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An HPLC Method for the Determination of Reactive (Available) Lysine in Milk and Infant Formulas

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A high performance liquid chromatographic procedure for the determination of reactive (available) lysine was developed for the analysis of samples of high carbohydrate content, such as milk or infant formulas. The sample is dialyzed, derivatized with 2,4,6-trinitrobenzenesulfonic acid, acid hydrolyzed, and the ϵ-TNP-lysine quantitated by HPLC. This procedure yielded values similar to those obtained by a conventional spectrophotometric method but did not require the use of reagent and sample blank corrections.

Excessive heat processing, or storage under adverse conditions, of a protein foodstuff of a high carbohydrate content will result in a loss of essential amino acids, particularly lysine (Henry and Kon, 1958; Mauron, 1961). The reaction of the free amino group of lysine in the protein with carbohydrate (Maillard reaction) renders the lysine nutritionally unavailable. Several chemical procedures for the estimation of available lysine (reactive lysine) have been based on the reaction of the free (unbound) ϵ-lysine amino group in the protein with a chromophoric reagent; the treated protein is then hydrolyzed and the concentration of the lysine derivative is determined spectrophotometrically. The reagent used in the classical procedure of Carpenter (1960) is fluorodinitrobenzene (FDNB); the procedure of Kakade and Liener (1969) utilizes trinitrobenzenesulfonic acid (TNBS), which was shown by the latter investigators to yield values similar to the Carpenter method.

In the spectrophotometric procedure, the solution of the hydrolyzed derivatized protein is extracted with organic solvents to remove excess reagent and colored byproducts of the reaction. A reagent blank is required to correct for incomplete extraction. An additional blank, containing the sample without the reagent, corrects for the colored products produced during the hydrolysis of the protein. Corrections are also applied for incompleteness of the reaction induced by high levels of carbohydrate (Posati et al., 1972).

The high performance liquid chromatographic (HPLC) procedure described here was developed for the assay of samples of milks and infant formulas, samples with a high lactose to protein ratio. The samples are dialyzed to remove the interfering lactose (Greenberg et al., 1977), derivatized, and hydrolyzed, as in the Kakade–Liener method, and the aqueous solution is injected directly onto the chromatographic column. Extraction with an organic solvent, or the necessity for reagent or sample blanks are eliminated. This simplified procedure yielded results essentially similar to the spectrophotometric procedure of Kakade and Liener.

EXPERIMENTAL SECTION

Apparatus. The HPLC instrument was a Hewlett Packard 1084B, and the spectrophotometer, a Perkin Elmer Lambda 3A; a laboratory autoclave or household pressure cooker was used for the hydrolysis; the dialysis tubing was Spectrapor, No. 3787-D32, 12 000 MW cutoff.

Purified Proteins. Bovine serum albumin, lysozyme, and ovalbumin were purchased from Sigma, and zinc–

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insulin crystals, beef, from Elanco.

TNP-Lysine Derivatives. ϵ-Trinitrophenyllysine was purchased from Sigma. α,ϵ-Bis(trinitrophenyl)lysine (noncrystalline) was prepared by the method preparation of Okuyama and Satake (1960).

Reagents and Solutions. The solutions used were 2,4,6-trinitrobenzenesulfonic acid (Sigma) (0.1% aqueous solution), sodium bicarbonate (4% solution, adjusted to pH 8.5 with NaOH), lysine standard stock solution (50 μg/mL, 0.0313 g of lysine·HCl (Sigma) in 500 mL of the above 4% bicarbonate, assay standards of 10, 20, 30, and 50 μg/mL prepared daily by appropriate dilution of the stock solution with the bicarbonate solution.

Sample Preparation. Samples containing carbohydrates are dialyzed with constant stirring for 72 h at 4 °C against distilled water with twice daily changes of the dialysis water. The dialyzed solution is transferred quantitatively and diluted to 100 mL. Nitrogen (Kjeldahl) is determined on the dialyzed sample and, if appropriate, on the sample before dialysis to determine the presence of dialyzable nitrogen. Aliquots of the dialyzed solution may be frozen for later analyses.

Reactive Lysine. HPLC Procedure. The conditions of derivatization and hydrolysis are essentially those described by Kakade and Liener (1969). The dialyzed sample is diluted with the bicarbonate buffer to a final protein concentration of 0.3–0.5 mg/mL. Sample (1 mL) and 1 mL of the TNBS solution are vortexed, capped, and incubated for 2 h in a 40 °C water bath. After the reaction has cooled, 3 mL of 12 N HCl is added, vortexed, and hydrolyzed at 120 °C at 18 psi for 1 h. H_2O (5 mL) is added to the cooled hydrolyzate, and, if cloudy, the solution is filtered through a Millex 0.45-μm filter unit. Samples are run in triplicate; the lysine standards are run in duplicate. The area or peak height of the ϵ-TNP-lysine of the standard solutions is plotted against lysine concentration, the regression equation is calculated, and the amount of lysine in the samples calculated from this equation.

