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ANALYSIS OF SOME TRYPTOPHAN AND PHENYLALANINE METABOLITES IN URINE BY A STRAIGHT-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUE

M. GHEBREGZABHER, S. RUFINI, M.G. CASTELLUCCI and M. LATO*

Istituto di Clinica Pediatrica della Università degli Studi di Perugia, Perugia 06100 (Italy)

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SUMMARY

A high-performance liquid column chromatographic technique is reported for the analysis of some tryptophan and phenylalanine acid metabolites in the urine. An acidified and NaCl-saturated urine sample is loaded on to a C_{18}-bonded silica microcolumn. After washing the microcolumn with clean and deionized water, the metabolites of interest are selectively extracted by successive elutions with organic solvents of variable polarity. Acids are eluted first and the neutral compounds with the next fraction. Basic compounds and other neutral substances of higher polarities were eliminated during the washing procedure.

The chromatography was performed in the straight-phase isocratic elution mode utilizing 5-μm silica-gel columns loaded with a triethanolammonium perchlorate—perchloric acid aqueous solution. The separations achieved have permitted the application of the chromatographic technique to the analysis of urinary metabolites with acceptable accuracy.

INTRODUCTION

Interest in techniques for the separation, identification and quantitation of tryptophan and phenylalanine acid metabolites stems from specific requirements in clinical diagnosis. The analysis of hydroxyindole acid metabolites and some mandelic acid derivatives in urine is not only important for the diagnosis of carcinoid malignancies and catecholamine-secreting tumors [1—4], but it also serves as a monitoring system in the course of treatment of these diseases.

Furthermore, study of the vast number of affections in which abnormalities in the tryptophan excretory pattern have been noticed [5, 6], but in which no real insight into the relationship of cause and effect has yet been achieved, requires the introduction of new analytical methods in addition to the existing paper chromatographic [7], thin-layer chromatographic (TLC) [8—12], gas

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chromatographic [13] and electrophoretic [14] techniques, which have proved to be sometimes time-consuming, relatively insensitive and low in accuracy for the quantitative estimation of the compounds of interest.

For these reasons a large number of studies have recently been undertaken on the application of high-performance liquid chromatography (HPLC) to the analysis of biogenic amines and related metabolites [15—20]. Relative simplicity, good reproducibility as well as high efficiency are the practical advantages of HPLC over the techniques mentioned above and ion-exchange chromatography; moreover, HPLC does not require any chemical processing of the samples before analysis.

The reversed-phase mode of HPLC, when it is operated under gradient-elution conditions, affords excellent simultaneous separations of a large number of aromatic acids [17] so that urine extracts can be effectively assayed for their phenylalanine and tryptophan acid metabolites.

On the other hand, normal-phase ion-pair HPLC has been found to give rapid and fair separations of some biogenic amines and their metabolites [18]. Such a procedure, despite the reduced number of compounds separated, indeed has the advantage of being simpler as it is performed isocratically. However, the ion-pair partition mode requires a sample preparation step (i.e. ion-pair formation in an organic phase of the compounds being tested before injection on to the chromatographic columns), which may complicate the application of this technique to the assay of physiological materials, where direct injection of underivatized and if possible not pretreated samples is to be preferred.

In the present paper we describe a normal-phase HPLC method that allows the isocratic separation of a relatively high number of metabolites, and its application to the analysis of clinically important urinary acid metabolites of tryptophan and phenylalanine.

EXPERIMENTAL

Materials

For chromatographic purposes use was made of the following analytical-grade organic solvents, without any further purification: *n*-heptane, isobutanol and 2-propanol. Ethyl acetate and methanol of highest purity grade (ACS UvaSol certified grade from Carlo Erba, Milan, Italy) were used for the extraction procedures. Urine extraction was accomplished on Sep-Pak^{tm} C_{18}-bonded silica cartridges (Waters Assoc., Milford, MA, U.S.A.).

