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SEPARATION OF 5-HYDROXYINDOLE-3-ACETIC ACID AND INDOLE-3-ACETIC ACID IN URINE BY DIRECT INJECTION ON A REVERSED-PHASE COLUMN CONTAINING A HYDROGEN-ACCEPTING STATIONARY PHASE

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SUMMARY

The separation of 5-hydroxyindole-3-acetic acid and indole-3-acetic acid from urine is demonstrated. The urine is injected directly on a liquid chromatographic column which is coupled to a flow-through fluorescence detector or a UV detector. A hydrogen-accepting stationary phase, tributyl phosphate (TBP), which retains carboxylic acids selectively, was more selective than more common reversed-phase systems with alkyl-bonded stationary phases. The mobile phases were buffers, sometimes buffers containing methanol or acetonitrile.

A simple method of removing late peaks in the chromatogram, in order to reduce analysis time, is also presented.

INTRODUCTION

Determination of low-molecular-weight carboxylic acids in complicated matrices such as urine often requires time-consuming and laborious extraction, evaporation or pre-concentration steps. In addition, very hydrophilic acids may not be quantitatively extracted. Direct injection of urine on a liquid chromatograph with on-line separation and detection can greatly simplify the determination technique. However, due to the vast number of low-molecular-weight compounds in urine, the determination of an endogenous compound may place very high demands on the selectivity of both the separation and the detection systems. These demands will vary depending on the nature and concentration level of the compound in the urine.

Determination of carboxylic acids in urine, *e.g.*, the metabolites of serotonin and catecholamines, has often involved an initial extraction with solvents such as ethyl acetate or diethyl ether followed by various assay techniques such as the colorimetric measurement of 5-hydroxyindole-3-acetic acid[1] or gas–liquid chromatography for vanillylmandelic acid[2]. A notable simplification of procedures has been obtained by liquid chromatography with on-line UV absorbance detection after the extraction by ethyl acetate for vanillylmandelic acid[3,4]. The chromatograms are, however, often rather complex. The application of electrochemical detectors has also been discussed[4,5]. With an even more selective liquid chromatographic detection the urine can be directly

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injected as had been done for the determination of vanillylmandelic acid by an on-line post-column reaction detector[6].

This paper discusses the separation of 5-hydroxyindole-3-acetic acid (5-HIAA) and indole-3-acetic acid (IAA) from urine. The use of liquid chromatography in the determination of 5-HIAA has previously involved an initial extraction from the urine, either by liquid–liquid extraction[7], which was not quantitative, or by chromatography[8]. Chilcote[9] greatly simplified the determination of 5-HIAA and IAA by direct injection of urine on an anion-exchange column coupled on-line to a fluorimetric detector. However, 0.5 h was required for the separation of 5-HIAA and 1 h for IAA. Deproteinized urine has been injected on reversed-phase liquid chromatographic columns combined with a fluorometric detector, but 5-HIAA did not seem to be well resolved[10].

In the present work we have compared the selectivity of a few commonly used reversed-phase liquid chromatographic systems with a newly developed phase system which has a proton-accepting stationary phase, tributyl phosphate (TBP). On-line detection by UV and fluorescence was used. TBP has extensively been used as a stationary phase in separations of metal ions by extraction chromatography[11], but its use for the column liquid chromatographic separation of organic compounds has only recently been studied, namely for carboxylic acids[12], amino acids[12,13] and catecholamines[13]. The TBP column with a fluorimetric detector was the only system that gave resolution of 5-HIAA when urine was injected directly into the column. This clearly shows the need for phase systems with selectivities higher than that of the most commonly used systems in reversed-phase liquid chromatography.

Application of the TBP system to the determination of increased levels of 5-HIAA in urine, from patients suspected to be suffering from carcinoid tumors, will be discussed elsewhere[14]. The method is more rapid and simpler than existing methods for the determination of 5-HIAA in urine.

EXPERIMENTAL

Phosphate or citrate buffers with an ionic strength of 0.1 were used. Methanol and tributyl phosphate were of analytical grade from Merck (Darmstadt, G.F.R.). Acetonitrile was Baker (Phillipsburg, NJ, U.S.A.) analyzed HPLC solvent. 5-Hydroxyindole-3-acetic acid was from Sigma (St. Louis, MO, U.S.A.) and indole-3-acetic acid from Merck. Water was deionized and purified in a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.).

The separation columns (100 × 4.6 mm or 150 × 4.6 mm, 316 stainless steel; Handy & Harman) were packed with LiChrosorb RP-18, 5 μm (Merck), suspended in chloroform. Zero dead volume fittings with Altex 250-21 2-μm stainless-steel frits were used in both ends of the column.

