Journal of Chromatography, 311 (1984) 400—404
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2268

Note

Thin-layer chromatography of hydroxylysine for collagen analysis

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(First received April 10th, 1984; revised manuscript received July 16th, 1984)

Ultramicro methods of amino acid analysis based on thin-layer chromatographic (TLC) separation of dansylated derivatives [1, 2] provide a simple and convenient procedure for analyzing the amount and specific radioactivity of picomolar quantities of amino acids [3]. The application of this simple but sensitive technique has allowed analysis of minute quantities of amino acids associated with subcellular amino acid pools and individual protein bands eluted from analytical polyacrylamide gels, as well as a number of other applications [4]. In a previous publication outlining optimal approaches to methodologies, we pointed out that the technique would also be well suited for routine determination of modified amino acids such as hydroxyproline [4]. We report here on the use of this radioisotope dilution method to rapidly and specifically quantitate hydroxylysine. The method is based on the derivatization of free amino acid with [^{14}C]Dns chloride to form [^{14}C]di-Dns-hydroxylysine which can be readily separated from other amino acids by TLC on polyamide plates. Indirect quantitation of non-radiolabeled hydroxylysine in acid hydrolysates of crude tissue and body fluid samples is carried out by mixing these with a precise amount of [^{3}H]hydroxylysine prior to dansylation. The degree to which the specific activity of the latter isotopic species is lowered allows quantitation of picomolar quantities of hydroxylysine. Because previously available methods of analysis of this amino acid are based on liquid chromatography or multi-step analytical procedures (for review, see ref. 5), the application of TLC provides significant improvement in convenience while increasing the sensitivity to the picomolar (10^{-10} to 10^{-12} *M*) range.

0378-4347/84/$03.00

EXPERIMENTAL

[^{3}H]Hydroxylysine was prepared by custom catalytic exchange in tritiated aqueous medium (Amersham, Arlington Heights, IL, U.S.A.) using δ-hydroxylysine hydrochloride obtained from Sigma (St. Louis, MO, U.S.A.). The resulting tritiated hydroxylysine (generally but not uniformly labelled) had a specific radioactivity of 32 dpm/pmol as determined by dansylation and confirmed gravimetrically. We obtained methyl[^{14}C]Dns chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) from Research Products International (Mount Prospect, IL, U.S.A.), diluted it with [^{12}C]Dns chloride to a specific radioactivity of 7.2 dpm/pmol, and stored it in acetone at 4° C until used. The concentration of the final Dns chloride solution was 4.2 m*M*. Standard mixtures of nineteen amino acids with or without hydroxylysine were obtained from Pierce (Rockford, IL, U.S.A.).

To quantitate hydroxylysine content of urine and crude tissue, samples were hydrolyzed overnight in redistilled hydrochloric acid (Ultrex, J.T. Baker, Phillipsburg, NJ, U.S.A.) at 110° C under vacuum, evaporated to dryness, taken up twice in deionized water and redried. The dried sample was brought up in deionized water. A 10-μl aliquot was mixed with 10 μl (300 pmol unless otherwise stated) of [^{3}H]hydroxylysine ("probe"), dried under vacuum and solubilized in 20 μl of 1 *M* sodium carbonate—sodium bicarbonate buffer (pH 9). The pH of the resulting sample was tested, and 10 μl additional buffer were added if the pH remained less than 9.

An equal volume of [^{14}C]Dns chloride solution in acetone was added to the sample and reacted at 37° C in a humidified atmosphere for 30 min or until the yellow color disappeared. The reacted sample was dried down and extracted three times in 100 μl of water-saturated ethyl acetate. The pooled samples in ethyl acetate were dried down and the sample reconsitituted in 10 μl deionized water. Chromatography was performed as previously described [3]. In brief, the reaction mixture was applied near the corner of a 7.5 × 7.5 cm^2 polyamide plate (Cheng Chin Trading, Taiwan, or Accurate Chemical and Scientific Company, Hicksville, NY, U.S.A.) by repeated spotting. The plates were developed first by ascending chromatography in covered glass tanks in formic acid (88%)—water (2:100), then dried. The plates were turned 90° and rechromatographed in benzene—glacial acetic acid (90:10). For optimal separation of hydroxylysine, chromatography was repeated in the second dimension with the same solvent system. Development in each tank was continued until the solvent front ran all the way to the top of the plate. Dns-amino acids were visualized under short-wave ultraviolet light (see Fig. 1), and identified based on patterns published in the literature [3, 4]. Individual dansylated derivatives were cut from the plate and placed in 7-ml scintillation vials to which were added 0.15 ml fresh NCS solubilizer (Amersham) and 3 ml Econofluor (New England Nuclear, Boston, MA, U.S.A.). Radioassay was performed on a Packard Tri-Carb liquid scintillation spectrometer with average counting efficiencies of 0.253 for ^{3}H and 0.505 for ^{14}C. The tritium-specific radioactivity of hydroxylysine (dpm/pmol) was calculated as:

