THIN LAYER CHROMATOGRAPHY OF INDOLYL-ACRYLYL-GLYCINE AND OTHER URINARY INDOLES

I. L. HANSEN AND M. A. CRAWFORD*
Department of Physiology, Makerere University College Medical School, Kampala (Uganda)
(Received September 27th, 1965)

It has been shown that apparently healthy people in both East and West Africa utilizing the plantain (*Musa sp.*) as a staple food, excrete indoles to an extent which would be considered as pathological in individuals living on a European type of diet[1-2].

The plantain contains large amounts of 5-hydroxytryptamine (5-HT) (*ca.* 50 μg/g pulp) which is excreted mainly as 5-OH-indolyl-acetic acid (5-HIAA). The presence in the urine of other 5-HT metabolites such as 5-HIAA-glycine and -glucuronide, N-acetyl-5-HT-glucuronide and 5-OH-tryptophol has been reported in the literature[3,4,5]. Further it has recently been shown that urine from banana-eaters frequently contains indolyl-acrylyl-glycine[6], a substance which so far only has been found in large amounts in the urine of Hartnup disease[7].

In order to investigate the reasons for the presence of the indolyl-acrylyl-glycine in the urine it therefore became necessary to establish a simple and rapid technique for identification of this and other indoles.

Routine determinations of urinary indoles have so far mainly been carried out by paper chromatography[8]; however, the development of thin layer chromatography offers excellent possibilities for efficient and rapid separations. The present investigation was carried out to separate indolyl-acrylyl-glycine and some of the most common urinary indoles by thin layer chromatography.

MATERIALS

Adsorbents

Kieselgel G according to Stahl;
Kieselgel GF_{254} according to Stahl;
Kieselguhr G according to Stahl;
Aluminiumoxyd G according to Stahl.

Solvents

Analytical grade reagents were used without further purification.

Standards

The non-conjugated indoles examined (5-HT as creatinine-sulphate and tryptamine as HCl) were available from commercial sources.

* Present address: Department of Biochemistry, Nuffield Institute of Comparative Medicine, Regent's Park, London, N.W. 1.

Standards for the conjugated indoles were prepared biologically from primates and lower animals: The IAA-glycine and IAA-glucuronide were obtained after oral administration of 0.5 g IAA to a guinea pig[7] and the glutamine-conjugate after interperitoneal injection of 0.75 g IAA to a Vervet monkey[9].

Indolyl-acrylyl-glycine was obtained from urine from a banana-eater after ingestion of indolyl-acrylic acid.

Location-reagents

The spots were detected on the chromatograms by the following: (a) U.V. light; (b) Ehrlich-reagent[10]; (c) ninhydrin/acetic acid[8]; and (d) cinnamaldehyde[8].

METHODS

Chromatoplates were prepared according to STAHL[11]. Non-activated (*i.e.* air-dried) plates of Kieselgel G were used in a few cases, but were not found suitable. In all experiments described below the plates were activated by heating at 105° for 30 min.

During attempts to find systems for separation of indolic amino acids and amines the suitability of Kieselguhr G and Aluminiumoxyd G was also examined; however, with all the solvents studied both these adsorbents caused "streaking".

RESULTS AND DISCUSSION

It was not found possible to obtain efficient separation of all the urinary indoles examined with one single solvent system. Instead an attempt was made to separate the indoles within the four following groups: (A) Indolic acids; (B) Conjugates of indolic acids; (C) Indolic amines; (D) Indolic amino acids.

By dividing the indoles in these groups a reduction in the number of indoles on each chromatoplate was obtained leading to better separation and thus facilitating elution for eventual quantitative determination.

The systems which were considered suitable are given in Table I and those applied for each of the groups A–D are discussed separately below.

TABLE I

COMPOSITION OF SOLVENT SYSTEMS

All chromatograms were run over a distance of 11–12 cm except where both media II and III were employed; in this case the chromatogram was run over a distance of 15 cm.