Distilled and deionized water was used. Triethanolammonium perchlorate ($TETOLA^+ClO_4^-$) was formed by neutralizing an aqueous perchloric acid solution of a given molarity by an equal amount of triethanolamine.

The standard compounds listed in Table I were purchased from Sigma (St. Louis, MO, U.S.A.).

Instrumentation

Chromatography was performed on a Hewlett-Packard liquid chromatograph Model 1010A, equipped with an UV absorption detector (Model HP 1032A) at two interchangeable fixed wavelengths (280 and 254 nm), a linear potentio-

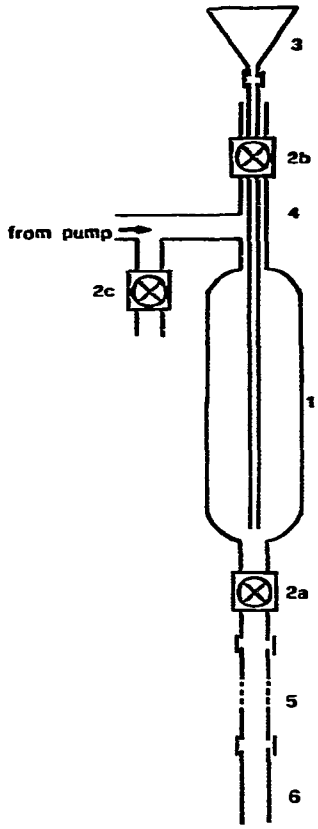

Fig. 1. Diagram of the packing apparatus. 1 = slurry reservoir; 2a, 2b, 2c = two-way high-pressure valves (Whitey); 3 = glass funnel; 4 = PTFE tube; 5 = precolumn; 6 = column.

metric recorder (Model HP 7127A) and with a high-pressure on-line injection block.

Throughout the investigation 24.75 × 0.4 cm I.D. stainless-steel columns, packed with LiChrosorb silica-gel particles (5 μm) were used. The column was packed using the equipment represented in Fig. 1. Pressure was applied to the packing system by one of the liquid chromatograph pumps, operating at the maximum feed capacity (about 240 bars).

Packing procedures

Columns were wet-packed using the slurry method with isopropanol—stationary phase (5:3, v/v) as dispersing solvent. The stationary phase consisted of an aqueous solution of 0.5 *M* $TETOLA^+ClO_4^-$—0.3 *M* $HClO_4$.

A 1.8-g amount of LiChrosorb SI-100-5 (E. Merck, Darmstadt, G.F.R.) is dispersed by mechanical agitation in the isopropanol—stationary phase mixture (10 ml). The opalescent suspension formed is then heated in a water-bath at 50—60°C for 3—4 h to remove any gas residue, and cooled at ambient temperature. Using a pasteur pipette the suspension is rapidly delivered into the column and previously connected precolumn; then the precolumn is assembled on to the reservoir via the two-way ballvalve 2a (Fig. 1). By means of a glass

funnel and a flexible PTFE tube attached to the funnel stem, 75 ml of the same solvent as that contained in the solvent reservoir (ethyl acetate) are gently poured in.

The funnel is now completely withdrawn. With valve 2b open and valves 2a and 2c closed, the pump is started at its maximum pressure feed. When the first drop of ethyl acetate has emerged through valve 2b, this valve is shut and valve 2a opened. Now the system is allowed to run until 120—150 ml of liquid have passed through the column.

Chromatographic procedure

All chromatographic experiments were performed at ambient temperature, in the isocratic elution mode. The mobile phase was *n*-heptane—isobutanol—2-propanol (10:10:30, v/v/v) saturated with 2.5—3.0 ml of stationary phase.

Equilibration of the chromatographic system with the mobile phase was carried out by allowing 150—200 ml of eluent to pass through the column at a flow-rate of 0.60—0.75 ml/min.

