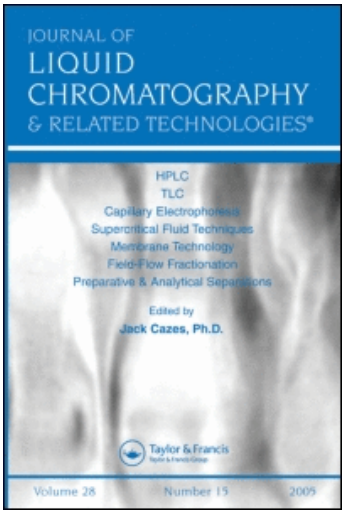

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STABILITY-INDICATING HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ASSAY FOR L-LYSINE

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ABSTRACT

A high-pressure liquid chromatographic method for the assay of L-Lysine is described. L-lysine was derivatized by reacting with 2,4-dinitrofluorobenzene. A fixed wavelength detector (254 nm) and a LiChrosorb RP-18, 10 um, Hibar-II column from MCB Manufacturing Chemists, Inc., was employed. The method is simple, precise, accurate and stability-indicating.

INTRODUCTION

For the last twenty years much work has been undertaken on the problem of gas-liquid chromatography (GLC) of amino acids. Many developments by several workers since 1961 when Johnson (1) succeeded in separating thirty-three amino acids as their n-amyl N-acetyl esters, involved separating either protein amino acids or non-protein biological amino acids (2) as n-butyl N-trifluroacetyl (BFTA) esters. Apart from some attempts with trimethylsilyl derivatives (3), most of the work by Gehrke's group on the

quantitative assay of amino acids has been done with n-butyl N-trifluoroacetyl ester derivatives (4-8). Other investigations were done on n-propyl N-acetyl esters of amino acids (9-11), but alkyl ester, N-heptafluorobutyrl derivatization nowadays appears to be more often used in the form of n-propyl (12-16), isoamyl (17, 18) and isobutyl ester of amino acids (19-22). High-pressure liquid chromatography has in recent years become an extremely valuable method for analysis of amino acids. It offers the advantage over the routinely used gas chromatographic method that it also allows one to identify easily the thermolabile or poorly volatile acids. Several elution programs for liquid chromatography of amino acids have been described (23-29).

Despite of all the work done on amino acids, there is no report on stability-indicating assay for L-lysine. In the present study, an assay method is described for the quantitative determination of L-lysine in L-lysine raw material or L-lysine stability samples.

EXPERIMENTAL

Reagents - methanol[1], glacial acetic acid[2], 2,4-dinitrofluorobenzene[3], tris(hydroxymethyl)aminoethane[4] (THAM), 2-naphthoic acid[5], and ethyl alcohol[6] USP anhydrous 200 proof were used as obtained.

Apparatus

The HPLC system consisted of a pump[7] and a UV monitor[8] operated at 254 nm. Samples were introduced using a loop injector[9] with fixed volume. The output of the detector was displaced on a recorder[10] having a full-scale range of 10 mv. The output signal was integrated and results were cal-

(1) Burdick & Jackson Laboratories, Muskegok, MI 49440.

(2) Reagent Grade A.C.S.

(3) Aldrich Chemical Co., Milwaukee, WI 53233.

(4) Fisher Scientific Co., Fair Lawn, N.J.

(5) Eastman Organic Chemicals, Rochester, N.Y. 14650.

culated using H.P. computer. The 25 cm x 4.6 mm i.d. stainless steel column contained reverse-phase packing[11]. The mobile phase was pumped at a flow rate of 1.5 ml/min.

Mobile Phase

Approximately 1 liter of mobile phase was prepared freshly daily by thoroughly mixing 600 ml of methanol, 400 ml of water and 2.5 ml of glacial acetic acid. The mobile phase after cooling to room temperature was degassed gently for 15 seconds. The pH of the mobile phase was ∿ 3.0.

Tris(hydroxymethyl)aminoethane (THAM) Solution Preparation

Weigh accurately approximately 1.44 g of THAM into a 100-ml volumetric flask. Dissolve and dilute to volume with distilled water.

2,4-Dinitrofluorobenzene Solution Preparation

Weigh accurately approximately 760 mg of 2,4-dinitrofluorobenzene into a 50-ml volumetric flask. Dissolve and dilute to volume with absolute ethanol. CAUTION! This material is hygroscopic and the weighing should be done quickly or in a dry box. This material may be carcinogenic.

Internal Standard Preparation

Weigh accurately approximately 27 mg of 2-naphthoic acid into a 25-ml volumetric flask. Dissolve and dilute to volume with THAM solution. Sonication for about 5 minutes may be necessary to dissolve it.

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(6) U.S. Industrial Chemicals.

(7) Model 6000A, Waters Associates, Milford, Mass. 01757.

(8) Model 440, Waters Associates, Milford, Mass. 01757.

(9) Valco loop injector, 7000 psi, 20-ul loop, Valco Instrument Co, Houston, Texas.

