{14}C]benzene[2].

General comments on the method

Extraction. A serious problem in extractions I and II is the occasional formation of very stable emulsions, especially with urines from diseased persons. Diethyl ether alone forms emulsions with urine that may be stable for many days. In this respect, the ternary mixture proved to be superior, although not perfect. If emulsions are still formed, the addition of cyclohexane–*tert.*-butanol (1:1) in 10-ml portions is usually sufficient to break them. A last resort is centrifugation.

In addition to tryptophan and indican, extract III contains a number of other compounds, especially those resulting from the oxidative degradation pathway of

TABLE V

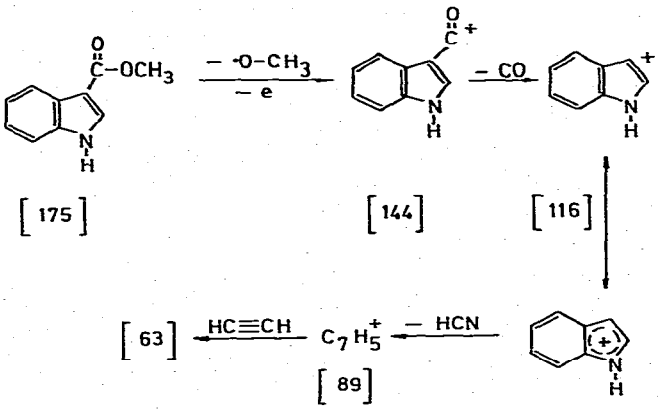
EXCRETION OF INDOLIC TRYPTOPHAN METABOLITES IN URINE BY SUBJECTS LISTED IN TABLE IV

Subject	*Amount excreted in urine (mg/24 h)*					
	I-3CA	*IAA*	*IAA-5OH*	*Try*	*Indican*	*IAAm*
No. 1						
b	1.72	0.00	0.00	9.58	7.22	0.07
No. 2						
b	2.30	0.59	0.00	4.76	6.88	0.00
l_1	2.78	1.82	0.14	7.35	8.32	0.00
l_2	2.63	2.29	0.16	6.80	10.95	0.00
No. 3						
b	0.86	0.00	0.00	0.85	7.52	0.38
l_1	2.24	0.00	0.00	10.20	15.00	0.53
l_2	6.06	0.00	0.00	5.82	10.90	0.28
No. 4						
b	1.01	0.00	0.00	0.00	0.00	0.00
l_1	4.29	0.88	0.05	4.68	6.12	0.00
l_2	1.73	0.00	0.00	0.00	0.00	0.00
No. 5						
b	0.00	0.00	0.00	0.00	3.62	0.00
l_1	1.41	0.59	0.33	3.83	13.40	0.00
l_2	0.00	0.00	0.00	0.00	5.99	0.00
No. 6						
b	0.97	0.78	0.23	1.18	5.88	0.00
l_1	0.98	0.76	0.32	2.34	15.80	0.00
l_2	1.56	0.68	0.21	2.54	15.90	0.00

tryptophan, such as kynurenine, anthranilic acid and kynurenic acid, and amino acids such as phenylalanine. Tracer studies with tryptophan–[^{14}C]benzene[2] have shown that several additional tryptophan metabolites of unknown structure are not extracted in this procedure and remain in the final salt residue.

Thin-layer chromatography. Commercial silica gel plates were chosen in preference to self-coated plates for several reasons: homogeneity of the silica gel thin layer, absence of starch binders and relative stability towards mechanical damage during storage for the purposes of documentation. Stained commercial plates can also be stored for years if they are protected from air and especially from light. This is achieved by simply stacking them on top of one another in the dark. Care should be taken that grains of glass or other matter are brushed off the surfaces, as they might prevent the plates from lying flat and allow air or gaseous reagents to enter from the edges and cause discolouration.

Application of samples. Band-type application of samples is essential for good separation of all metabolites. A simple apparatus is used for this technique, consisting of a T-shaped platform with a smooth undersurface and edge. To this platform an arm (lever) is hinged, which can be raised and lowered in the plane of the T-stem through use of the index finger. Capillary tubing of sufficient length is attached vertically to the forward tip of the arm in such a manner that, when the finger is removed, the tube