The standard compounds were prepared by dissolving weighed amounts in 1 ml of either *n*-butanol—dichloromethane—6 *N* HCl (5:5:0.1) or 2-propanol—diethyl ether—0.15 *M* HCl (4.5:4.5:1.0), depending on the solubility of the substances. Five microlitres of these solutions, containing 0.05—2.0 μg of each solute, were injected into the column. The standard solutions had to be prepared fresh before injection because most of the compounds are easily degraded in organic solvents.

Sample preparation

Urine samples were obtained from ten of our laboratory technicians and students, from five children admitted to the Pediatric Department for diseases not correlated with any of the tryptophan or the phenylalanine metabolic anomalies, from one patient affected with neuroblastoma, from two cases of phenylketonuria, and from one patient affected with a hypochromic microcytic anemia. The specimens were in every case 24-h urines collected under controlled nutritional conditions and kept frozen after being acidified to about pH 2 with hydrochloric acid.

Isolation of aromatic acids and neutral compounds

Two C_{18}-bonded silica cartridges were prepared for each urine sample to be extracted by inserting the longer end into the tip of a 10-ml disposable plastic syringe barrel (after removal of plunger).

The C_{18} microcolumns are then pre-wetted by allowing to pass successively through the cartridges 2.5 ml of isopropanol and 5 ml of clean distilled and deionized water.

Then 5 ml of urine, previously acidified to pH 2 and saturated with NaCl, are poured into the syringe barrel of one of the two cartridges and, after the liquid has passed completely through the microcolumn, it is washed with 3.0 ml of water and the effluents are discarded.

Then 5 ml of a glycine—NaOH buffer (pH 9.0) [21] are allowed to pass through the cartridge and the effluent collected in a 10-ml test-tube and care-

fully acidified to pH 1.5—2.0 with 125 μl of 3 *N* HCl. The acidified solution is then poured into the second cartridge and the effluent discarded. Now the column is washed with 3.0 ml of clean water and finally with 0.5 ml of ethyl acetate.

Acid metabolites are now extracted by eluting with 2.5 ml of ethyl acetate—methanol (1:1). The eluate is collected and evaporated to dryness under a stream of dry nitrogen under mild temperature conditions (50—60°C); the residue is redissolved in 100—500 μl of ethyl acetate—2-propanol (1:1) and constitutes fraction A.

The residual neutral substances on the first microcolumn are now extracted by 2.0 ml of ethyl acetate—methanol (1:1) of which the first 0.5 ml are discarded, while the successive effluent liquid, collected and evaporated as above, constitutes fraction B.

Another fraction (C) may still be obtained by subjecting the first microcolumn to further elution with methanol.

The fractions A, B and, eventually, C are kept frozen until they are subjected to chromatographic analysis.

In all cases, the passage of the liquids through the cartridges may be accelerated by gently pushing the syringe plunger. Any air bubbles that may be formed inside the narrow bore between the syringe and the cartridge must be removed by a pasteur pipette.

RESULTS

The chromatographic parameters of twenty tryptophan and phenylalanine metabolites are reported in Table I, where a wide range of k' values is displayed that permits the separation of a group of indole and catechol derivatives (Fig. 2). The number of compounds separated is even higher when urine extracts were subjected to analysis (Figs. 3—7).

In some urine extracts identification of the peaks corresponding to homovanillic acid (HVA), vanilmandelic acid (VMA), 5-hydroxyindole-3-acetic acid (5HIAA) and 5-methoxyindole-3-acetic acid (5MIAA) was tentatively carried out by co-chromatography with standard compounds once the effective presence of the considered metabolites has been ascertained by specific detection reagents on TLC [22].

For quantitative purposes, plots of peak heights versus amount of injected sample were performed for 5HIAA, indole-3-acetic acid (IAA), HVA, VMA and tryptophan (Try). They were found to be linear over the range of the quantities tested: 0.15—1.5 μg/μl for 5 HIAA and IAA; 0.45—3.5 μg/μl for HVA, VMA and Try.

The reproducibility of the retention times of the compounds tested over repeated injections of reference solutions and urine extracts (ten and five times, respectively) gave relative standard deviations of 1.3%. The standard deviation for peak height was 1.1%.