(10) Hewlett Packard 7130A, 5858 East Malloy Road, Syracuse, N.Y. 13211

(11) LiChrosorb RP-18, 10 um MCB Manufacturing Chemists, Inc., Division of E. Merck, Elmsford, N.Y. 10523.

Standard Preparation

Weigh accurately approximately 36 mg of L-lysine standard[12] into a 50-ml volumetric flask. Dissolve and dilute to volume with distilled water. Pipet 2.00 ml of the L-lysine solution into a 10-ml volumetric flask. Add 2.00 ml of THAM solution and 2.00 ml of 2,4-dinitrofluorobenzene solution. Cap tightly. Shake to mix well. Suspend in a 50°C water bath for 30 minutes. Allow the flask to cool to room temperature and then add 2.00 ml of internal standard solution. Dissolve to volume with methanol. Mix well. Inject 20 µl.

Sample Preparation

Weigh accurately approximately 36 mg of sample into a 50-ml volumetric flask. Dissolve and dilute to volume with distilled water. Pipet 2.00 ml of sample solution into a 10-ml volumetric flask and follow the same procedure as the standard preparation.

Calculation

Measure the area by peak height x width at half-height method or by computer.

$$\text{Factor. (F)} = \frac{\text{mg/ml of L-lysine x purity of std. x area of int. std.}}{\text{mg/ml of int. std. x area of lysine x 100}}$$

$$\text{Sample mcg/mg} = \frac{\text{F x mg/ml int. std. x 50 x 10 x area of L-lysine x 1000 mcg/mg}}{\text{area of int. std. x mg of sample x 2}}$$

RESULTS & DISCUSSION

The time required for derivitization of L-lysine was studied by varying the length of reaction time, temperature, concentrations of THAM and 2,4-dinitrofluorobenzene. The derivitization was complete after 30 minutes at 50°C and the area response was constant for 30 minutes even at reflux. A typical sample chromatogram is shown in Fig. 1.

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(12) Bristol Reference Standard, Bristol Laboratories, Syracuse, N. Y. 13201.

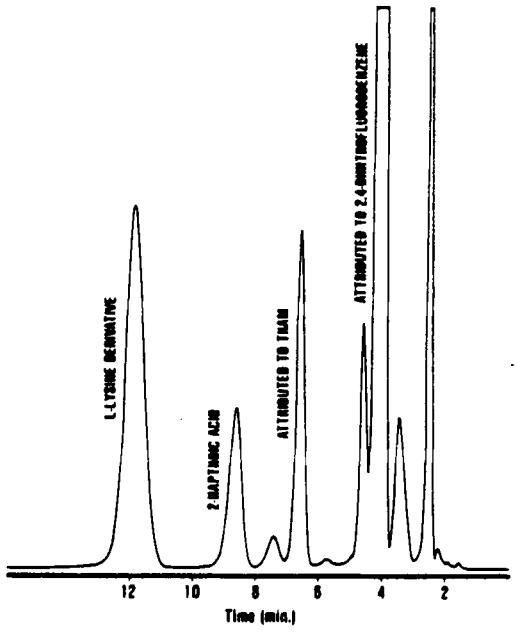

FIGURE 1

Linearity response was determined at 0.07 mg/ml to 0.3 mg/ml for L-lysine. Detector response was linear at these concentrations.

Standard deviation (s%), relative standard deviation (2s%) for chromatographic variability, standard variability, sample variability and procedural variability for L-lysine are shown in Table I.

Accuracy of the assay was determined by spiking and assaying in duplicate a L-lysine sample at the 50% and 100% of target value levels. Table 2 contains the % recoveries obtained for each spike and the average % recovery.

The assay is stability-indicating. Two 10 ml aliquots of aqueous L-lysine solution (1.47 mg/ml) were mixed with 10 ml of 1N HCl and 1N NaOH separately. Both solutions were refluxed for 30 minutes, neutralized, derivatized. A solid sample of L-lysine was also irradiated with 2500Å light in a photochemical reactor for 6 days. Table 3 contains the results of stability studies. There were no

TABLE 1

	Standard Deviation (s%)	Relative Standard Deviation (2s%)
Chromatographic Variability	0.0033	3.81
Standard Variability	0.0018	2.05
Sample Variability	0.621	1.32
Procedural Variability	1.48	3.16

new peaks in the acid or base treated samples, and only a small peak following the L-lysine peak was observed in the irradiated sample.

In summary, the described HPLC method is reliable and easily pertormed. It can be applied to the stability-indicating studies. The method can easily be adapted for determination of L-lysine in pharmaceutical products.

TABLE 2

Spike Recoveries

% Recoveries of L-lysine	
50% Level	100% Level
105.4	103.8
104.3	100.9
$\bar{X}$ = 104.8	$\bar{X}$ = 102.3

TABLE 3

Stability Result

mcg/mg of L-lysine			
Sample Used For Stability	1N HCl	1N NaOH	2500Å
950.0	903.3	893.3	1024.3

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