The liquid chromatograph consisted of a Milton-Roy Minipump with pulse dampener (LDC Model 711-26), a Schoeffel FS 970 fluorescence detector with continuously variable excitation wavelength and a cut-off filter to isolate the emitted light and a SpectroMonitor III (LDC) variable-wavelength UV detector. Two sample valve injectors, one Rheodyne 7120 with a 1.1-ml loop connected in series before a Valco CV-6UHPa injector with 10-μl loop, were used. A HETO Model 02 PT 923 water-bath (HETO, Birkerød, Denmark) with external circulation was used to thermostat

the eluent reservoir and the chromatographic column, by means of a water-jacket, to 25.0 ± 0.1°C.

The eluent for the TBP phase system was prepared by mixing one volume of methanol with nine volumes of phosphate buffer. This mixture was saturated at 25.0 ± 0.1°C with TBP and nine volumes of the saturated solution were mixed with one volume of a corresponding unsaturated solution, which gave an eluent with 90% saturation of TBP.

The stationary TBP phase was applied by the injection technique[12]. After coating, the column was conditioned overnight.

Urine samples were filtered through 0.5–2-μm filters or centrifuged prior to injection.

RESULTS AND DISCUSSION

Choice of phase system in the separation column

The separation column consists of LiChrosorb RP-18 as the support coated with tributyl phosphate (TBP) as the stationary liquid phase. The eluent is a buffer mixed with 10% methanol and is partly saturated with TBP. The stationary phase is a strong hydrogen acceptor which can form hydrogen bonds to carboxylic acids such as 5-HIAA and IAA. A more detailed discussion of the properties of this phase system and of its ability to retain carboxylic acids is given elsewhere[12]. Due to its hydrogen-accepting properties this phase has a selectivity different from conventional reversed-phase systems where only the RP-18 phase is used as the stationary phase.

The advantage of using the TBP phase system is obvious from Fig. 1, which gives chromatograms of a urine pool obtained in different chromatographic phase systems and with fluorometric detection. Three of the phase systems are combinations of LiChrosorb RP-18 with eluents of methanol–buffer, acetonitrile–buffer or buffer only. In these systems a high noise level is obtained in the elution region of 5-HIAA due to the presence of fluorescent compounds in the urine, and 5-HIAA cannot be resolved at its normal concentration level in urine (about 10 μmol/l). Some experiments with different concentrations of methanol and acetonitrile and at different pH values of the buffer did not show any improvement of the resolution. In the fourth system, where LiChrosorb RP-18 is coated with TBP, most of the fluorescent compounds are eluted early whereas 5-HIAA and IAA are retained selectively, resulting in complete resolution of 5-HIAA at the normal concentration level.

Choice of detector

Detection by UV absorption and by fluorescence was tested. In the concentration range of interest for 5-HIAA, *i.e.*, above the normal level of about 10 μmol/l urine, it can be detected with the UV detector at the absorption maximum of 280 nm. However, as shown in Fig. 2 for a urine sample from a healthy individual, 5-HIAA was not completely resolved and the chromatogram was rather complicated. The advantage of using the more selective fluorescence detector, as in Fig. 1D, is obvious. It gave a much simpler chromatogram and 5-HIAA was completely resolved. Although this chromatogram was from a pooled urine sample, the conclusion is correct since the individual urine sample of Fig. 2 showed the same main peaks as in Fig. 1D when fluorescence detection was used.

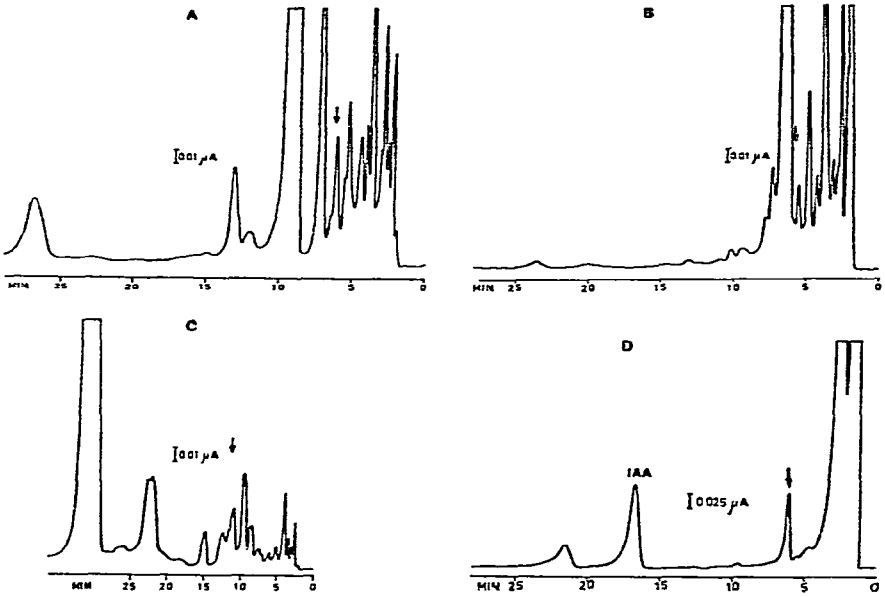

Fig. 1. Chromatograms of urine samples in different phase systems with fluorescence detection. Samples: 10 μl pooled urine from five healthy individuals. Stationary phases: RP-18 (A, B, C) and TBP (D). Eluents: A = buffer pH 5.0–methanol (85:15); B = buffer pH 3.0–acetonitrile (85:15); C = buffer pH 6.0; D = buffer pH 6.0–methanol (90:10) with 90% saturation of TBP. Excitation: 280 nm. Emission: 370 nm cut-off. Column length: 150 mm (A, B, C) and 100 mm (D). Flow-rate: 0.5 ml/min. Arrows indicate the retention time of 5-HIAA.