The efficiency of the present extraction procedure was tested for IAA, HVA and VMA. The recovery was approximately 98%. Similarly, the selectivity was found to be fairly high (see Table II).

TABLE I

CHROMATOGRAPHIC PARAMETERS AND SENSITIVITY LIMITS OF SOME TRYPTOPHAN AND PHENYLALANINE METABOLITES

Solvent system: *n*-heptane—2-propanol—isobutanol + 2.50 ml of stationary phase. Flow-rate: 0.71 ml/min. t_0 = 2 min 21 sec.

Substance	Abbreviation	t_R	k'*	Sensitivity limit (μg/ml)**
3-Hydroxyethylindole***	3HEI	7 min 22 sec	2.13	40
5-Hydroxyindole***	5HI	9 min 42 sec	3.13	40
Indole-3-acetamide***	IAM	23 min 02 sec	8.80	60
Indole-3-acetic acid	IAA	7 min 14 sec	2.08	60
Indole-3-acrylic acid	IAcrA	5 min 21 sec	1.27	35
Indole-3-glyoxylic acid	IGA	8 min 34 sec	2.64	40
Indole-3-lactic acid	ILA	12 min 21 sec	4.25	85
5-Hydroxyindole-3-acetic acid	5HIAA	22 min 48 sec	8.70	60
5-Methoxyindole-3-acetic acid	5MIAA	10 min 12 sec	3.34	60
Tryptophan	Try	27 min 48 sec	10.83	60
5-Hydroxytryptophan	5HTry	50 min 10 sec	20.34	
Anthranilic acid	An	8 min 54 sec	2.78	30
3-Hydroxyanthranilic acid	3Han	12 min 17 sec	4.15	100
Xanthurenic acid	XA	12 min 45 sec	4.30	25
Kynurenic acid	KA	18 min 30 sec	6.87	70
Vanillic acid	VA	5 min 41 sec	1.42	20
Homovanillic acid	HVA	9 min 32 sec	3.05	90
Vanilmandelic acid	VMA	21 min 50 sec	8.29	150
3,4-Dihydroxycinnamic acid	DHCA	6 min 44 sec	1.86	20
3-Methoxy-4-hydroxycinnamic acid	MHCA	6 min 44 sec	1.86	20

*$k' = \frac{t_R(\text{sec}) - t_0(\text{sec})}{t_0(\text{sec})}$

**μg of compound in 1 ml of unconcentrated urine.

***Compounds extracted in fraction B. Remaining compounds are extracted in fraction A.

TABLE II

EXTRACTION SELECTIVITY OF THE C_{18}-BONDED SILICA CARTRIDGES

Five millilitres of an acidified (pH ≈ 2) aqueous solution containing indoleacetamide (5.2 μg), indole-3-ethanol (5.2 μg), indole-3-lactic acid (6.2 μg) and homovanillic acid (6.2 μg) were extracted as described in the Experimental section, and the amounts of these substances in the resulting fractions A and B determined. Standard errors (S.E.) have been calculated from the means of six repeated determinations.

Substance	Fraction A			Fraction B		
	μg	± S.E.	%	μg	± S.E.	%
Indole-3-ethanol	0.052	0.008	1.02	5.096	0.009	98.8
Indoleacetamide	0.425	0.062	8.34	4.60	0.071	90.2
Indole-3-lactic acid	6.07	0.035	98.00			
Homovanillic acid	6.10	0.035	98.00			