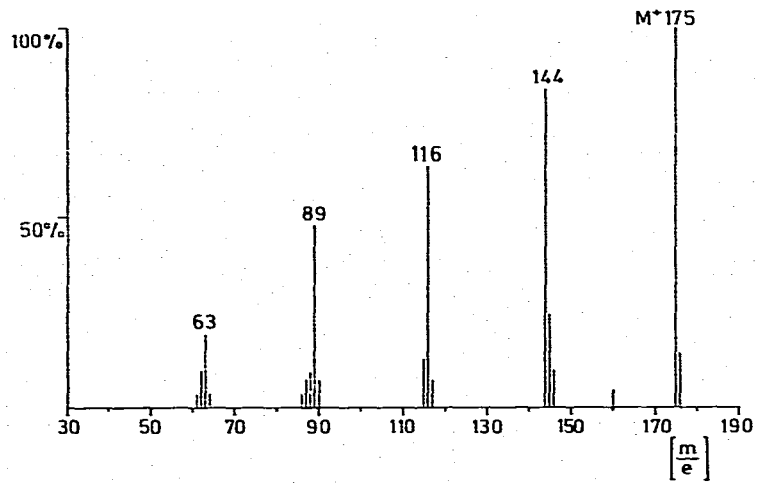


Fig. 3. Mass spectrum of the methyl ester of indole-3-carboxylic acid after isolation from urine. Conditions: combined gas chromatography–mass spectrometry, Varian-MAT 311,70 eV. Column: Gas-Chrom Q – 3% XE-60; temperature programmed from 130° at 10°/min to 225°.

and arm fall gently forward. During application, the cross of the T is pushed flush against the edge of the plate, the tubing is allowed to make contact with the surface of the plate and the platform is moved with sufficient speed along the edge. Volumes of 5–100 μl can be applied in thin bands of approximately 2–3 mm width without difficulty.

Variability of R_F values in TLC. In solvent system 3, a large difference is observed between the R_F value of IAA-5OH in urine samples and that of the authentic test compound. This discrepancy also occurs when authentic IAA-5OH is added to the urine sample. The higher R_F value of the IAA-5OH in the urine sample is probably

due to the presence of urea, which exerts a "pushing" effect. No difference in R_F values is observed between the sample IAA-5OH and the authentic compound in solvent system 2.

ILA exhibits considerable tailing in solvent system 3, but this leads to no further disadvantages in quantitation. The tailing is less when solvent system 4 is used, but there is then a decrease in the separation between IAA-5OH and Try-N.Ac.

Solvent system 5 is employed to determine whether unidentified metabolites are basic, neutral or acidic. Acidic indoles of low polarity are rendered neutral at the pH of the layer impregnated with 0.05 *M* ammonium formate (pH 4.5) and are easily chromatographed in the relatively non-polar butanone-2–*n*-hexane system. However, if the atmosphere of the tank is made alkaline with ammonia fumes, the acidic compounds are held as salts at the start. Indoleamines, on the other hand, exhibit the exactly opposite behaviour. Neutral indoles are not affected by the ammonia atmosphere.

Quantitative measurement. For each quantitative measurement, a fresh dilution series chromatogram is prepared simultaneously with that of the sample under exactly the same conditions. This was found to be necessary as the absorption maxima and the extinction coefficients of the spots may vary between assays and with time. This may be explained by the heterogeneity of the Van Urk's reaction product. Solvent system 2 separates several different coloured components, the composition of which appears to change with time. In this connection, the investigation by Dibbern and Rochelmeyer[3] is very interesting.

Comparison of the present method with previous methods for the determination of indican and indole-3-acetic acid

A comparison of the present method with previously published methods appeared to be necessary because of the relatively low excretion rates found for indican and indole-3-acetic acid. As all of the published methods could not be considered, three were selected that have been utilized most often and that are similar to other general methods. The methods suggested by Bryan[4] and by Curzon and Walsh[5] for the determination of indican and that suggested by Weissbach *et al.*[6] for the determination of indole-3-acetic acid were compared with the present method using the same urine sample. The quantitative results for indican are presented in Table VI.

Indican determination by the method of Bryan[4]. A 5-ml volume of urine was diluted with 1 ml of 2 *N* hydrochloric acid and 40 ml of water. The solution was allowed to elute from a 17 × 1.5 cm Dowex 50W (H^+) column, which was then washed with two 20-ml portions of 0.1 *N* hydrochloric acid. From the total eluate of 85 ml, two aliquots of 3 ml were taken and transferred into test-tubes: 0.2 ml of 0.25% sodium nitrite solution was added with shaking and, after 5 min, 0.2 ml of 10% ammonium sulphamate solution was added with shaking, followed after 3 min, by 0.2 ml of a 0.25% solution of N-(1-naphthyl)ethylenediammonium chloride with shaking. The mixture was allowed to stand for 3 h, during which period it became blue in colour. Test solutions of 5, 10 and 25 μg of indican in 3 ml of 0.1 *N* hydrochloric acid were treated in the same manner. The samples and test solutions were assayed at 550 nm against 1 *N* hydrochloric acid and the results are given in Table VI. The remainder of the eluate from the Dowex column was evaporated to dryness and taken up in 2.0