Chromatography conditions: The column was a Regis Hi Chrom Reversible Spherisorb S5C8, Octyl, 5μ (4.6 × 250 mm); the column temperature was 50 °C; sample size 50 μL. The stock solvents were (A) 5 mM trifluoroacetic acid (0.385 mL TFA/1 L of H_2O) and (B) 5 mM TFA in 40% *n*-propyl alcohol. The elution solvent consisted of 75% A and 25% B; the solvent was degassed under vacuum for 15 min and the flow rate was 1.5 mL/min. Analyses were routinely run isocratically at a flow rate of 1.5 mL/min with UV detection at 346 nm.

Spectrophotometric Method. The procedure of Kakade and Liener (1969) using TNBS as the derivatizing agent, as detailed above, was the method employed for comparison of a spectrophotometric method with the HPLC method with the exception that lysine·HCl was used as the standard rather than preformed ϵ-TNP-lysine.

RESULTS AND DISCUSSION

Derivatization and Hydrolysis of the Lysine Standard. As demonstrated in the HPLC chromatograms of Figure 1, the derivatization of lysine·HCl with TNBS produces the α,ϵ-bis(TNP)lysine which is then completely converted to the ϵ-TNP-lysine upon acid hydrolysis. Lysine·HCl was used for the standard curve, rather than commerically available ϵ-TNP-lysine; the assumption was that incompleteness of derivatization or destruction during hydrolysis would be the same for the samples and standards. In actuality, equimolar concentrations of lysine·HCl and ϵ-TNP-lysine carried through the complete assay procedure gave nearly identical regression lines in either the spectrophotometric or HPLC procedure.

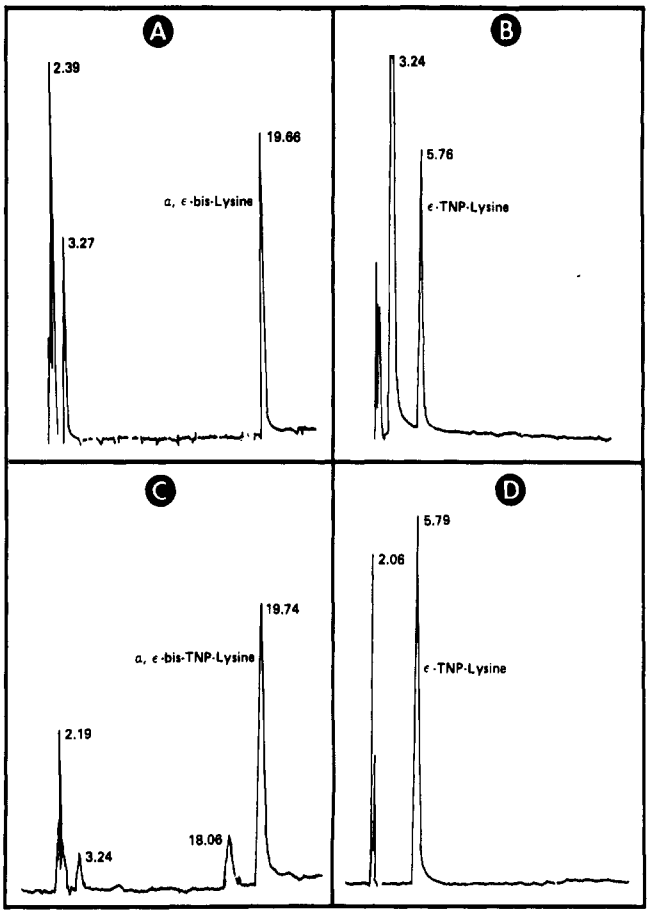

Figure 1. Derivatization of lysine to α,ϵ-bis(TNP)lysine and its conversion to ϵ-TNP-lysine by acid hydrolysis. (A) Derivatized lysine. (B) Derivatized and hydrolyzed lysine. (C) Synthesized α,ϵ-bis(TNP)lysine (noncrystalline). (D) ϵ-TNP-lysine (Sigma). For the chromatograms of A and C, a gradient elution schedule with increased concentrations of solvent B was used: 25% from 0–5 min, 50% to 15 min, 100% to 20 min, 25% to 25 min. The peak at 3.24–3.27 min was identified as picric acid; TNBS elutes with the solvent front. For the chromatograms of B and D the routine isocratic conditions were used.

Table I. Influence of Infant Formula Sample Size on Analytical Results

sample 1[a]		sample 2[b]	
μg of protein/mL	reactive lysine (% of protein)	μg of protein/mL	reactive lysine (% of protein)
219	6.9	222	6.6
219	6.7	222	6.5
438	6.8	444	6.4
438	6.6	444	6.5
657	6.6	666	6.3
657	6.7	666	6.4

[a] Average 6.7 ± 1.74% relative standard deviation. [b] Average 6.5 ± 1.63% relative standard deviation.