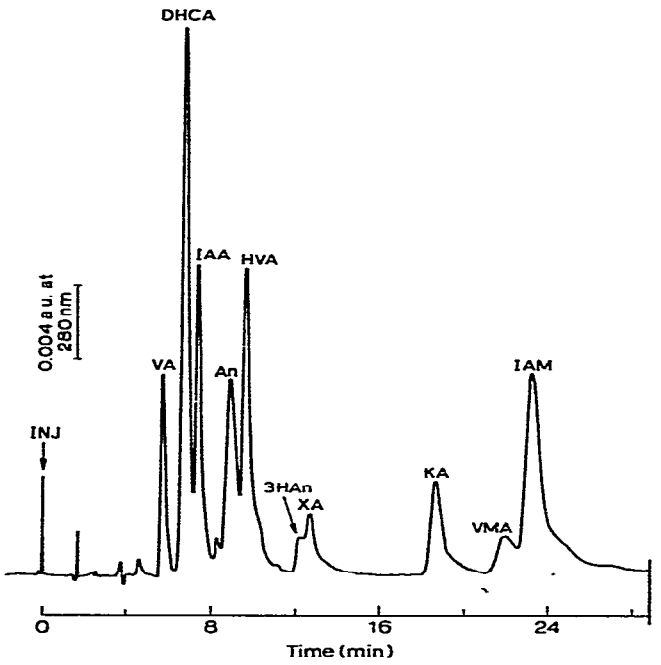

Fig. 2. Chromatogram of a standard mixture containing some indole and catechol derivatives. Volume injected was 2.50 μl, in which 0.08—0.5 μg of each compound was dissolved. Column: LiChrosorb SI 100 (Merck); the silica gel was loaded with a triethanolammonium perchlorate—perchloric acid solution (see text). Mobile phase: *n*-heptane—2-propanol—isobutanol (60:30:10) saturated with the stationary phase. Flow-rate: 0.71 ml/min. Temperature, ambient (18—22°C). Chart-speed: 0.25 in./min. For abbreviations see Table I.

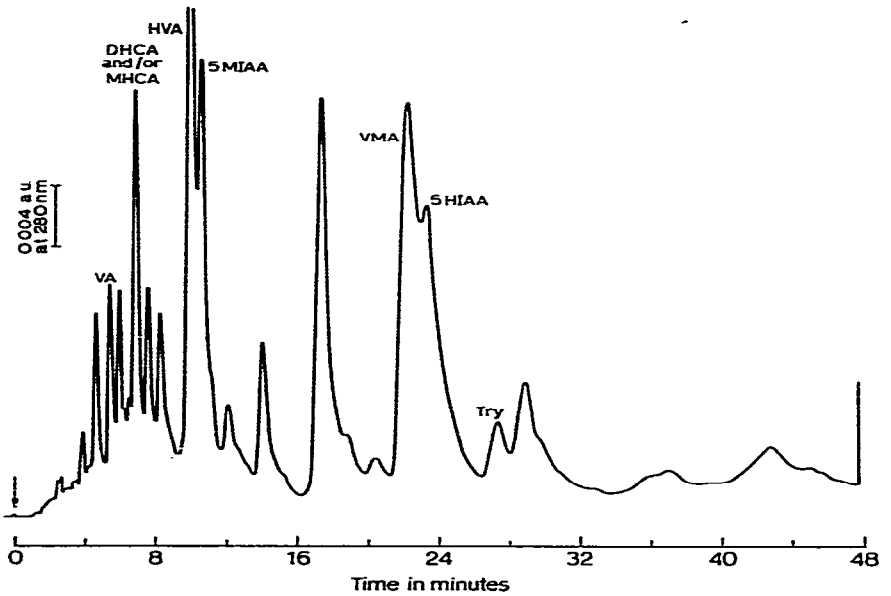

Fig. 3. Chromatogram of fraction A of the extract of a urine sample from a patient with neuroblastoma. Volume of extract injected: 2.5 μl. Chromatographic conditions as in Fig. 2. For abbreviations see Table I.