Medium	*Constituents*	*Proportions*	*Time (min)*
I	Ether–petrol ether (60–80°)–HCOOH	75:25:2	30
Ia	Ether–petrol ether (60–80°)–CH_3COOH	75:25:2	30
II	Acetone–NH_3 (25 %)	100:1	30
III	Acetone–$CHCl_3$–acetic acid–water	40:40:20:5	60
IV	Ether–acetone–acetic acid	2:2:1	45
V	Ethyl acetate–acetic acid	4:1	45
VI	Ethyl acetate–benzene–acetic acid	4:1:1	45
VII	Ether–acetic acid	100:1	30

(*A*) *Indolic acids*

Medium I: Diethyl ether–petrol ether (60–80°)–formic acid (75:25:2).
Medium Ia: Diethyl ether–petrol ether (60–80°)–acetic acid (75:25:2).
Medium VII: Diethyl ether–acetic acid (100:1).

Indolic acids were separated in acidic systems containing small amounts of either acetic or formic acids. It is known that the indole nucleus is unstable in presence of strong acid and it would be natural to prefer the weaker acetic to the formic. Since double chromatography (*i.e.* 2-dimensional chromatograms using the same solvent in both directions) did not show any decomposition of 4 of the 5 indolic acids examined, and since formic gives sharper spots than acetic acid, system I was preferred.

Two spots could be detected after chromatography of indolyl-acrylic acid with either of the systems I and Ia. Bands of the two substances located under U.V. were eluted from the Kieselgel GF_{254} layer with acetone–water (1:1). In both cases chromatography of each of the two fractions resulted in two spots identical with the original. Repetition of this procedure with either spot reproduced two spots at the original R_F values; on elution U.V. absorption peaks were identical for both spots. It was therefore assumed that the presence of the two spots on the chromatograms was due to isomerization rather than to decomposition.

The presence of two isomers could be explained by the fact that indole in solutions will normally be present in both the indole and indolenine form (see Fig. 1).

Since the double-bond in the side-chain in indolyl-acrylic acid is conjugated with the double bond in the pyrrole ring, it is possible that both the *cis* and *trans* form of the indolyl-acrylic acid could be found in a tautomeric equilibrium.

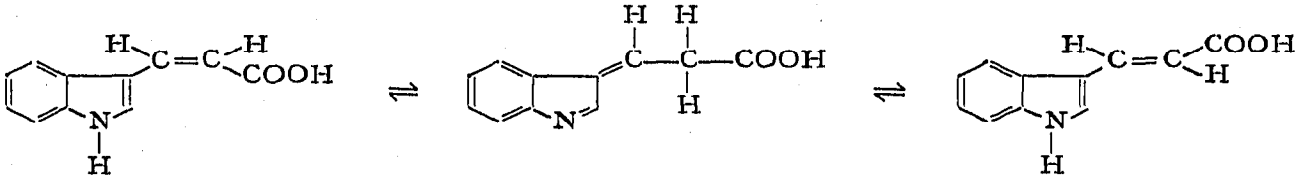

Fig. 1. Diagramatic representation of the possible tautomeric rearrangements of indolyl-acrylic acid which could give rise to the *cis–trans* isomers and double spots on the chromatogram.

It was obvious that the amount of the less polar and less stable of the two isomers increased during storage of a solution. A double chromatogram also suggested that isomerization took place during the chromatographic procedure. Attempts to further prove the assumed isomeric character of the two spots were considered to be outside the scope of the present investigation.

The solvent systems I and Ia were the only ones of the systems investigated which were able to separate both indolyl-propionic acid, IAA, indolyl-acrylic acid and 5-HIAA. It was, however, not found possible to obtain separation between indolyl-lactic acid and 5-HIAA. Separation of these two substances would be achieved using system VII.

These three systems will only allow migration of indolic acids whereas the other indolic groups B–D plus urea remain at origin.

(*B*) *Conjugated indoles*

Medium IV: Diethyl ether–acetone–acetic acid (2:2:1).
Medium V: Ethyl acetate–acetic acid (4:1).
Medium VI: Ethyl acetate–acetic acid–benzene (4:1:1).

Conjugated indolic acids, being more polar than the corresponding free acids, could be separated in more polar systems. Also in this case, sharper spots could often be obtained by substitution of acetic acid with formic acid.