Linearity of Response. The linearity of response of the lysine·HCl standards and of a milk protein sample, in amounts of 50% and 150% of the usual assay concentration, is demonstrated in the combined plots of Figure 2. Within the range studied, the sample size did not influence the analysis results. Duplicate analyses of two infant formula samples at three protein conentrations are shown in Table I.

Standard Addition Test. To determine if constituents in the matrix were interferring with the reaction, analyses were conducted, in triplicate, on samples containing 450

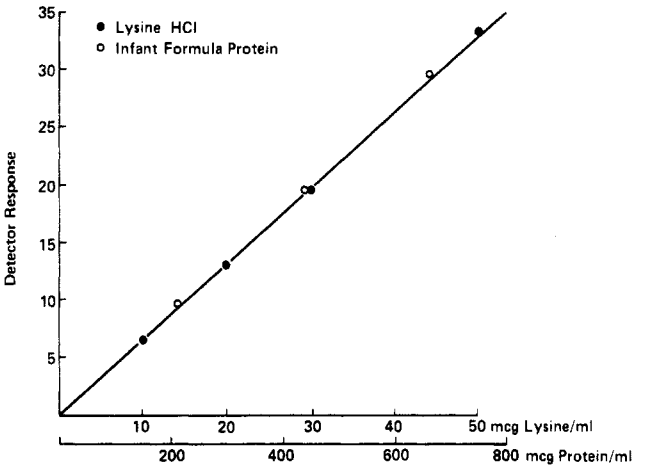

Figure 2. Linearity of response of lysine·HCl and protein solutions of increasing concentrations.

μg of protein/mL with the addition of either 0, 5, 10, 20, or 30 μg of lysine/mL. Calculations from the regression equation yielded a value for the protein sample of 5.44% with a 95% confidence limit of 5.01–5.92%. Direct analyses of the unfortified samples yielded values of 5.7, 5.8, and 5.5%, all within the confidence limits, indicating no statistically significant evidence of matrix interference (Ostle and Mensing, 1975).

Comparison with the Spectrophotometric Procedure. In the foregoing studies many of the samples were analyzed by both the HPLC and spectrophotometric procedure. As demonstrated in Table II, with most samples the values obtained by the two methods were similar, in those cases where there was a difference, the HPLC value was slightly lower, presumably because of the nonspecificity of the spectrophotometric method. Also included in Table II are the results of HPLC analysis of purified proteins. The values are in reasonable agreement with those reported for these proteins by Kakade and Liener (1969) and Finley and Friedman (1973) using the TNBS spectrophotometric procedure.

Reproducibility. The reproducibility of the HPLC procedure is demonstrated by the typical data of Tables I and II. The analyses of replicate aliquots had less than 2% average relative standard deviation. Repeated analyses of a sample over a several month period (B of Table II) had an average relative standard deviation of 4.8%. As evident from the data of Table II, the reproducibility of the HPLC method was superior to that of the spectrophotometric.

Interference by Lactose. The drastic interference of lactose with TNBS derivatization, as shown by Posati et al. (1972) in their analyses of whey samples, was also demonstrated in the present study by the results of analyses of infant formula samples with or without prior removal of lactose by dialysis. A typical analysis of an undialyzed infant formula sample yielded a value of 3.4% lysine compared to 6.4% when the same sample was dialyzed.

Table II. Comparison of the HPLC and Spectrophotometric Methods for the Determination of Reactive Lysine

sample	reactive lysine (% of protein) spectrophotometric[a]	HPLC[a]
formula, powder A	6.7 ± 13.8%[b] (4)	6.7 ± 3.4%[b] (9)
formula, powder B	7.1 ● 5.8%[b] (25)	6.7 ● 4.8%[b] (36)
formula, powder C	6.5 ± 7.1%[b] (25)	6.5 ± 2.3%[b] (3)
formula, liquid	6.2 ± 4.6%[b] (2)	5.7 ± 1.3%[b] (2)
bovine serum albumin	12.8%,[c] 12.9%[d]	12.9% (2)
insulin	2.6%,[c]	2.9% (2)
lysozyme	5.7%,[c] 5.8%[d]	5.3% (2)
ovalbumin	6.4%,[c] 5.1%[d]	5.9% (2)

[a] The number in parentheses is the number of analyses. [b] Relative standard deviation. [c] Kakade and Liener data. [d] Finley and Friedman data.

In the HPLC method described here the measurement of the chromatographically isolated TNP derivative affords a specificity not available in the conventional colorimetric procedure, and in addition, requires no corrections for irrelevant colored reaction byproducts. Dialysis of high carbohydrate samples, while an additional step, is recommended since it avoids the uncertainty inherent in applying correction factors for reaction interference.

Studies now in progress have indicated that total lysine may be determined with this HPLC procedure by first hydrolyzing the protein before derivatization with TNBS.

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Registry No. Lysine, 56-87-1; lysozyme, 9001-63-2; insulin, 9004-10-8.

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