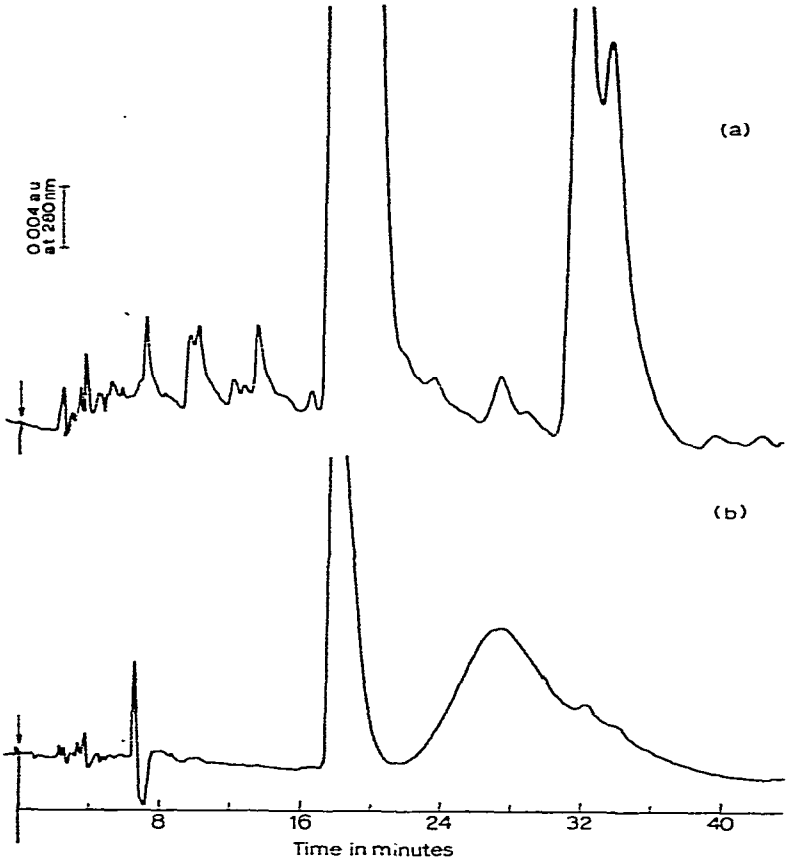

Fig. 4. Chromatograms of fraction B (a) and fraction C (b) of the extract of the same urine sample as in Fig. 3. Volume injected: 2.5 µl. Chromatographic conditions as in Fig. 2.

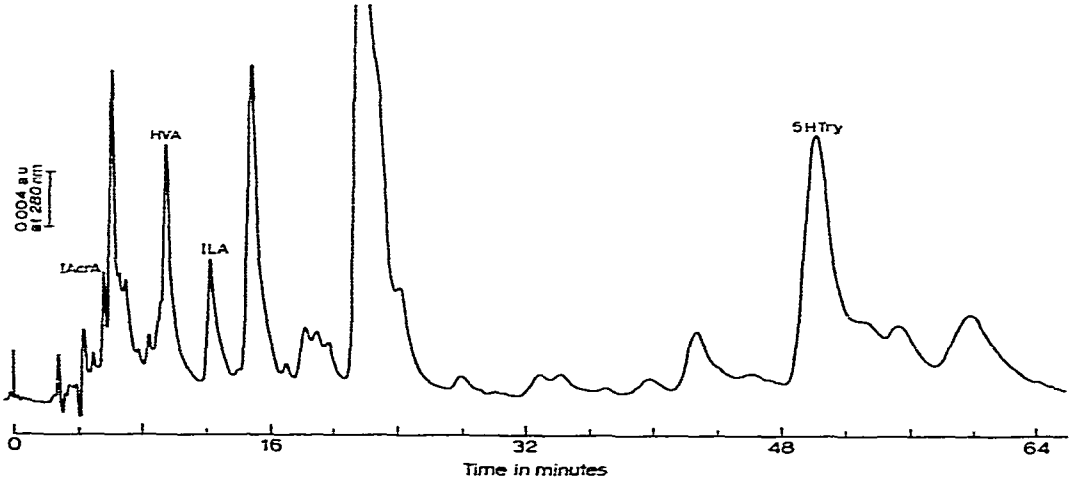

Fig. 5. Chromatogram of the extract (fraction A) of a urine sample from a case of phenylketonuria. Volume injected: 2.5 µl. Chromatographic conditions as in Fig. 2. For abbreviations see Table I.