$$\frac{^{3}\text{H dpm} \times 2}{^{14}\text{C dpm} \div 7.2 \text{ dpm/pmol}} \quad (1)$$

where 7.2 dpm/pmol is the specific radioactivity of [^{14}C]Dns chloride. The two-fold correction is made because of the didansylated nature of the hydroxylysine species isolated.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatographic separation of didansylated hydroxylysine from other amino acids. Under the conditions of chromatography chosen, most other amino acids were allowed to migrate further from the origin in both dimensions in order to provide optimal separation of di-Dns-hydroxylysine from Dns-hydroxide and other neighboring derivatives.

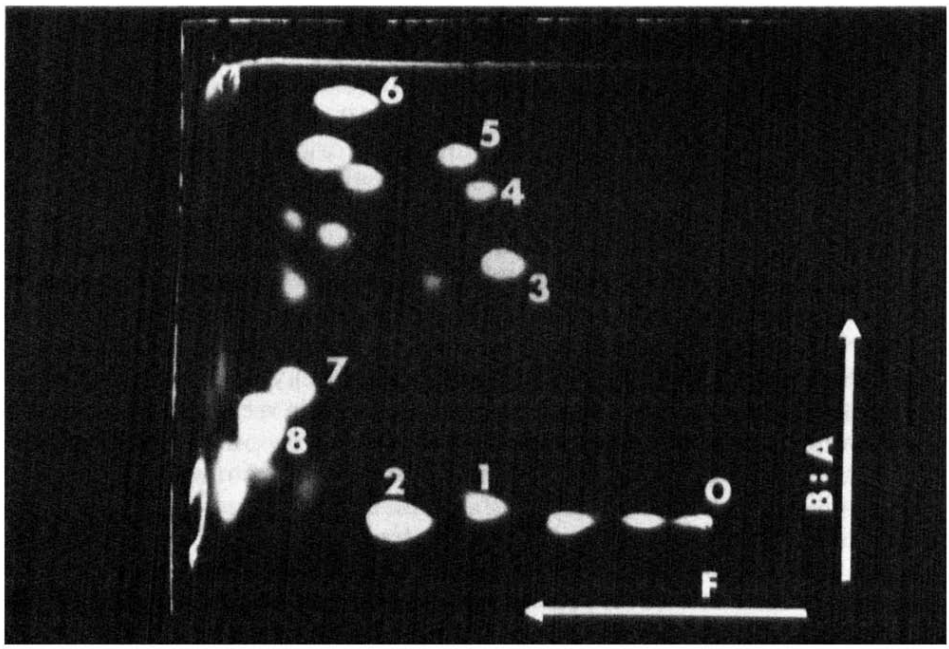

Fig. 1. TLC of Dns-amino acid derivatives. Identification of individual spots is based on patterns provided in previous publications [3, 4]. Individual dansylated amino acids are readily visualized under short-wave ultraviolet light by their yellow fluorescence. Approx. 100 pmol of hydroxylysine were chromatographed in the presence of a mixture of twenty other amino acids. 0 = Origin, 1 = di-Dns-hydroxylysine, 2 = Dns hydroxide, 3 = Dns-phenylalanine, 4 = Dns-leucine, 5 = Dns-isoleucine, 6 = Dns-proline, 7 = Dns-glycine, and 8 = Dns-hydroxyproline. Arrows indicate ascending chromatography in formic acid (F) and benzene—acetic acid (B:A).

