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TRYPTOPHAN AND KYNURENINE DETERMINATION IN UNTREATED URINE BY REVERSED-PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A fast and sensitive method for the analysis of tryptophan and some of its metabolites is discussed. A reversed-phase chromatographic system with water mobile phase can separate tryptophan, N-formalkynurenine, kynurenine and 3-hydroxykynurenine in less than 15 min at a flow-rate of 1 ml/min. The application of the method to the analysis of tryptophan and kynurenine in untreated urine of a patient loaded with tryptophan is described. The ease and speed of analysis makes the method very attractive for clinical purposes. Among other things, it was found that tryptophan in untreated urine degrades with time, even if the sample is frozen at −11°.

INTRODUCTION

Important clinical information can be obtained from the analysis of various amino acids and their related metabolites in serum and urine. With the advent of high-performance liquid chromatographic (HPLC) systems, the opportunity exists for quick and reliable assays with minimum work up and minimum analysis times. An example of the application of HPLC to a biologically important system is the analysis of uric acid as described by Pachla and Kissenger [1].

The measurement of urinary tryptophan and its metabolites has considerable clinical importance. Abnormally large amounts of kynurenine and 3-hy-

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droxykynurenine are excreted in most patients with hemoblastic diseases such as myeloid or lymphoid leukemia, Hodgkin's disease, and multiple myeloma [2], as well as in many patients with bladder cancer [3]. Elevated tryptophan with low kynurenine following tryptophan loading has been reported in cases of congenital tryptophanuria with dwarfism [4] and Hartnup's disease [5]. Increased excretion of kynurenine, 3-hydroxykynurenine, kynurenic acid, and xanthurenic acid has even been reported in patients with infantile spasm due to pyridoxine deficiency, pyridoxine dependency, or abnormalities of kynureninase [6].

It is thus desirable to develop a fast and simple method of analyzing tryptophan and some of its metabolites in urine. Rable [7] has demonstrated the separation of a synthetic mixture of some tryptophan metabolites on a Pellidon column. Some metabolites have been determined by ion-exchange systems [8—11], but most analyses have been accomplished by thin-layer chromatography [11—15]. Several spectroscopic methods were also utilized in the analysis of tryptophan metabolites [10, 16, 17]. These papers represent only a fraction of the published work which can be found in the literature.

Considerable interest has recently been focused on the tryptophan—kynurenine—nicotinic acid pathway. The importance and scope of this metabolic pathway has been aptly discussed in a recent review by Allegri and De Antoni [18]. The present work describes a quick and highly sensitivity assay for urinary tryptophan, kynurenine, N'-formylkynurenine, and 3-hydroxykynurenine. The utilization of this method in the quantitative determination of the first two compounds in normal and loaded urine will be demonstrated.

EXPERIMENTAL

Liquid chromatograph

The liquid chromatograph consisted of a Waters Assoc. 6000A pumping system, and a Waters Assoc. 440 dual wavelength absorbance detector, which was capable of monitoring 254 nm and 280 nm simultaneously. The detector was set at 0.5 a.u.f.s. Injections were made via a Waters Assoc. Model U6K injector. Data were collected on a Laboratory Data Control dual-pen chart recorder. The columns were a 25 cm × 4.6 mm I.D. Partisil 10 column, and a 25 cm × 4.6 mm I.D. Partisil ODS column (Whatman, Clifton, N.J., U.S.A.). The solid support size was 10 μm. All chromatograms were obtained at ambient temperature.

Reagents and chemicals

The mobile phase was distilled and deionized water. Solutes were obtained from various sources. Urine specimens were collected from a normal adult male before, and at two-hour intervals after receiving a tryptophan load of 100 mg/kg body weight (5.66 g in the present study). Samples were frozen at −11° until use. Standard mixtures were made by dissolving either 0.5 or 1 mg/ml of mobile phase. Five or ten microliters of untreated urine were injected onto the various columns.

Data treatment

The amounts of tryptophan and kynurenine in the urine were determined from peak heights which were compared with those of known standards. The determined weights are believed to be accurate to ±5% since the columns were not thermostatted.

RESULTS AND DISCUSSION

Several systems for the separation of tryptophan and its metabolites were tested before a suitable one for real urine samples was selected. For example, a Partisil 10 column, conditioned to heptane using the series of solvents recommended by Scott and Kucera [19], and a mobile phase consisting of 1% acetic acid—1% sodium chloride—water gave good separations of synthetic standard mixtures. Unfortunately, this and other attempted normal phase systems proved to be unsuitable for the analysis of real samples owing to interfering substances from urine.

