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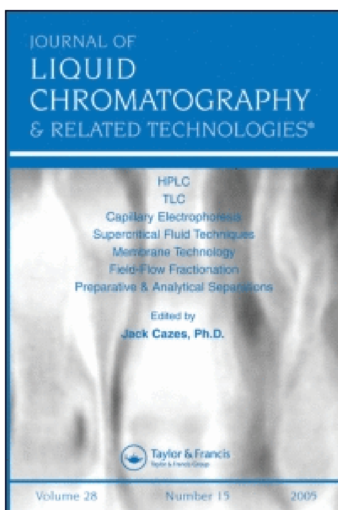

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A Thin Layer Chromatographic Procedure for the Separation of Proline and Hydroxyproline from Biological Samples

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A THIN LAYER CHROMATOGRAPHIC PROCEDURE FOR THE SEPARATION OF PROLINE AND HYDROXYPROLINE FROM BIOLOGICAL SAMPLES

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ABSTRACT

Studies of collagen metabolism often require the analysis of proline and hydroxyproline which necessitates their separation. A methodology was developed which: 1)separates proline and hydroxyproline using thin layer chromatography; 2) locates the imino acids by autoradiography and; 3) recovers them by dialysis in high yield with great precision.

INTRODUCTION

The separation of proline (Pro) and hydroxyproline (Hyp) is essential to studies of the incorporation of radioactive Pro into animal tissues or cells in tissue cultures.[1,2] Numerous separation techniques are currently available.[2-21]

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These methods utilize various approaches (i.e. HPLC, supercritical fluid extraction, capillary zone electrophoresis, paper and thin layer chromatography). We report here a thin layer chromatographic procedure that separates Pro and Hyp, which is unique in that it demonstrates no interference from plasma hydrolysates and affords excellent recovery of Hyp. The procedure is simple, reliable and relatively inexpensive.

EXPERIMENTAL

Chromatography of Amino Acids Standards

Twenty-two amino acids (Sigma Chemical Co., St. Louis, MO) were dissolved in distilled water at a concentration of 5 mg/mL. Five microliter aliquots were spotted 2.5 cm from the lower edge of silicic acid gel impregnated glass fiber sheets (20 cm x 20 cm ITLC-SA, Gelman Sciences Inc., Ann Arbor, MI) at 3.5 cm intervals from the left edge. Warm air was blown under the sheet during the spotting procedure for rapid evaporation of the solvent, to minimize the spot size. The sheets were equilibrated for one hour, then lowered into the solvent (isopropanol:water, 7:3) and developed for eight hours. The dried chromatograms were sprayed with a 0.25% solution of ninhydrin (Pierce Chemical Co., Rockford, IL) in 95% ethanol and heated at 105^0C for five minutes for visualization of the amino acids.

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Chromatography of ^{3}H-Pro and ^{3}H-Hyp in Plasma Hydrolysates

Reconstituted human plasma samples (Sigma) were made 6M with HCl and heated at 110^0C for 24 hours. The hydrolysates were desiccated under vacuum at 40^0C over sodium hydroxide and calcium chloride, then brought to the volume of the original plasma sample with distilled water. Thin layer chromatography sheets which had been spotted with 0.2 μCi of ^{3}H-Hyp (ICN, Irvine, CA) were overspotted with 0, 2, 4 or 6 microliters of plasma hydrolysate and chromatographed as described. The dry chromatograms were autoradiographed for two weeks at -20^0C using Kodak X-Omat film, which was subsequently developed in an automatic developer.

Sixteen gingival crevicular fluid and connective tissue hydrolysates were overspotted with 0.2 μCi 3H-Hyp standards (ICN), chromatographed and autoradiographed as described. The location of the tritiated imino acids in the chromatograms was determined by the image in the developed autoradiograms.