TABLE VI

COMPARISON OF ASSAYS OF INDICAN IN THE SAME URINE SAMPLE BY THE TLC-*IN SITU* METHOD AND TWO COMMONLY USED METHODS

Sample	*Indican concentration (μg/ml)*		
	Bryan[4] *method*	*Curzon and Walsh*[5] *method*	*Present method*
Urine after elution from Dowex 50W(H^+)	69.92		3.81*
Whole urine		99.96	4.67**
Urine after extraction (extract III)	70.50***		5.34
			2.57§

* After TLC of concentrated eluate in solvent system 6 and staining with the modified diazotization-coupling reagent (see text) and scanning of spot 2 (Fig. 4a).

** After TLC of light petroleum extract and quantitation of band A4 corresponding to indican (see Fig. 5).

*** Diazotization and coupling of untreated aliquot of extract III in 3 ml of 0.1 *N* HCl.

§ After TLC of extract III in chloroform–methanol–glacial acetic acid (65:30:5) and staining with modified diazotization-coupling reagent as in the first footnote).

ml of methanol–hydrochloric acid (1:1)*, and an aliquot of 100 μl was subjected to TLC (silica gel, solvent system 6). The chromatogram as seen under ultraviolet light is shown in Fig. 4a. A second aliquot of 200 μl was chromatographed with a modification of solvent system 4 (65:30:5) and was stained with a suitably modified diazotization and coupling reagent: (a) spraying with a 0.25% solution of sodium nitrite in water; drying of the plate by ventilation in the hood; (b) spraying with a 10% solution of ammonium sulphamate in water–ethanol (1:1); drying in the hood; and (c) spraying with a 2% solution of N-(1-naphthyl)ethylenediammonium chloride in 25% hydrochloric acid–ethanol (1:3). The stained chromatogram is shown in Fig. 4b, where it can be seen that in addition to indican (spot 9), several spots are stained blue, violet or bluish green.

Indican determination by the method of Curzon and Walsh[5]. A 1-ml volume of whole urine was treated with 3 ml of a 4% solution of 4-dimethylaminobenzaldehyde in 25% hydrochloric acid–ethanol (1:1); the mixture became milky pink in colour. After 5 min, 3 ml of 40% potassium hydroxide solution were added (precipitation of reddish solid material), and the warm mixture was cooled in an ice-bath, then extracted for 30 min by shaking with 10 ml of light petroleum (b.p. 60–90°). A test solution of 100 μg of authentic indican in 1 ml of water and a blank sample of 1 ml of water without added indican were treated in the same manner. Aliquots of 3 ml of the urine sample and the test solution were measured against light petroleum at 465 nm and compared, and the results are shown in Table VI.

The light petroleum extracts were then evaporated to dryness and the reddish residues were weighed: extract from urine, 0.74 g; extract from 100 μg of test indican, 0.13 g. The solid material was dissolved in 2.0 ml of isopropanol (ruby red solution)

* Dark brown solution.