Owing to the polar nature of the solutes, it became apparent that a reversed-phase column would be more advantageous. The initial studies with an ODS (C_{18}

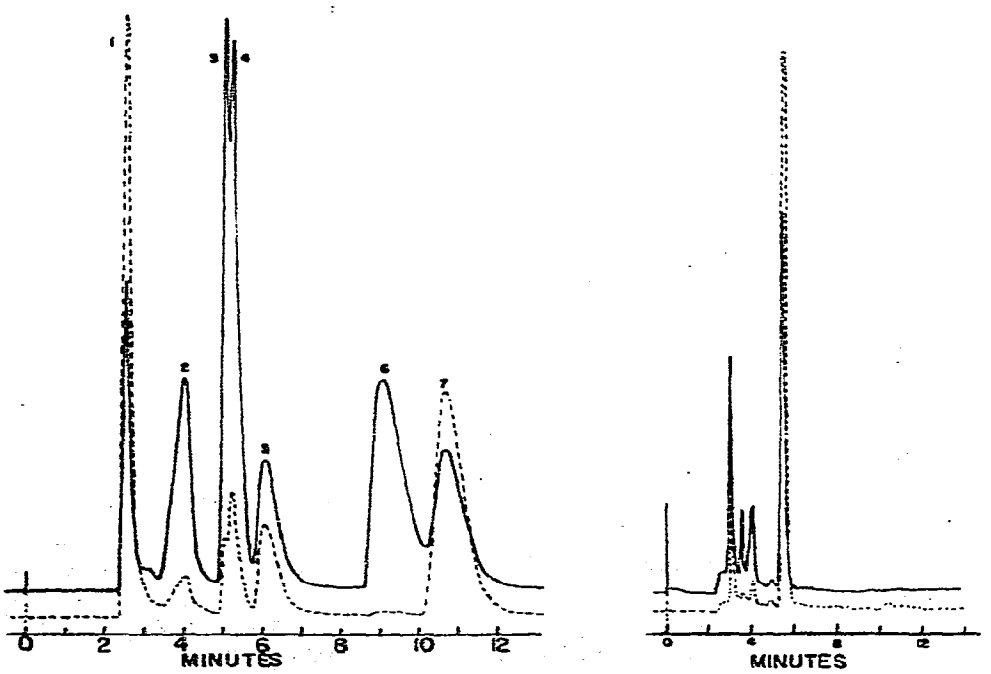

Fig. 1. Separation of tryptophan metabolites and several other compounds of interest. Column: Partisil ODS 25 cm × 4.6 mm I.D. Mobile phase, water; flow-rate, 1 ml/min; temperature, 26°; sensitivity, 0.5 a.u.f.s. ———, 254 nm; — — —, 280 nm. 1 = Orotic acid (3 μg); 2 = N-formylkynurenine (3 μg); 3 = uracil (1 μg); 4 = uridine (3 μg); 5 = 3-hydroxykynurenine (3 μg); 6 = kynurenine (5 μg); 7 = tryptophan (5 μg).

Fig. 2. Control urine profile, 5-μl sample. Conditions, see Fig. 1. ———, 254 nm; — — —, 280 nm.

bonded to the silica gel support) column, indicated that such a column could give better and more reproducible separations. Fig. 1 shows the separation of a synthetic mixture of four tryptophan metabolites and orotic acid, uracil and uridine on an ODS column with distilled deionized water as mobile phase. (The orotic acid, uracil and uridine were present as a part of another study not related to tryptophan metabolism.) The total analysis time is about 12 min.

Fig. 2 shows a chromatogram of control urine injected directly without any prior work up. At 0.5 a.u.f.s. no tryptophan or kynurenine could be seen. Fig. 3 shows a profile of a urine sample collected 2 h after the patient received a tryptophan load. Peaks for kynurenine and tryptophan are clearly evident. Fig. 4 shows the chromatogram of a urine specimen taken 4 h after the load. The kynurenine and tryptophan levels have increased.