The portions containing the ^{3}H-Hyp were cut out and each chip was placed in a piece of dialysis tubing (10 mm flat width, 70 mm long). One end was sealed with a closure (Spectra/Por, Spectrum, Los Angeles, CA) and 0.5 mL of distilled water or 1M acetic acid was added to each sample. As much air as possible was excluded and the other end of the tubing was sealed with a second closure. Dialysis was carried out against two changes of 10 mL of the same solvent as had been added to the bag, i.e. water or 1M acetic acid, in one ounce capped polypropylene vials, which were agitated at approximately 30 reciprocations per minute. The combined dialysates and a 5 mL rinse (25 mL total) were lyophilized in 50 mL polypropylene tubes. Each sample was then dissolved in 5 mL of distilled water, transferred to a scintillation vial and 10 mL of scintillation cocktail (ACS, Amersham Co., Arlington Heights, IL) added. Counts per minute were determined for the chromatographed samples and 0.2 μCi ^{3}H-Hyp standards (ICN). Recovery of the test samples was calculated by the following formula:

CPM of test sample/ CPM of 0.2 μCi ^{3}H-Hyp x 100 = % recovery.

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RESULTS

Chromatography of Amino Acid Standards

The chromatographic system described separated Hyp and Pro without interference from complex biological matrices such as plasma hydrolysates. While Hyp and Pro were completely separated other amino acids co-migrated with the imino acids. Aspartic acid, alanine, glutamic acid, glutamine, which is converted to glutamic acid on hydrolysis, and glycine co-migrated with Hyp. Asparginine, which is converted to aspartic acid on hydrolysis, co-migrated with Pro (Table I).

Chromatography of ^{3}H-Pro, ^{3}H-Hyp in Plasma Hydrolysates

Development of the chromatograms for eight hours gave a clear separation of Pro and Hyp. The addition of human plasma hydrolysate did not interfere with this separation in quantities as high as six microliters.

Table 1

Chromatographic Mobility of Assayed Amino Acids

Group	R_f Range	Amino Acids
A	.89-.81	leucine, tryptophan, tyrosine, isoleucine, methionine, phenylalanine, valine, cys-H, threonine, serine
B	.78-.75	hydroxyproline, aspartic acid, alanine, glutamic acid, glutamine*, glycine
C	.68-.69	proline, asparginine**
D	.55	histidine
E	.11-.10	arginine, lysine, 5-hydroxylysine

* Converted to glutamic acid during hydrolysis.
** Converted to aspartic acid during hydrolysis.

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Elution of Hyp

Dialysis of the developed chromatograms for elution of Hyp gave a recovery of 84.4% +/- 4.3% (avg. +/- SD). Extractions using water as the solvent contained substantial quantities of a fine particulate material (presumably silica) whereas extractions using 1M acetic acid resulted in no detectable particulates on complete desiccation.

DISCUSSION

Resolution of Pro and Hyp was accomplished by thin layer chromatography using silicic acid gel impregnated glass fiber sheets and isopropanol:water (7:3) as the mobile phase. Chromatograms had to be developed for 8 hours to insure a clean separation of Pro and Hyp. The presence of human plasma hydrolysate had no effect on the separation of the imino acids. In addition, this system completely separated histidine from the other 21 amino acids that were examined.

When ^{3}H-Pro is used in animal or tissue culture experiments, about 10% is incorporated into glutamic acid, aspartic acid, alanine, arginine, valine and tyrosine.[1] In our system glutamic acid, aspartic acid and alanine co-migrate

with Hyp. Elimination of the co-migrating amino acids can be most easily accomplished by deamination of the sample prior to chromatography, a procedure that does not affect the imino acids.[22]

When elution of Hyp from the developed chromatograms was done by placing the chromatogram chips containing the imino acid in water or a weak acid the recovery was only 40 - 50%. In addition, the extracts contained significant amounts of particulate material. Elution of Hyp by dialysis of the chromatogram chips consistently gave an 85% recovery of the Hyp. When water was used as a solvent, the dialysates contained substantial quantities of a fine particulate material (presumably silica) that precipitated when the volume of the dialysates was reduced. The particulate material posed serious problems for spectrophotometric determination of Hyp using Ehrlich's reagent.[4] The particulates were eliminated by using 1M acetic acid for the dialysis procedure.

The presented chromatographic procedure effectively separates Pro and Hyp which can subsequently be localized by autoradiography and extracted by dialysis from the developed chromatograms for quantitation by standard methods such as scintillation counting or Ehrlich's reagent. This is a simple, accurate and inexpensive method for the separation of Pro and Hyp from complex biological samples.

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