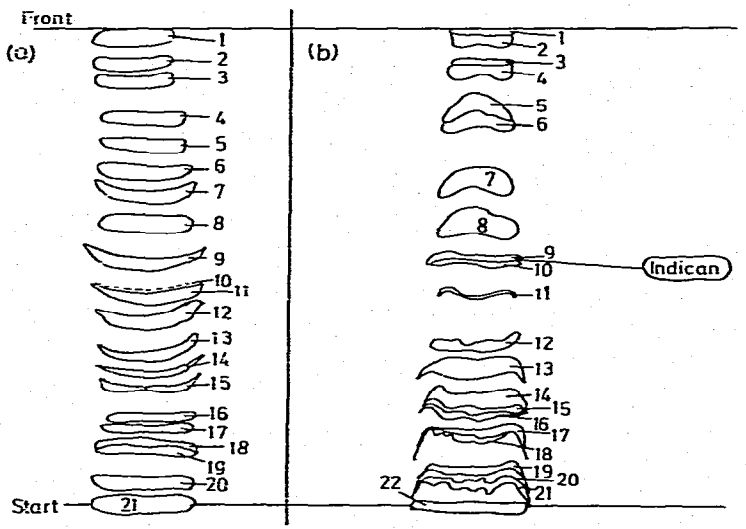


Fig. 4. (a) Chromatogram of 100 μl (= 0.24 ml of urine) of concentrated (2 ml) eluate from the Dowex 50W (H^+) column as seen under UV light (254 and 360 nm). Silica gel, solvent system 6. Dark spots: 1, 2 (indican), 3, 4, 5, 6, 8, 16, 17, 18, 20 and 21. Fluorescent spots: 7 (rose), 9 (rose), 10 (bluish white), 11, 12, 13, 14, 15 (all bluish). (b) Chromatogram of 200 μl (= 0.48 ml of urine) of the same sample as in (a). Silica gel, solvent system chloroform–methanol–glacial acetic acid (65:30:5) stained with modified diazotization–coupling reagent (see text). Colours: 1 = green; 2 = violet; 3 = rose; 4 = bluish green; 5 = blue; 6 = reddish brown; 7 = brown; 8 = reddish brown; 9 = indican (violet); 10 = green; 11 = greenish brown; 12 = blue; 13 = brown; 14 = rose; 15 = brown; 16 = rose; 17 = rose; 18 = brown; 19 = intense green; 20 = rose; 21 = intense greenish blue; 22 = intense blue.

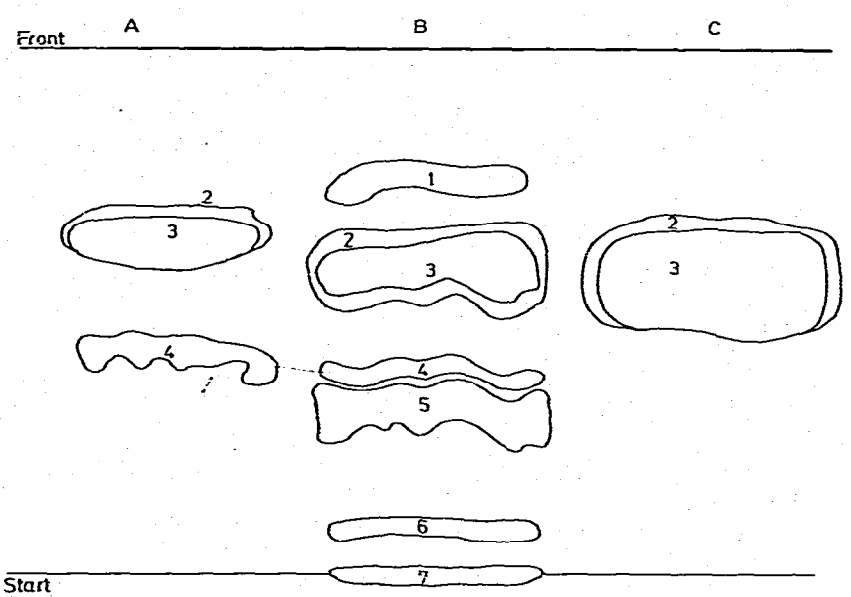


Fig. 5. Chromatograms of light petroleum extract of (A) test indican, (B) urine and (C) blank sample (distilled water after fluorindal reaction) (after Curzon and Walsh[5]). Silica gel, solvent system diethyl ether–*n*-hexane (3:1). Colours: 1 = yellow, fluorescent blue; 2 = violet (appears after exposure to air); 3 = intense yellow, fluorescent yellow; 4 = ruby red, intense fluorescent red, corresponding to indican; 5 = intense orange, strong fluorescent orange; 6 = yellow; 7 = yellow.

and aliquots were subjected to TLC (silica gel, diethyl ether–*n*-hexane (3:1) (Fig. 5).

Staining of the chromatogram of extract III (this method) with fluorindal reagent and the modified reagent described by Bryan[4]. A 15-μl volume of extract III was subjected to TLC and the chromatogram (Fig. 6a) was stained with Van Urk's reagent in

