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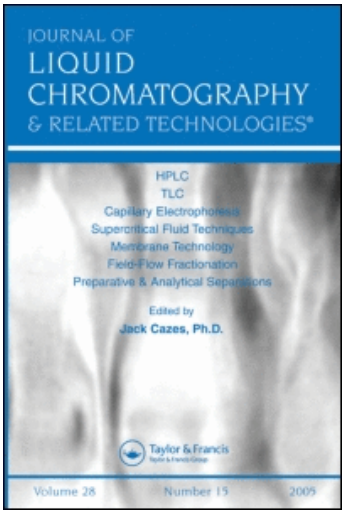

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QUICK AND SIMPLE SIMULTANEOUS DETERMINATION FOR SOME AMINO ACIDS BY REVERSED-PHASE HPLC WITH UV DETECTION

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ABSTRACT

A simple and rapid high pressure liquid chromatographic method using an ultraviolet detector for simultaneous analysis of histidine, tyrosin and tryptophan, is presented.

Chromatographic separation is achieved on Spherisorb-5 RP-18 5μm reversed phase column and the mobile phase is the isocratic mixture of acetonitrile, methanol and water (5:30:65). The eluted amino acids are detected at 220 nm. The retention time is 1.55 min for histidine, 2.21 min for tyrosin and 2.80 min for tryptophan. The correlation of the integrated peak areas with the concentration of amino acids showed a linear relationship between 0.40 to 9.43 ppm for histidine, 0.24 to 22.6 ppm for tyrosin, and 0.20 to 12.8 ppm for tryptophan per 10 μl injection.

Simultaneous analysis of histidine, tyrosin and tryptophan gave reproducible results with a mean coefficient of variation 1.93 pc for tryptophan, 2.29 pc for tyrosin and 3.51 pc for histidine and r^2=0.999. The proposed technique was applied to the analysis of these amino acids in urine samples.

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INTRODUCTION

It is well known that amino acids are not readily analyzed by high--performance liquid chromatography (HPLC) (1), because of their low response to ultraviolet or fluorescent detection and high polarity. Tyrosin, tryptophan and histidine appear to have a high response to ultraviolet detection and so, can be determined employing an ultraviolet detector in the presence of other amino acids.

Pre-column and post-column derivatization have been used to the analysis of amino acids. So phenylthiohydantoin (2-6), o-phthalaldehyde (7-16), 5-dimethylaminonaphthalenesulfonyl (17,18) derivatives have rendered the technique sensitive which is particularly desirable under constraints of low concentration or small sample size. However these derivatives generally require removal of reaction solvents and sometimes use of two columns for HPLC analysis (19,20). The lengthy preparation required for these derivatives limits their usefulness for routine amino acid analysis in clinical laboratories. Also these derivatives were found to lack sufficient specificity for amino acid analysis in samples of biological interest.

In the present paper a simpler, fast-this method reduces chromatographic analysis time to about one tenth of that required for the analysis of amino acid ' s derivatives- and sensitive technique for the simultaneous determination of histidine, tryptophan and tyrosin is developed.

This procedure is then used in the analysis of urinary amino acids.

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EXPERIMENTAL

Reagents

Histidine, tyrosin and tryptophan were from Merck (Darmstadt W. Germany). These reagents were used as provided without further purification. All standard solutions of these compounds were prepared by dissolving the appropriate amount in distilled water and stored at $4^{o}C$. HPLC-grade methanol and acetonitrile were obtained from Merck (Darmstadt W. Germany). Glass--distilled water was used throughout and all solvents used were of analytical grade.

Eluent System

A number of eluent systems were examined, these systems being selected on the basis of their relative polarities and low absorption at 220 nm. The

most suitable of them being Acetonitrile, Methanol and Water (5:30:65). All eluting solvents were ultrasonically degassed under vacuum before use and by using helium during the analysis time.

Apparatus

UV spectra were recorded in distilled water in a 1-cm cell on a VARIAN DMS 100S double beam ultraviolet spectrophotometer fitted with an EPSON LX-800 recorder module.