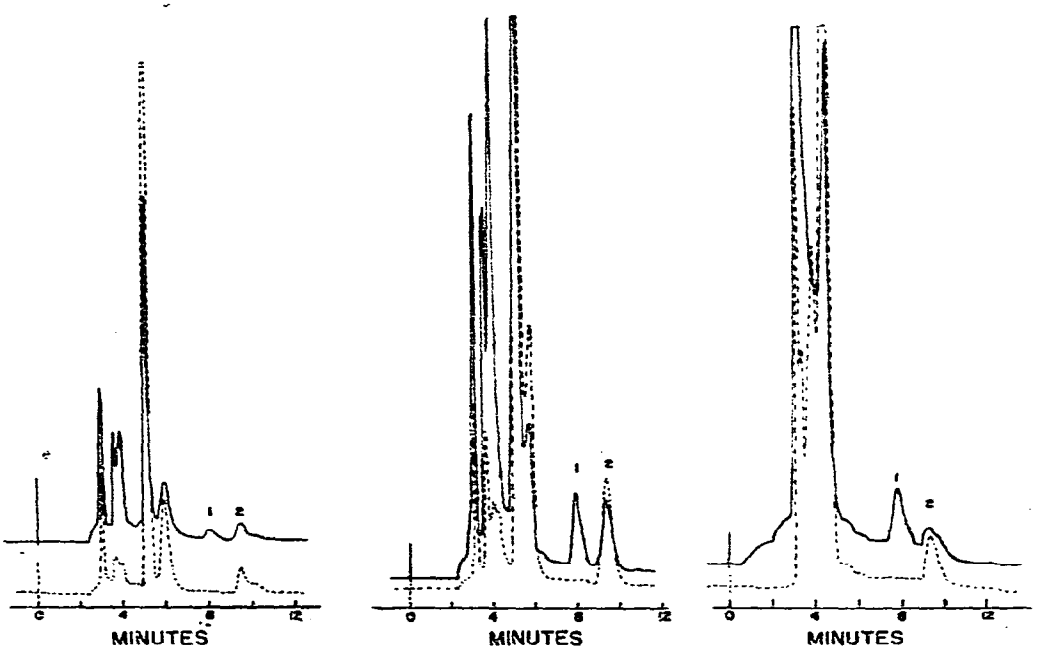

Fig. 3. Urine profile of a 2-h sample, 5-μl injection. Conditions, see Fig. 1. ———, 254 nm; — — —, 280 nm. 1 = Kynurenine; 2 = tryptophan.

Fig. 4. Urine profile of a 4-h sample, 10-μl injection. Conditions, see Fig. 1. ———, 254 nm; — — —, 280 nm. 1 = Kynurenine; 2 = tryptophan.

Fig. 5. Urine profile of a 4-h sample, 1 week old (frozen at −11°). Conditions, see Fig. 1. ———, 254 nm; — — —, 280 nm. 1 = Kynurenine; 2 = tryptophan.

The identification of these two compounds was confirmed by spiking the urine samples with pure standards. Position and relative intensity of the absorbances at 254 nm and 280 nm matched those obtained with standard mixtures.

The general pattern of the urine changed drastically upon administration of the tryptophan load. A number of additional peaks are present in Figs. 3 and 4. No identification of these was attempted; however, most likely some of these are tryptophan metabolites which were not characterized in this study. The

complex elution pattern around 4 min did not allow measurements of N-formylkynurenine. The very small shoulder appearing slightly after 6 min in Fig. 4 is thought to be 3-hydroxykynurenine. This identification, however, is only tentative at this point.

All the above mentioned chromatograms were run one day after the administration of the tryptophan load. Fig. 5 shows a chromatogram of the 4-h sample after being stored frozen (at −11°) for one week. A drastic change in the urine chromatographic profile is evident along with a drop in tryptophan and kynurenine levels.

The standard mixture was rerun in order to determine if the observed changes were due to deterioration of, or variations in, the system's performance. The standards showed, for all practical purposes, no significant variations in column performance. The initial samples that we analyzed were collected 2—4 h after the tryptophan loading, and delivered to our laboratory on the following day. However, urine samples were collected every 2 h for 12 h, and the whole set of specimens was frozen at −11°. Our laboratory received these samples a week after administration of the load. As shown in Table I there is a drastic difference in the amounts of tryptophan and kynurenine in the day old samples and in the week old ones. These data indicate that analysis should be carried out as quickly as possible. Even freezing immediately after urine collection has not assured constant levels of tryptophan and its metabolites with passing time. Such freezing and long term standing is frequently common in the clinical laboratory. Although this point was not investigated carefully, it seems that freezing at dry ice temperature will arrest the destruction of tryptophan. The tryptophan degradation was observed in all the urine samples independent of their sources.