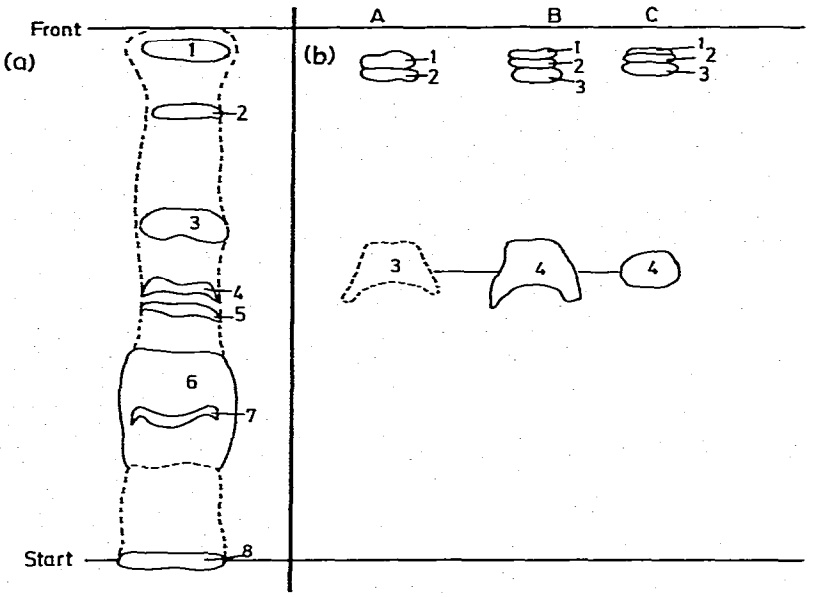


Fig. 6. (a) Chromatogram of extract III. Silica gel, solvent system chloroform–methanol–glacial acetic acid (65:30:5). Stained with fluorindal reaction (see text). Colours: 1 = intense orange, fluorescent reddish orange, completely removed by light petroleum; 2 = as (1); 3 = intense orange, fluorescent reddish orange, partial extraction; 4 = indican, red orange, fluorescent ruby red, partial extraction; 5 = as indican; 6 = urea, yellow; 7 = intense orange, fluorescent orange, partial extraction; 8 = orange, fluorescent orange, complete extraction. For elution with light petroleum, see text. (b) Chromatograms of (A) extract III, (B) extract III + 5 μg of indican and (C) test indican; same conditions as in (a). Stained with the modified diazotization–coupling reagent after Bryan[4] (see text). A: 1 = yellow; 2 = violet; 3 = indican, pale blue. B and C: 1 = violet; 2 = blue with yellow edge; 3 = violet; 4 = indican, deep blue.

the usual manner. After drying, the chromatogram was sprayed with a 2% solution of sodium acetate in isopropanol–water (8:2). As indicated in Fig. 6a, several spots were stained yellow with Van Urk's reagent. Several of these spots changed to a reddish orange colour after the treatment with sodium acetate, and the spots then exhibited the typical reddish fluorescence of indican after the fluorindal reaction when viewed under ultraviolet light (360 nm). The stained chromatogram was immersed in light petroleum and left overnight in the dark. Those spots which exhibited the fluorindal reaction were either completely or partially eluted from the silica gel.

Two aliquots of 15 μl of extract III were spotted; to one sample, 5 μg of authentic indican were added. The plate was chromatographed and then stained with the modified reagent after Bryan[4], and the results are shown in Fig. 6b. The spot of indican was quantitated by *in situ* photometry (Table VI).

Analysis of the method for the determination of indole-3-acetic acid described by Weissbach et al.[6]. A 0.36-ml volume of 12 *N* hydrochloric acid and 10 ml of chloroform were added to 4 ml of urine to which 100 μg each of indole-3-carboxylic acid, indole-3-acetic acid, indole-3-lactic acid, 5-hydroxyindole-3-acetic acid and N-acetyltryptophan had been added. The mixture was shaken for 5 min and then centrifuged. The chloroform layer was transferred into a flask containing 1 ml of 0.5 *N* phosphate buffer of pH 7.0. The mixture was shaken for 5 min and the chloroform layer removed; the buffer layer was acidified with dilute hydrochloric acid and extracted three times with 3 ml of diethyl ether. The ether extract was evaporated to dryness and the residue taken up in 500 μl of methanol, and 250 μl of the solution were subjected to TLC in solvent system 2 and stained with Van Urk's reagent (Fig. 7a). The chromatogram

