Journal of Chromatography, 231 (1982) 166—172
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1294

Note

Separation of indole metabolites from urine with an ODS type resin by high-performance liquid chromatography

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(First received January 11th, 1982; revised manuscript received February 22nd, 1982)

The major pathway of degradation of tryptophan in man begins with the cleavage of the indole ring by tryptophan dioxygenase to yield kynurenine, and only a few per cent of administered tryptophan have been reported to be excreted as indole metabolites [1]. In metabolic disorders such as Hartnup disease [2,3], phenylketonuria, etc., large amounts of indole metabolites have been reported to be excreted in the urine [4].

In the present paper, a new ODS type of column (Hitachi No. 3053), which is octadecylsilane-treated silica gel, is introduced for rapid analysis of various indoles in urine by high-performance liquid chromatography (HPLC). Proper extraction of the metabolites from urine in the case of siblings manifesting malabsorption and malelimination of plasma tryptophan may be applicable to detect increased amounts of indole metabolites for the investigation of tryptophan metabolism.

Ether extraction of indole metabolites [5] is recommended to detect all the indole metabolites except for indoleacetic acid, which was efficiently recovered by chloroform [4] prior to phosphate buffer extraction.

MATERIALS AND METHODS

Twenty-four-hour urines were collected from siblings in whom malabsorption of tryptophan from the intestine was observed along with retarded elimination of plasma tryptophan upon oral loading of tryptophan. A tryptophan loading test (100 mg/kg body weight) was conducted on three siblings 3, 4 and 5 years old. In the youngest case, in whom the symptoms were most dominant, niacin was administered for two months at 100 mg a day, then another tryptophan loading test was conducted. Aliquots of the collected urine was treated according to the method of Weissbach et al. [4] to detect urinary indole-3-acetic acid (IAA). This procedure has been reported to extract mainly IAA and indolelactic acid (ILA) without extracting appreciable amounts of other indole metabolites, corresponding to chloroform-extractable indoles. 5-Hydroxyindoleacetic acid (5-HIAA) and other indole metabolites have been reported to be extractable in ether according to Udenfriend et al. [5], corresponding to ether-extractable indoles.

Briefly, for the extraction of indole metabolites, to 4 ml of urine 0.36 ml of concentrated HCl was added and heated for 15 min at 100°C in a stoppered tube to hydrolyze bound metabolites. Then 10 ml of chloroform or ether were added and shaken for 5 min; then 8 ml of the added solvent were transferred to another stoppered tube to which 0.5 ml of 0.5 *M* phosphate buffer (pH 7.0) was added and shaken for 5 min. For ether extraction, NaCl was added to saturation, then 0.4 ml of aqueous phase was pipetted out and 50 μl were applied to the ODS column (Hitachi 3053 gel) in the high-performance liquid chromatograph (Hitachi Model 635A). The column size was 4 × 150 mm, particle size range 4—6 μm, elution was carried out by 1% of 1 *M* sodium acetate, 4% of 1 *M* acetic acid, 10% of 1 *M* Na_2SO_4 and 25% of methanol in water. The flow-rate was 1.0 ml/min; detection was by ultraviolet (UV) light at 280 nm.

RESULTS AND DISCUSSION

The elution profiles of various indole metabolites are shown in Fig. 1. Authentic metabolites eluted are sharply differentiated except for tryptamine and ILA, the latter substance being eluted as a broader peak after the tryptamine peak. Indolepyruvic acid (IPA) is also eluted late as a small peak due to low sensitivity to UV light. Both ILA and IPA were applied in amounts five times as high as other metabolites giving lower and broader peaks. Urinary indoles were extracted and analyzed from the youngest siblings before and after tryptophan loading, as shown in Fig. 2. The elution profile of urinary indoles extracted in chloroform is shown in Fig. 2a, while the profile of those extracted in ether is shown in Fig. 2b. Unlike other indole metabolites, IAA is far less extractable from urine by ether than by chloroform, as has been reported by others [4]. In the detection of indole substances derived from tryptophan, chloroform-extractable IAA is considered to be a main metabolite in normal subjects; therefore, in the present study of tryptophan metabolism other than by the kynurenine pathway, the primary choice of extraction procedure may be with chloroform. Two major indole metabolites in urine