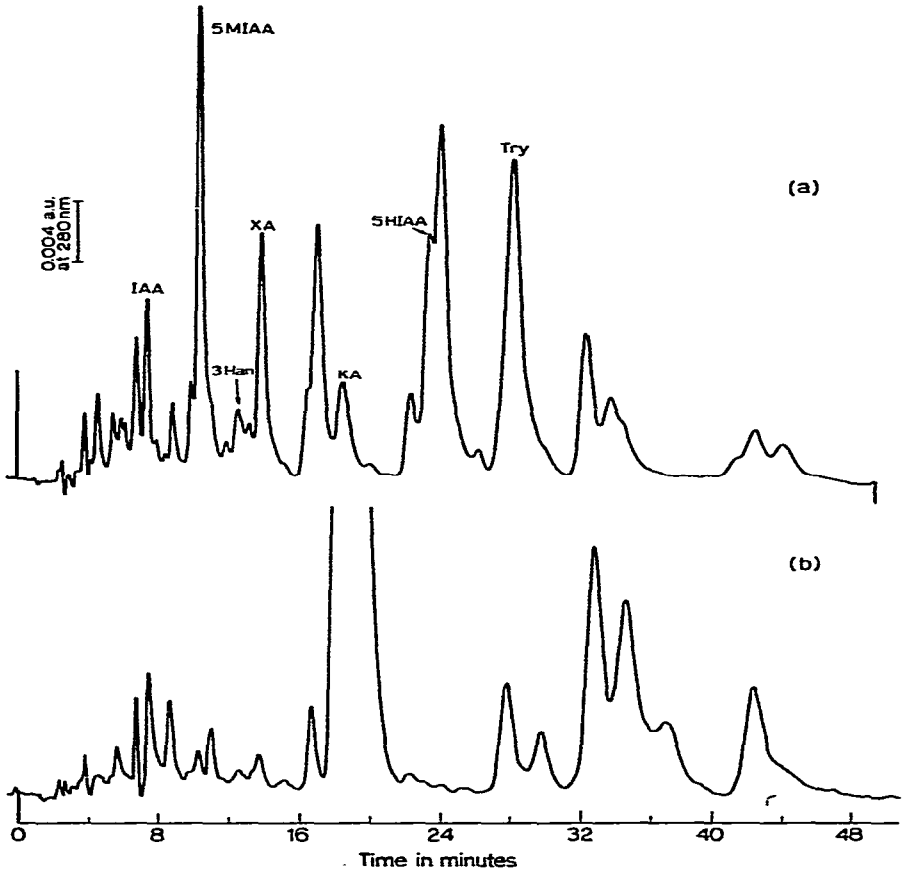

Fig. 6. Chromatograms of fraction A (a) and fraction B (b) of the extract of a urine sample from a patient affected with hypochromic microcytic anemia. A 24-h urine sample was collected after loading the patient with tryptophan. Volume injected: 2.5 μl. Chromatographic conditions as in Fig. 2. For abbreviations see Table I.

DISCUSSION

The present chromatographic system was selected from several we tested in a preliminary study (unpublished results) because it proved to be fully adaptable for the separation of (a) the major tryptophan metabolites of the kynurenine and serotonin pathways, (b) homovanillic acid, vanilmandelic acid and other related compounds, and (c) 5HIAA, 5MIAA, HVA and VMA. In fact, this kind of separation should enable one to examine the pathological conditions which involve the acid metabolites of tryptophan and phenylalanine.

We have tested the adaptability of this chromatographic technique to the solution of practical problems in clinical pathology, subjecting to analysis real urine samples from healthy adult subjects as well as from infants affected with various diseases. The results obtained (Figs. 3—6) show that the technique is capable of resolving a discrete number of compounds with acceptable efficiency and selectivity even without gradient elution, so that it can be profitably used for testing the aromatic constituents of the urine.