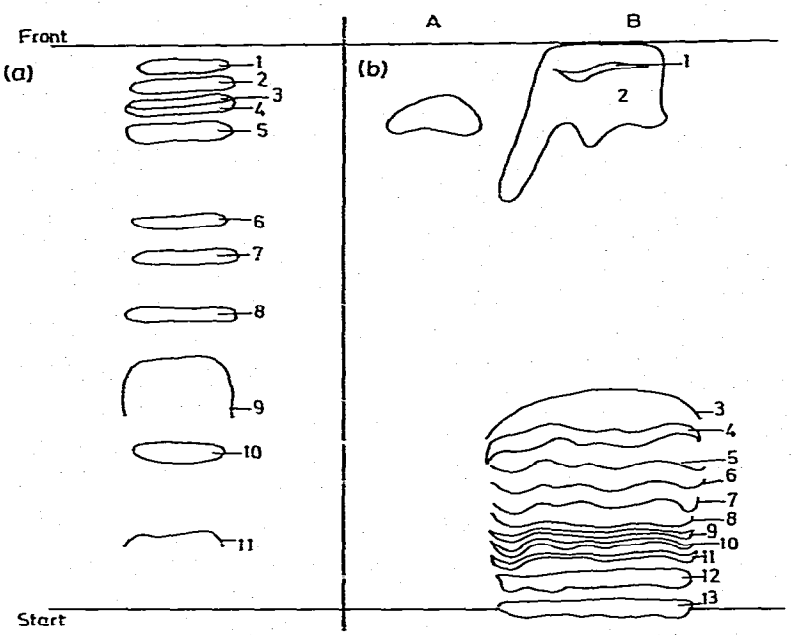


Fig. 7. (a) Chromatogram of ether extract of phosphate buffer from work-up of urine (after Weissbach *et al.*[6]) to which authentic IAA, I-3CA, ILA, IAA-5OH and Try-N,Ac had been added. Silica gel, solvent system 2. Colours: 1 = blue; 2 = blue; 3 = I-3CA, pink; 4 = IAA, violet; 5 = orange-yellow; 6 = orange-yellow; 7 = IAA-5OH, violet; 8 = Try-N,Ac, blue; 9 = urea, yellow; 10 = yellow-orange; 11 = blue-violet. (b) Chromatogram of evaporated phosphate buffer extract after reaction with 4% Van Urk's reagent (after Weissbach *et al.*[6]). A: coloured product of authentic IAA. B: Colours: 1 = pale blue; 2 = yellow; 3 = brown; 4 = blue; 5 = brown; 6 = violet; 7 = brown; 8 = blue; 9 = pink; 10 = intense blue; 11 = as 10; 12 = blue; 13 = blue-violet.

contains, in addition to indole-3-acetic acid, also indole-3-carboxylic acid, 5-hydroxyindole-3-acetic acid and N-acetyltryptophan. Near the start are several spots with blue and brown colours. At the front, two additional spots are observed which are stained with an intense blue colour.

A 4-ml volume of urine was treated as above with the exception that the phosphate buffer that remained after the removal of chloroform was treated with 3 ml of 4% Van Urk's reagent. The solution was evaporated to dryness and the residue

extracted with 10 ml of methanol. The methanol extract was reduced in volume to *ca.* 500 μl and was then chromatographed in chloroform–methanol–acetic acid (65:30:5). A sample of authentic indole-3-acetic acid was treated in the same manner. The chromatograms of the coloured products are shown in Fig. 7b. In the urine chromatogram only a faint spot was found at the R_F value (spot 1) corresponding to the coloured product of indole-3-acetic acid. On the other hand, many coloured bands were found near the start.

DISCUSSION

A review by Sandler[7] gives a good summary of the methods used up to 1968 for the determination of indolic compounds in biological materials. A brief comparison of the results obtained by the present method and of those published elsewhere is presented below.

Although a number of papers concerning the TLC separation of synthetic mixtures of indolic tryptophan metabolites has been published, this method has been used only rarely for the study of human urinary indoles. Some developments in this direction have been described by Diamantstein and Ehrhart[8], Schmid *et al.*[9], Gibbs *et al.*[10], Tanimukai[11] and, more recently, by Haworth and Walmsley[12].

TLC is superior to paper chromatography in several respects: it is less time consuming, requires less apparatus and has, in most instances, greater resolving power and sensitivity. The last aspects are important when the procedure is used to search for new metabolites in pathological urines. A review of the paper chromatographic analysis of indoles in urine has been published by Jepson[13].

A very important prerequisite for the TLC analysis of urine is the preliminary treatment of the sample. Ideal conditions are that the urine is extensively desalted, that the bulk of interfering substances are eliminated and, when a group of metabolic compounds such as the indoles is being studied, that a pre-separation according to functional groups or other chemical characteristics is possible. Most desalting procedures, such as those using charcoal[14–17] or ion exchangers (batch)[18], usually result in the loss of some of the compounds (for example, indole-3-carboxaldehyde is strongly retained by Amberlite resin). Extraction with either ethyl acetate[19,20] or diethyl ether[21] has the advantage of simultaneously removing the indoles both from salts and much of the interfering material in urine. It was found that extraction with diethyl ether is more complete than with ethyl acetate[22]. Sprince *et al.*[23] found that differential extraction with diethyl ether and with butanone at acidic and alkaline pH permitted the pre-separation of indolic metabolites, which was advantageous for paper chromatography.