Table I seems to indicate that the maximum amount of kynurenine in the urine occurs several hours after the maximum amount of tryptophan is observed. More work is needed to quantitate precisely the amounts of these compounds in urine as a function of time immediately after a tryptophan load, and as a function of elapsed time from administration of the load.

The lower limit of detection, at present, is estimated to be about 0.05 μg/μl of urine. No attempts were made to improve the sensitivity of the method.

TABLE I

TRYPTOPHAN (Trp) AND KYNURENINE (Ky) LEVELS IN URINE SAMPLES

Time after load (h)	1 day old		1 week old		Change (%)	
	μg/10 μl Ky	μg/10 μl Trp	μg/10 μl Ky	μg/10 μl Trp	Ky	Trp
Control	∿0	∿0	∿0	∿0	—	—
2	0.24	0.82	0.12	0.60	50	27
4	0.95	1.9	0.88	0.44	7	77
6	—	—	1.32	0.47	—	—
8	—	—	0.16	0.05	—	—
10	—	—	0.16	0.05	—	—
12	—	—	0.12	∿0	—	—

Based on the chromatogram of the standard mixtures, however, it is believed that a tenfold increase in the sensitivity could be attainable if needed.

The chromatographic column has been used for over 125 h with direct injections of untreated urine. No degradation in column performance has been observed. It is conceivable that, after prolonged use, urinary proteins and other macromolecules might destroy the column efficiency. In the present study, this did not present a problem.

After the compilation of this study, the column was stored for several weeks in distilled water. The column was then re-installed in the chromatograph in order to analyze urine of a mentally retarded patient. It was found that the retention characteristics of the column changed considerably. An examination of the previous history of the column revealed that it was conditioned by the manufacturer with methanol—water. Apparently some of the methanol was adsorbed on the surface of the support. Eventually, after a long time of usage with, and storage in, water, the methanol was depleted, causing a change in the retention times. However, adding about 0.5—1% methanol to the water mobile phase restored the previously observed elution pattern.

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REFERENCES

1 L.A. Pachla and P.T. Kissenger, *Clin. Chim. Acta*, 59 (1975) 309.
2 L. Musajo, C.A. Benassi and A. Parbajola, *Clin. Chim. Acta*, 1 (1956) 229.
3 R.R. Brown, J.M. Price, E.J. Satter and J.B. Wear, *Acta Unio Int. Contra Cancrum*, 16 (1960) 299.
4 K. Tada, H. Ita, Y. Wada and T. Arakawa, *Tohoku J. Exp. Med.*, 80 (1962) 118.
5 M.D. Milne, M.A. Crawford, C.B. Giaro and L.W. Longhridge, *Quart. J. Med.*, 29 (1960) 407.
6 J.H. French, R.B. Grueter, R. Druckman and D. O'Brien, *Neurology*, 15 (1965) 101.
7 F.M. Rabel, *Anal. Chem.*, 45 (1973) 957.
8 D.D. Chilcote and J.E. Mrochek, *Clin. Chem.*, 18 (1972) 778.
9 N.C. Chen and R.K. Gholson, *Anal. Biochem.*, 47 (1972) 139.
10 G.G. Guilbault and P.M. Forelich, *Clin. Chem.*, 20 (1971) 812.
11 K.M. Morris and R.J. Moon, *Anal. Biochem.*, 61 (1974) 313.
12 M. Binazzi, P. Calandra and P. Lisi, *Acta Vitaminol. Enzymol.*, 28 (1974) 185.
13 P. Calandra, *Acta Vitaminol. Enzymol.*, 28 (1974) 189.
14 G. M. Anderson, *J. Chromatogr.*, 105 (1975) 323.
15 P. Baumann, *J. Chromatogr.*, 109 (1975) 313.
16 L.V.S. Hood and J.D. Winefordner, *Anal Biochem.*, 27 (1969) 523.
17 J. Buxto and G.G. Guilbault, *Clin. Chem.*, 20 (1974) 765.
18 G. Allegri and A. De Antoni, *Acta Vitaminol. Enzymol.*, 28 (1974) 223.
19 R.P.W. Scott and P. Kucera, *J. Chromatogr. Sci.*, 12 (1974) 473.