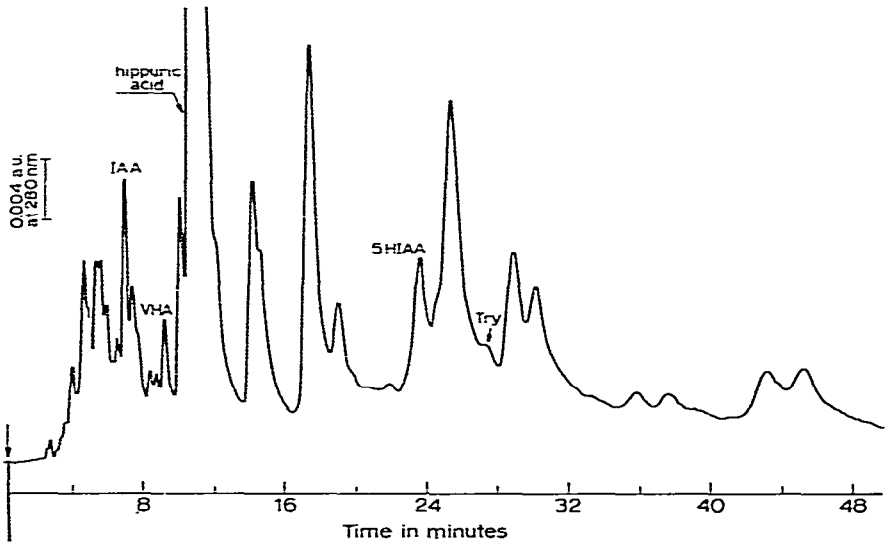

Fig. 7. Chromatogram of the extract (fraction A) of a urine sample from a healthy adult subject. Volume injected: 2.5 μl. Chromatographic conditions as in Fig. 2. For abbreviations see Table I.

It was necessary to employ extraction procedures for the substances of interest in the urine prior to chromatography since the number of UV-absorbing urinary constituents was very high. However, these procedures do offer the advantage that such a multicomponent mixture is fractionated into subgroups of given chemical affinities, for example neutral compounds, acids and bases, so as to obtain simpler chromatographic responses.

For this purpose we have devised a method for the extraction of organic compounds from urine using C_{18}-bonded silica microcolumns, which appears to us less laborious and more flexible than ethyl acetate extraction of acidified and NaCl-saturated urine [17, 22, 23].

Though both extraction methods are based on the same principle, i.e. liquid—liquid partition of the substances between two immiscible phases, the use of the C_{18} cartridges offers a more versatile system in as much as more than one fraction may be collected from a single specimen by multiple successive elutions with solvents of different strengths. Thus we obtained three fractions, substantially different from each other, from the urine of a case of neuroblastoma (Figs. 3—5). HVA and VMA were present in the first of these fractions (A), while the remaining two fractions yielded chromatograms with some high peaks, which have not yet been identified.

The fraction A obtained from the urine of a phenylketonuric patient was found to contain indole-lactic acid and 5-hydroxytryptophan, as well as some unknown substances (Fig. 5). Similarly, HVA was always present in the first fraction from urine samples of healthy subjects (Fig. 7).

As far as the selectivity of the present extraction method is concerned, we have obtained a clear-cut separation of some acids and neutral compounds (Table II), in fractions A and B, respectively, while the basic and very polar substances were not retained by the bonded phase.

The drawback of the chromatographic method described is that the strongly retained compounds, which yield relatively broad peaks, give poor detection responses if sensitivity is correlated only with peak height. Generally, the sensitivity limits, expressed in terms of peak height, were lower than in reversed-phase HPLC, probably because of bathochromic or ipsochromic effects of the organic mobile phase on the UV-absorbance maxima of the metabolites studied.

In conclusion, we believe that: (A) the HPLC technique described here, although still open to further improvement, constitutes a valuable system for the rapid and simple analysis of the metabolites considered; and (B) the use of the C_{18}-bonded silica cartridges further simplifies the analytical procedure and offers the possibility of obtaining more highly purified and fractionated samples for chromatographic analysis.

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