As seen from Table II it was found difficult to obtain good separation between indolyl-acetyl-glutamine/urea (IV and V) and urea/indolyl-acetyl-glycine (IV and VI). Since, however, IAA-glycine and IAA-glutamine will rarely be present in large amounts in the same urine sample, one of the three systems should be suitable for any urine sample.

TABLE II

R_F VALUES × 100

The column labelled II/III represents the final R_F value reached after consecutive chromatography in these two systems (see Fig. 2).

Indole group	*Solvent system*								
	I	*Ia*	*II*	*III*	*II/III*	*IV*	*V*	*VI*	*VII*
A									
Indolyl-acetic acid	61	59	—	93	93	88	85	85	96
Indolyl-acrylic acid	50	43	—	98	98	98	85	85	84
Indolyl-lactic acid	24	4	—	75	75	74	59	57	22
Indolyl-propionic acid	68	64	—	96	96	98	86	85	84
5-OH-Indolyl-acetic acid	28	28	—	86	86	78	83	77	73
B									
Indolyl-acetyl-glucuronide	—	—	—	45	45	30	15	8	—
Indolyl-acetyl-glutamine	—	—	—	68	68	58	42	21	—
Indolyl-acetyl-glycine	—	—	—	82	82	72	66	52	—
Indolyl-acrylyl-glycine	—	—	—	83	83	72	69	55	—
C									
Tryptophan	—	—	—	38	38	16	—	—	—
5-OH-Tryptophan	—	—	—	24	24	11	—	—	—
D									
Tryptamine	—	—	55	51	64	10	—	—	—
5-OH-Tryptamine	—	—	36	38	46	7	—	—	—
E									
Indoxyl sulphate	—	—	24	33	73	54	27	17	—
Kynurenine sulphate	—	—	—	25	30	—	—	—	—
Skatole	96	97	98	100	100	100	90	91	100
Urea	—	—	23	73	73	63	56	49	10

After chromatography of the standard sample of indolyl-acrylyl-glycine with medium VI two spots giving the characteristic red colour with Ehrlich's reagent appeared on the chromatograms. It was assumed that this phenomenon again could be explained by *cis–trans* isomerism.

(*C*) *Indolic amines*

Medium II: Acetone–ammonia (25 %) (100:1).

Indolic amines could be separated in many different systems. One of the most simple of these media, acetone containing 1 % of a concentrated NH_3 solution, gave a rapid and efficient separation of tryptamine and 5-HT leaving the indole groups (A, B and D) at the origin. Indoles containing neither NH_2- nor COOH-groups were taken to the solvent front.

(*D*) *Indolic amino acids*

Medium II–III.

The greatest difficulties were met during the attempts to establish a system for separation of 5HT, tryptophan and 5-OH-tryptophan. It was possible to separate the two latter compounds in several systems, but most of these did not give sufficient separation of 5HT and tryptophan.

An interesting combination led to the required result: in many acidic systems a good separation was obtained between 5HT and tryptophan and between 5HT and tryptamine (*e.g.* in system III). If after this separation the chromatoplate was dried and then developed with system II the amino acids would remain stationary whereas the amines would migrate further. Consequently a complete separation between all the four compounds discussed was achieved (see Fig. 2). It is essential to dry the chromatogram thoroughly before development in the second system; heating at 110° for 20 min apparently did not destroy any of these four compounds. Also the free acids can be separated by this procedure, but since they are partly decomposed during the heat treatment separation with system I is still to be preferred for these substances.