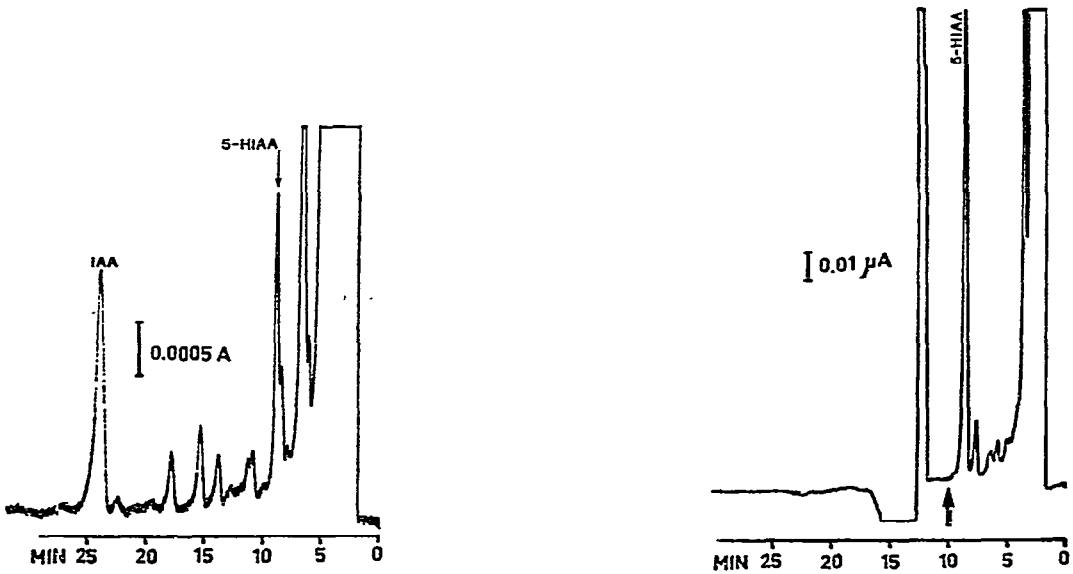

Fig. 2. Chromatogram of urine with UV-detection at 280 nm. Sample: 10 μl urine from a healthy individual. Stationary phase: TBP. Column length: 150 mm. Other conditions as in Fig. 1D.

Fig. 3. Removal of late peaks by a step change of pH of the eluent. Sample, phase system, column and flow-rate as in Fig. 2. Fluorescence detection conditions as in Fig. 1. The arrow indicates injection of 1 ml of buffer pH 8–methanol (9:1).

Stability of columns

The chromatographic phase system, based on TBP as the stationary phase, has a very high long-term stability, provided that the saturation degree of TBP in the eluent is carefully controlled, as discussed earlier[12]. No special precautions had to be taken when the urine samples were injected except that they were filtered or centrifuged before application. No deterioration in column performance due to the urine samples was observed on application of a total volume of urine of 1.0 ml corresponding to 100 samples.

Removal of late peaks by change of pH of the eluent

If only one of the components in the chromatogram has to be determined, those peaks that are eluted later increase the time intervals between sample injections. The retention time of the late peaks can be reduced by introducing a relatively small volume of a stripping eluent through the sample loop, provided that a fast equilibration is obtained on the column.

If only 5-HIAA is to be determined, IAA and some smaller peaks must be eluted before the next sample injection can be made. The retention of carboxylic acids can be decreased by increasing the pH of the mobile phase[12], *e.g.*, by injecting a 1-ml plug of buffer pH 8–methanol (9:1). At this pH IAA will have a capacity ratio 100 times smaller than at pH 6 and migrates with the front of the plug as in Fig. 3. A clean baseline is obtained within a much shorter time than in Fig. 1D, taken into account the difference in column size.

The minimum volume of stripping solvent that had to be applied to the columns was 1 ml. With smaller volumes the method was not effective. The technique was evaluated by injecting a series of 21 urine samples with intermittent injections of a pH 8 plug; 20 min were allowed between each urine injection. The retention time of 5-HIAA was unaffected by the pH 8 plugs provided that two column void volumes of eluent were passed down the column between the injection of the plug and that of the sample.

This principle can be used in many similar cases and can be automated easily.

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