High performance liquid chromatographic analyses were conducted with a ternary gradient system Spectra Physics SP 8800 pump, a Spectra Physics spectra chrom 100 variable wavelength UV-Vis detector operated at 220 nm and a sensitivity setting of 0.05 absorbance units f.s.d. A Rheodyne 7125 injection valve was fitted with a 10 μl loop. A Spherisorb-5 C18 reversed-phase column 5 μm particle size, 220x4.6 mm of I.D. stainless steel from Spectra Physics. The flow rate was 1.0 ml/min at a pressure of 2300 psi. The peak areas were integrated by using a Spectra Physics SP 4290 integrator.

Computations were carried out using a PC 1512 AMSTRAD computer.

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Detection Limits

Pure amino acids were accurately weighed and then dissolved in distilled water to give solutions 1.00×10^{-3}M for histidine, 2.50×10^{-4}M for tyrosine and 2.56×10^{-4}M for tryptophan. These solutions were diluted and 10 μl of each dilution injected onto the HPLC system. The limits at which amino acids could be detected, as represented by a peak height, twice the size of background noise on this HPLC system were considered to be the detection limits.

Standard calibration curves for the determination of amino acids

Calibration curves for each amino acid, in distilled water, were constructed by plotting the peak areas produced by the injection of 10 μl of each amino acid solution prepared over the ranges 0.40 to 9.43 ppm for histidine, 0.24 to 22.6 ppm for tyrosin and 0.20 to 12.8 ppm for tryptophan. Each calibration curve was prepared commencing at twice the detection limit. This procedure was repeated seven times for statistical evaluation.

Extraction of aminoacids from urine

Urine (500 μl) was transferred to a centrifugation tube and 2.5 ml of 0.1M perchloric acid was added. The resulting solution was then centrifuged

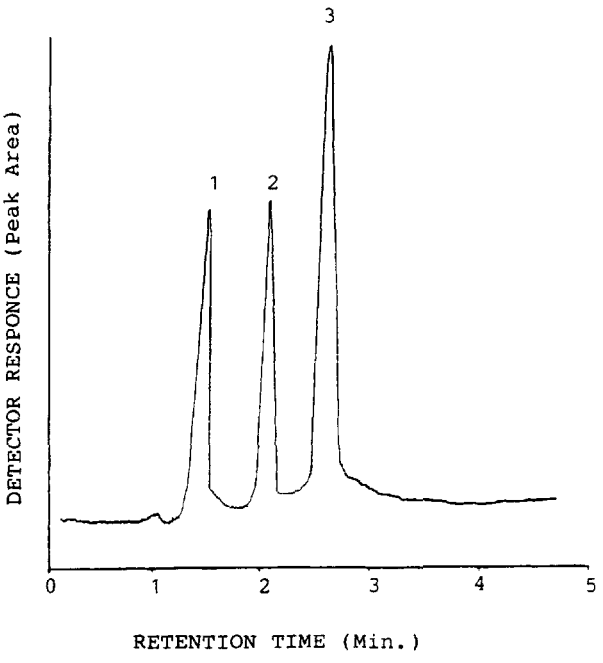

Figure 1. High-Performance Liquid Chromatogram of Histidine, Tyrosin and Tryptophan.
Peaks: 1=Histidine, 2=Tyrosin, 3=Tryptophan.
For chromatographic conditions see in the text.

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at 4000 rpm for 15 min. To 200 μl of the supernatant, 100 μl of standard amino acid solutions 6,9,12 ppm were added and 10 μl of the final solution was injected onto the column.

Employing the described chromatographic conditions and following direct extraction it was possible to determine tryptophan in under 3 min time with a recovery ranging from 84 to 91 pc.

The analysis of histidine and tyrosine was not acceptable because of the interferences from endogenous compounds which prevented the distinct separation and determination of these amino acids. Therefore derivatives of histidine and tyrosine had to be done. For this purpose phenylthiohydantoin, o-phthalaldehyde and 5-dimethylaminonaphthalenesulfonyl were used. The obtained results did not improve those existing in literature (2-18), the retention

times were over 30 min, thus a new derivatizing agent 1-fluoro-2,4 dinitro benzene was selected but again the retention times were of about 25 to 27 min.

Using the described chromatographic conditions and following a single extraction step, we can determine tryptophan in urine samples in under 3 min time.

Following this quick and simple procedure, calibration curves were constracted for the determination of tryptophan in urine samples.