In the present method, the extraction with diethyl ether under alkaline and acidic conditions seems to be the most suitable procedure. The basic and neutral indoles are separated from the acidic compounds and indican and tryptophan are separated together with other ether-insoluble compounds with a separate extraction mixture. Thus salts are eliminated; larger amounts of sample can be investigated and the identification of the individual compounds is simplified.

The *in situ* quantitation of indoles in TLC had not been extensively investigated. Berry and Krupanidhi[24] used *in situ* densitometry to determine 5-hydroxyindole-3-acetic acid on paper chromatograms of urine, and McNeil *et al.*[25] studied the characterization of indoles through *in situ* fluorometry on TLC plates as a preliminary to

TABLE VII

BASAL EXCRETION VALUES OF URINARY INDOLIC TRYPTOPHAN METABOLITES TAKEN FROM THE LITERATURE IN COMPARISON WITH VALUES OBTAINED BY THE PRESENT METHOD

Metabolite	*mg/24 h*	*Range (mg/24 h)*	*Reference*	*Method***
IAA (free)		3.1–8.1	6	Ref. 6
	17.6±8.6	6.1–38.0	36	Ref. 6
	Free: 6.3	4.8–8.0	37	Extraction after ref. 6, colorimetry (Fischl's tryptophan reagent)
	Total: 10.1	6.4–13.0	37	
	Females: 14.4±7.5			
	Males: 20.6±6.2		35	Ref. 6
	Children: 6.6±3.4			
	7.2±3.1 (urine pH 5–7)		38	Refs. 6 and 39
	15.7±6.2 (alkaline urine)			
	Children: 4.3	1.48–5.4	40	Ref. 41
		0.0–0.78	This work	
IAA-5OH	4.8±1.9*	2.2–8.6*	36	Amberlite CC, $CHCl_3$ extraction, colorimetry (1-nitroso-2-naphthol)
	5.7±4.3*		38	Ref. 42
	3.5±0.2	0.7–8.6	43	Automatic CC, fluorimetry (*o*-phthalaldehyde)
		0–40 μg ml	24	Extraction with ethyl acetate, PC, staining with *p*-dimethylaminocinnamaldehyde, *in situ* quantitation
		0.0–0.23	This work	—
Sero	0.13±0.05*	0.06–0.24	36	Amberlite CC, fluorimetric, pH 3.0, oxidation (metaiodate), measurement of residual fluorescence
		0.03–0.12	44	Amberlite CC, elution with 1 *N* HCl, fluorimetry
	Not detectable in normal subjects		This work	—
Indican	55.9±31.1	12.5–136	36	Ref. 5
	Children: 36.9±25.1			
	Females: 76.6±44		35	Ref. 45
	Males: 126 ± 50.5			
	83±36		4	See text
		32–142	46	Phenol elution from charcoal, colorimetry (Van Urk's reagent)
	77.8±21.7		47	Dowex 50 CC, colorimetry (Van Urk's reagent)
	48.2±19.1		5	See text
	ca. 50		48	PC
	Children: 14.6	0–38	40	Ref. 40
		0–7.5	This work	—

TABLE VII *(continued)*

Metabolite	*mg/24 h*	*Range (mg/24 h)*	*Reference*	*Method***
TryAm	0.17±0.14	0.04–0.61	36	Ref. 49, fluorimetry
		0.1–0.2	50	Ref. 49
	Not detectable in normal subjects		This work	–
Try	20	5–35	40	PC, visual comparison
	18.0	12.8–22.9	51	Microbiological
	24.6±11.3	8.5–56.0	52	Microbiological
	8.8±7.3*	0–30.7	53	PC, visual comparison
	19.56±8.61	9.26–35.1	10	PC, visual comparison
		0–9.58	This work	–

* Calculated from data given.
** PC = paper chromatography; CC = column chromatography.

quantitative work. The theory and versatility of this method of quantitation has been extensively studied with other compounds and it has been shown to give precise results[26–34]. The precision depends mainly on the care taken in the application of the sample, the quality of the chromatographic separation, the positioning of the sample in the light beam and identical treatment of the calibration and sample chromatograms. In preliminary experiments, it was found that cutting out and weighing the peak areas was to be preferred to planimetry. In the hands of a less experienced operator, the latter method can be extremely time consuming and inaccurate, while the former method introduces no large errors.