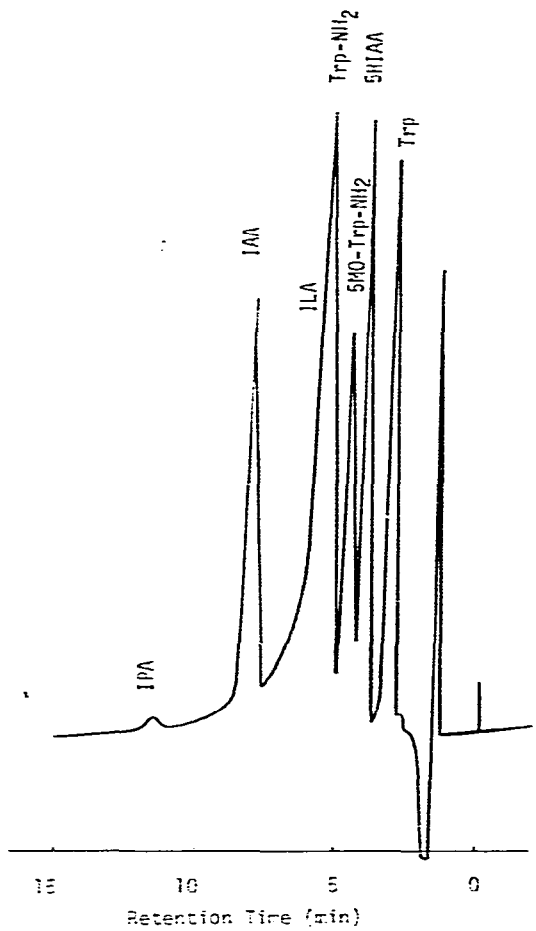

Fig. 1. HPLC elution profiles of indole derivatives. The mixture of authentic derivatives was applied on Hitachi ODS type resin (Hitachi 3053 gel), column size 150 × 4 mm I.D., eluted with 1% of 1 *M* sodium acetate, 4% of 1 *M* acetic acid, 10% of 1 *M* Na_2SO_4, and 25% of methanol in water, detected by UV light at 280 nm; 1 μg of each substance was applied except ILA and IPA (5 μg of each). Trp = tryptophan; Trp-NH_2 = tryptamine; 5MO-Trp-NH_2 = 5-methoxytryptamine.

of three siblings are quantitated by this method as shown in Table I. In these cases, about 3% of orally administered tryptophan is recovered as IAA over basal excretion before loading, while an increasing amount of ILA is observed in the youngest sibling in whom tryptophan absorption from intestine and elimination from plasma were considerably disturbed, and yet no influence of niacin treatment was observed. However, the excreted amount of ILA was lower after niacin treatment, probably because the plasma level of tryptophan did not attain even half the maximal value of the control, which was worse than the previous loading test. On the other hand, ILA was less extractable from older siblings: less than 1% of administered tryptophan was recovered in case 3 in whom almost normal absorption and elimination of tryptophan was observed.

TABLE I

URINARY EXCRETION OF INDOLE ACIDS IN 24-h URINE AFTER TRYPTOPHAN LOADING (100 mg/kg)

Patient	Dose (g)	Indoleacetic acid			Indolelactic acid		
		Pre-load (mg)	Post-load (mg)	Yield (%)	Pre-load (mg)	Post-load (mg)	Yield (%)
Case 1-I	1.5	65.4	109.0	2.9	4.9	110.2	7.0
1-II*	1.5	36.5	106.6	4.6	8.6	59.8	3.4
Case 2	1.8	31.1	83.4	2.9	n.d.**	40.0	2.2
Case 3	2.0	52.0	112.3	3.0	n.d.	15.0	0.8

*After niacin treatment (100 mg/day for two months).
**n.d. = not detectable.