RESULTS AND DISCUSSION

A systematic study of the HPLC separation of histidine, tyrosin and tryptophan, in the presence of other amino acids, with several solvent systems was performend. From the eluting systems examined, the most suitable was found to be acetonitrile, methanol and water (5:30:65). This system gives good separation of the three amino acids in very good retention times, Figure 1.

Using solutions of the amino acids prepared in distilled water the recommended procedure gave a linear relationship between the peak area and the amount of amino acid injected onto the column. The range of linearity was found to be 0.40 to 9.43 ppm for histidine, 0.24 to 22.6 ppm for tyrosin, and 0.20 to 12.8 ppm for tryptophan.

The mean coefficient of variation is 1.93 pc for tryptophan, 2.29 pc for tyrosin and 3.51 pc for histidine.

The detection limits for the amino acids examined were found to be 2.0 ng for histidine, 1.2 ng for tyrosin and 1.0 ng for tryptophan.

Statistical Analysis

Solutions of amino acids in distilled water.

Amino acid	Regression Equation	Correlation Coefficient
Histidine	$y = 0.6948 + 0.1882\ x$	0.9991
Tyrosin	$y = 0.2204 + 0.8199\ x$	0.9994
Tryptophan	$y = 0.1001 + 0.4089\ x$	0.9993

Results obtained for the simultaneous determination of a mixture of the three amino acids are given in Table 1.

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TABLE 1

Experimental results for the simultaneous analysis of histidine, tyrosin and tryptophan after separation by HPLC

Amino acid	Retention Time (min)	Added (ng)	Found[a](ng)
Histidine	1.55	48.00	49.14±1.73
		96.00	94.84±3.30
Tyrosin	2.21	36.00	35.23±0.80
		72.00	70.61±1.62
Tryptophan	2.80	28.00	29.07±0.55
		56.00	56.29±1.06

[a] Average value of six determinations ± standard deviation

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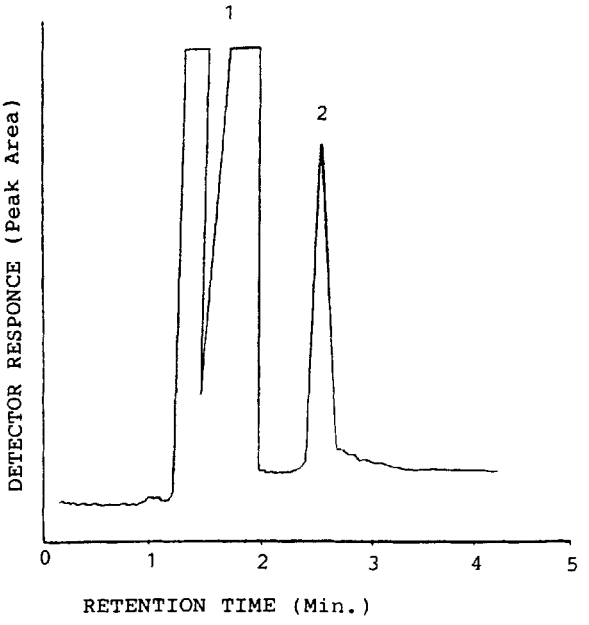

Figure 2. High-Performance Liquid Chromatogram of Tryptophan extracted from Urine.
Peaks: 1=Unknown, 2=Tryptophan.

TABLE 2

Experimental results for the analysis of tryptophan in urine samples.

Amino acid	Added (ng)	Found[a](ng)	Recovery (pc)
	28.00	23.52	84
Tryptophan	42.00	38.22	91
	56.00	49.84	89

[a] Average value of six determinations

The method was applied to the determination of tryptophan in urine samples, Figure 2.

Employing the standard addition technique it was possible to determine tryptophan, in under 3 min time, in urine samples with no interferences from endogenous compounds.

Results taken for the determination of tryptophan in urine samples are given in Table 2.

CONCLUSION

The present reversed phase HPLC assay provides a reliable and rapid method for the identification and simultaneous determination of histidine, tyrosin and tryptophan in the presence of other amino acids. The technique is also applicable to the determination of tryptophan in urine samples at a level of 1.0 ng injected onto the chromatographic column.

Additionally the fact that no special equipment is required -a UV detector, common in all analytical laboratories suffices for the determination- render the technique to be widely applicable.

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