The qualitative results for normal urine, with the exception of I-3CA, agree with those obtained by Sprince *et al.*[23] by paper chromatography under basal conditions. They found the metabolites IAA, IAA-5OH, indican, Try and the conjugates indoleacetylglutamine and indoleacetylglucuronic acid or IAAm, which is formed from the conjugates under alkaline conditions. After tryptophan loading, Try-N.Ac and ILA were occasionally detected in trace amounts. In our work, the two conjugates of IAA were not found owing to the alkaline conditions used in the first extraction step[1]. So far we have identified Try-N.Ac and ILA only under pathological conditions. Armstrong *et al.*[20] found, in addition to these metabolites, I-3CA and a number of unidentified compounds positive to Van Urk's reagent. These authors had access to a large number of healthy and diseased persons, but they made no distinction between normal and pathological states.

Although the quantitative results are not fully representative owing to the small number of subjects considered, they deviate considerably from published values. In general, the values found in our work are lower. Table VII presents a summary of the published quantitative data on urinary indole metabolites. Almost all of the results on indole-3-acetic acid were obtained by some modification of the method of Weissbach *et al.*[9]. The extraction procedure used in that method is not very specific for free indole-3-acetic acid and may be complicated by the presence of 5-hydroxyindole-3-acetic acid, indole-3-carboxylic acid, indoleacetamide[35] and N-acetyltryptophan[1]. The finding of comparatively large amounts of indole-3-carboxylic acid in most of the urines (normal and pathological) studied in this laboratory complicates the results.

The fact that this metabolite is also extracted under the conditions employed in the method of Weissbach *et al.*[6] casts doubt on the quantitative validity of the results obtained by that method.

The greatest divergence is found with indican. In our opinion the extremely high values given in the literature result from the use of non-specific methods on whole untreated urine or on raw fractions obtained from inadequate column chromatographic procedures. That compounds other than indican may be responsible for the high excretion rates given in the literature has been demonstrated by the comparison of the methods of Bryan[3] and of Curzon and Walsh[5] with the present method. Indican accounts for only a small fraction of the blue-coloured products formed when the Dowex eluate is first chromatographed by TLC and then diazotized and coupled. Bryan's reagent, when modified for spraying and used in combination with TLC, gives values for indican that are comparable with those obtained by the present method. The modified reagent is satisfactory for the detection of indican on thin-layer chromatograms down to a concentration of *ca.* 0.5–1 μg per spot and is superior to Van Urk's reagent in that urea is not stained. However, the maximum colour intensity is reached only after several days. A further complication is that the chromatograms become uniformly blue in colour when exposed to air overnight.

At present, there is no obvious explanation of the fact that Bryan's method gives the same values when applied to the Dowex eluate and to the unresolved extract III, even though indican is the main spot stained on chromatograms of the extract.

The higher values obtained by the method of Curzon and Walsh[5] may be explained through the presence of compounds in urine, other than indican, which react in a manner identical with that of this substance in the fluorindal reaction and whose reaction products are extractable into light petroleum. A further indication that these compounds interfere is evident from the fact that the reaction products from urine and from authentic indican are not completely identical. The size of the residue from the light petroleum extract shows that a large amount of contaminant is also extracted. The main contaminant, which is obviously a reaction product of 4-dimethylaminobenzaldehyde and sodium hydroxide, appears as a bulky yellow spot on the chromatograms of the residues. The yellow colour becomes more intense with time. The basal tryptophan values show more agreement with the literature values but they are, however, in the lower regions of the published ranges. The isolated basal values for 5-hydroxyindole-3-acetic acid are too few for comparison. They do show, however, that this substance is not an obligatory urinary metabolite in normal subjects.

The method as described in this paper has a sensitivity such that 0.1 μg of a substance can be detected by TLC. This means that the detection limit is *ca.* 50 μg per 24-h urine, which holds if *ca.* 1% of the extract is applied on to the plate. In the case of healthy persons and in most cases of metabolic diseases, the entire alkaline extract, equal to 100 ml of urine, can easily be chromatographed, which means that *ca.* 1 μg per 24-h urine of an indolic compound can be detected. The acidic extract contains more substances in larger amounts, and therefore a maximum of only 7.5% of the total extract can be used: for example, IAA-5OH can be determined in amounts down to *ca.* 50 μg per 24-h urine. The amount of extract III (ether-insoluble substances) that can be spotted is limited to about 2% of the final extract.

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