It may be concluded that the metabolic disorder in the present study is the unusual excretion of ILA in the urine at the younger age, rather than of IAA which has been reported to be the main indole metabolite of tryptophan.

Other indole metabolites were extracted in ether and quantitated as shown in Table II. From these analyses, tryptophan itself was not always recovered in larger amounts after tryptophan loading than the preloading basic urinary excretion level, but is recovered as other indole metabolites especially in the form of ILA in this study.

As has been reported, IAA was mostly extractable by chloroform but poorly recovered by ether (cf. Tables I and II), whereas in the case of ILA ether extraction was found to be more efficient: as much as 2—3 times was recovered compared to chloroform extraction.

In these studies, administered tryptophan was constantly recovered as IAA, which was around 3% of loaded tryptophan (Table I), and a fairly large amount of ILA was detected in the urine after tryptophan loading, which is probably due to retarded absorption of tryptophan from the intestine where it is presumably metabolized to indole derivatives by intestinal bacteria rather than absorbed and excreted in the urine without being utilized as a nutrient. Or the metabolic pathway of indoles rather than kynurenine formation may be more active in the present subjects and recovered normal metabolic ability as their age advances which are clinically manifest as retarded physical development accompanying urinary odors of indoles in their younger stage.

It has been reported that in phenylketonuria a large proportion of the chloroform-extractable indoles was ILA, but the highest unusual chloroform-extractable indole is IAA in diabetes, neuromuscular disorders and idiopathic sprue [4].

ILA was excreted in all the cases in the present study, as a basic excretion of indoles besides IAA, and rapid and sensitive analyses of these metabolites by HPLC may be recommended to investigate such metabolic disorders previously detected by thin-layer chromatography or measurement of indoles by colorimetry using xanthydrol [4] or Ehrlich's aldehyde reagent [6].

It is also interesting to note that other indole derivatives such as 5-HIAA, 5-methoxytryptamine or tryptamine, which are metabolic derivatives of 5-hydroxytryptamine, are less influenced by the tryptophan loading test in the present study.

TABLE II

URINARY EXCRETION OF INDOLE METABOLITES IN 24-h URINE BEFORE AND AFTER TRYPTOPHAN LOADING (100 mg/kg)

Patient	Dose (g)		Tryptophan (mg)	5-HIAA (mg)	5-Methoxytryptamine (mg)	Tryptamine (mg)	ILA (mg)	IAA (mg)	IPA (mg)
Case 1-I	1.5	Pre	11.81	1.78	2.42	0.43	1.04	1.1	—
		Post	4.79	3.98	2.11	—	331.20	4.22	—
Case 1-II*	1.5	Pre	3.57	1.61	1.98	0.50	2.44	1.60	1.95
		Post	6.47	3.95	2.79	2.26	88.17	3.40	2.99
Case 3	2.0	Pre	3.40	3.26	3.56	1.31	7.60	2.33	—
		Post	9.66	4.92	3.20	—	27.43	3.82	2.08

*After niacin treatment (100 mg/day for two months).