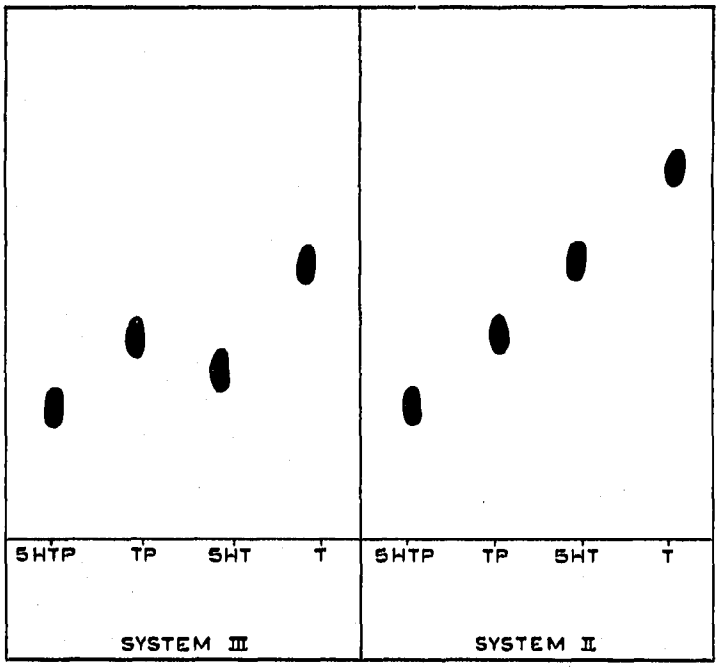

Fig. 2. Diagramatic representation of the separation of the amino acids from the amines by developing the results of solvent III in II. System III separates the amino acids and amines from other indoles but does not separate amines and amino acids. System II then causes further migration of the amines, separating them from the amino acids.

The method described here has the advantage over the procedure earlier reported by SCHMID *et al.*[12] in that it also separated the indolic amino acids from the free acids. The method of SCHMID[12] gives an excellent separation among the four compounds discussed here but all acidic indoles are concentrated in a very small area on the chromatogram.

The location reagents used were the same as those normally used for paper chromatography. Substitution of Kieselgel G with Kieselgel GF_{254} with incorporated fluorescence indicator made all the spots visible under U.V. but they all now appeared blue on a yellow-green background and fluorescent characterization of different indoles was therefore impossible.

All the systems described could be applied successfully for qualitative determination of indoles in neat urine, as even rather large concentrations of urea apparently did not disturb the separation of the indoles.

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Technical Co-operation and the Tropical Medical Research Board for financial assistance and guidance.

SUMMARY

Urinary indoles were separated on chromatoplates of Kieselgel G. The indoles were divided into four groups according to polarity and pK values, and solvent systems were established for separation of the compounds within these four groups. A weakly polar system was used for the free indolic acids, a more polar system for the corresponding conjugates and a strongly polar basic system for the amines. Separation of tryptophan, 5-HTP, 5-HT and tryptamine was achieved by application of an acidic system followed by an alkaline system in a one-way chromatogram; this procedure will separate most indoles present in an indole mixture.

The separation of indolyl-acrylic acid and its glycine conjugate from other urinary indoles was achieved; the double spot formation of indolyl-acrylic acid and its conjugates is discussed in terms of *cis–trans* isomerism.

REFERENCES

1 M. A. CRAWFORD, *Lancet*, 1 (1962) 352.
2 J. M. FOY AND J. R. PARRATT, *J. Pharm. Pharmacol.*, 12 (1960) 360.
3 W. M. MCISAAC AND I. H. PAGE, *J. Biol. Chem.*, 234 (1959) 858.
4 H. WEISSBACH, B. G. REDFIELD AND S. UDENFRIEND, *Federation Proc.*, 17 (1958) Abstr. 1653.
5 S. KVEDER, S. ISKRIC AND D. KEGLEVIC, *Biochem. J.*, 85 (1962) 447.
6 J. BANWELL AND M. A. CRAWFORD, *Biochem. J.*, 89 (1963) 69P.
6a M. A. CRAWFORD, *E. African Med. J.*, 41 (1964) 228.
7 M. D. MILNE, M. A. CRAWFORD, C. B. GIRAO AND L. LOUGHRIDGE, *Clin. Sci.*, 19 (1960) 165.
8 J. B. JEPSON, in I. SMITH (Editor), *Chromatographic and Electrophoretic Techniques*, Vol. I, Heinemann, London, 1960, pp. 183*ff*
9 R. Z. PATEL AND M. A. CRAWFORD, *Biochem. J.*, 89 (1963) 81P.
10 A. B. DURKEE AND J. C. SIROIS, *J. Chromatog.*, 13 (1964) 173.
11 E. STAHL, *Dünnschicht-Chromatographie*, Springer-Verlag, Berlin, Göttingen, Heidelberg, 1962.
12 E. SCHMID, L. ZICHA, J. KRAUTHEIM AND J. BLUMBERG, *Med. Exptl.*, 7 (1962) 8.