Eqn. 1 shows how the specific radioactivity of hydroxylysine can be determined. Fig. 2 shows how hydroxylysine in non-radioactive samples can be quantitated by mixture with [^{3}H]hydroxylysine (probe) or known specific activity. Known amounts of non-radioactive hydroxylysine were added to a fixed amount (300 pmol) of [^{3}H]hydroxylysine probe. The solid line shows

the expected reduction in specific radioactivity as increasing amounts of non-labeled hydroxylysine are added, and can be expressed by the equation:

$$\frac{\text{SA (probe + standard)}}{\text{SA (probe)}} = 1 + \frac{\text{pmol (standard)}^{-1}}{\text{pmol (probe)}} \quad (2)$$

where SA is specific radioactivity of hydroxylysine. We set up the assay in such a way that unknown samples containing 300 pmol of hydroxylysine fell on the middle of the curve. The observed data (closed circles) were obtained by adding known amounts of non-labeled hydroxylysine and closely fit this expected curvilinear relationship.

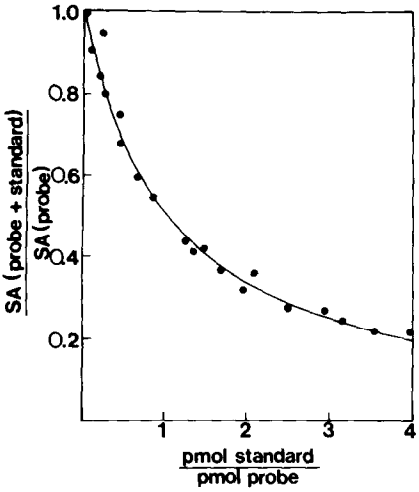

Fig. 2. Standard curve for quantitation of hydroxylysine. The progressive decrement of [^{3}H]hydroxylysine probe specific activity as increasing amounts of non-labeled hydroxylysine are added is shown. The solid line indicates the expected relationship between specific activity and amount of added hydroxylysine. Closed circles represent individual data points. The assay as set up is accurate for samples containing 100—1000 pmol of hydroxylysine.

Based on analysis of fifteen standard samples containing 0.5 to 3.0 times as much unlabelled hydroxylysine as the probe, we found that the measured value was 0.988 ± 0.041 (mean ± S.D.) of the expected hydroxylysine content. Because the relationship between specific radioactivity and amount is non-linear, we routinely dilute all unknown samples empirically such that they contain 0.5 to 3.0 times as much hydroxylysine as found in the probe. Where necessary, the amount of probe can similarly be varied.

To test whether the assay for hydroxylysine was similarly accurate in the presence of other amino acids, we constructed a standard curve by adding increasing amounts of a standard mixture of nineteen amino acids to 300 pmol of [^{3}H]hydroxylysine. The composition of the mixture was such that 38 pmol of amino acids were present for each pmol of hydroxylysine. Eqn. 3 describes the close linear relationship between the amount of hydroxylysine added (X) and the amount measured by radiodilution assay (Y):

$$Y = 0.015 + 0.997X \; (r^2 = 0.999, p < 0.001) \quad (3)$$

Approx. 19% of the starting hydroxylysine which was reacted with Dns chloride could be recovered in the carefully cut di-Dns-hydroxylysine spot. However, because quantitation is based on specific radioactivity determinations and because radiolabeled probe is added to the unknown sample before dansylation and separation, complete recovery is not necessary for accuracy.

ACKNOWLEDGEMENTS

We gratefully acknowledge the technical assistance of Jill Soltys and Patty Waitkus and the services of the Word Processing Center. William S. Stirewalt, Ph.D., provided helpful insight into experimental approaches. Supported by Grants PHS 5429-22-9 from the National Institutes of Health and from the Health Effects Institute.

Research described in this article is conducted under contract to the Health Effects Institute, an organization that supports the conduct of independent research and is jointly funded by the U.S. Environmental Protection Agency and automotive manufacturers. Publication here implies nothing about the view of the contents by HEI or its research sponsors. HEI's Health Review Committee may comment at any time and will evaluate the final report of the project. Additionally, although the work described in this document has been funded in part by the U.S. E.P.A. under assistance agreement with HEI, the contents do not necessarily reflect the views and policies of the agency; nor does mention of trade names or commercial products constitute endorsement or recommendations for use.

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