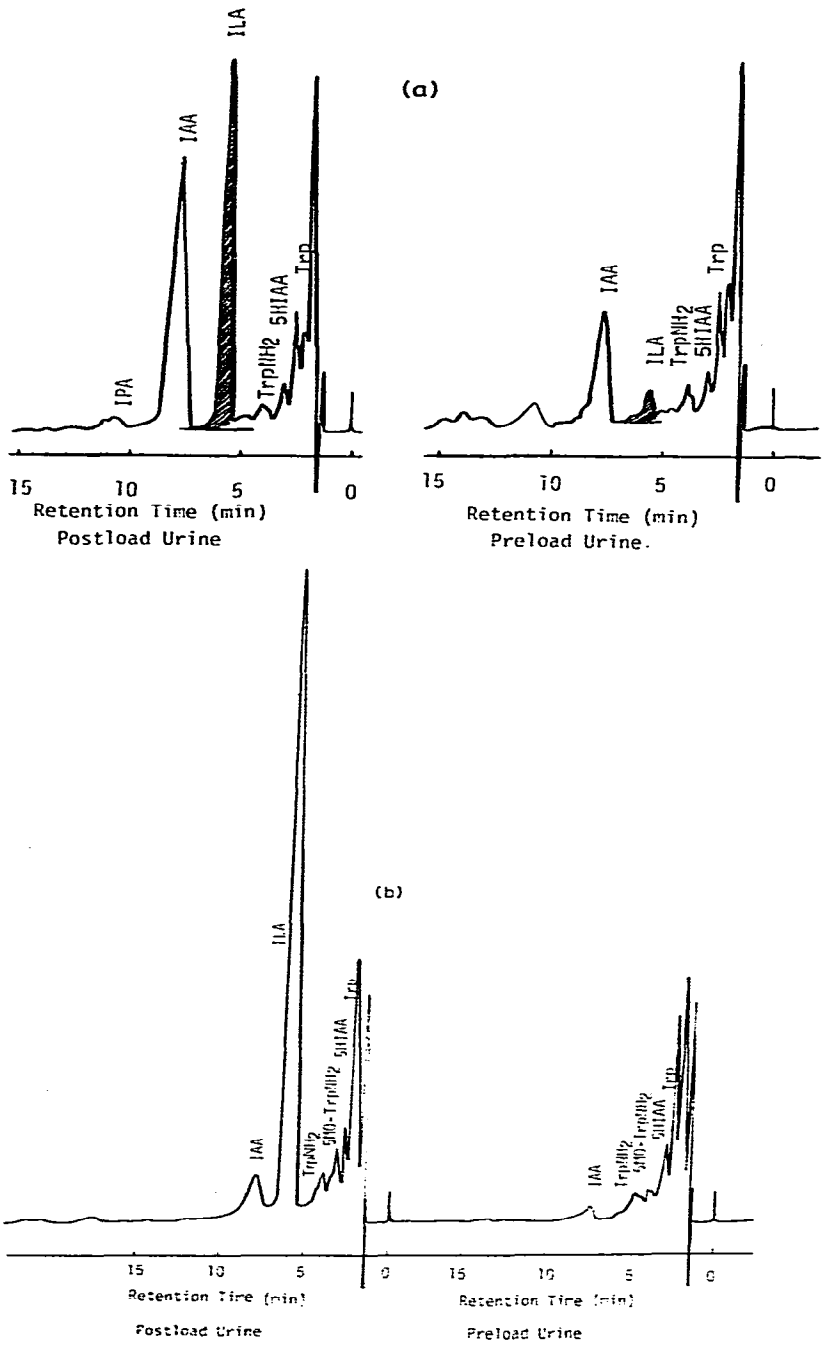

Fig. 2. (a) HPLC analysis of chloroform-extractable indoles in urine. An aliquot of urine was pretreated with chloroform, then extracted in phosphate buffer (see text) and applied on Hitachi 3053 gel, before and after tryptophan loading in a case of malabsorption and excretion of tryptophan. (b) HPLC analysis of ether-extractable indoles in urine. An aliquot of 24-h urine was pretreated with ether, then extracted in phosphate buffer (see text) and applied on Hitachi 3053 gel, before and after tryptophan loading in a case of malabsorption and excretion of tryptophan. For abbreviations see Fig. 1.

ACKNOWLEDGEMENTS

ODS type resin (Hitachi No. 3053 gel) was kindly provided by Nissei Sangyo Co.

Thanks are due to Professors Y. Matsumura and K. Furiya for their support.

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