Reversed-Phase High-Performance Liquid Chromatographic Separation of the Tautomers of Tryptophan Amadori Product

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The various tautomeric forms of the tryptophan Amadori rearrangement product, including the open-chain keto form, were separated at room temperature, for the first time by HPLC, on a C-18 Ultrasphere, 5 μm, 2.0 × 150 mm reversed-phase column using 5% H_3PO_4 (0.01 M) in CH_3CN (pH 2.8) as mobile phase. The flow rate was 0.6 mL/min, and the UV detector was set at 280 nm. At these conditions, the concentration of β-pyranose was 45.2%, α-furanose, 27.4%, β-furanose 12.5%, α-pyranose 10.1%, and keto form 4.7%. The effect of pH on the relative concentrations of each tautomer was also determined.

INTRODUCTION

Amadori compounds are the key intermediates in the nonenzymatic interaction of reducing sugars with amino acids; their thermal degradation (Nursten, 1980) is responsible for the formation of numerous compounds important in the formation of characteristic flavors, aromas, and brown polymers. In addition, it has been shown that such interactions also promote intramolecular cross-linking of proteins in biological systems, especially in patients suffering from diabetes (Monnier, 1989).

The widespread occurrence and biological importance of Amadori products necessitate development of HPLC separation methods for identification purposes.

Amadori compounds contain both amino and carboxylic acid groups, in addition to a carbohydrate moiety. Bonded aminopropyl columns are effective at separating mixtures with a wide range of polarities. They have been widely used to separate carbohydrates. Attempts to separate Amadori compounds using such columns from amino acids at neutral pH using acetonitrile–water mobile phases resulted in long retention times and tailing peaks. The ionized carboxyl groups present in the amino acids and in the Amadori compounds interact with the protonated amino groups on the aminopropyl columns to cause the long retention times observed. Decreasing the value of pH to 2.3 with phosphate buffer in the mobile phase afforded symmetrical peaks and shortened retention times. Reversed-phase (RP) columns have also been used successfully to separate Amadori compounds. The retention times for the amino acids were found to be affected by the degree of their ionization, that is, by the pH of the mobile phase. As the ionization of the amino acids increased, retention times decreased. Control of mobile phase pH can affect the degree of ionization of weak acids and bases and significantly influence the retention times. For amino acids, retention was minimal at pH levels where the zwitterion predominates. High pH levels (>9) cause the amino group to be less ionized, while low pH levels (<3) cause the carboxyl group to be less ionized. Thus, extreme pH conditions cause longer retention times on an RP column. Successful separation of tryptophan Amadori products (t_R = 9 min) from tryptophan (t_R = 15 min) was achieved on a μ Bondapak C-18 column with 30% acetonitrile–70% aqueous 0.01 M phosphate buffer at pH 2.3 containing 0.02% SDS counterion at a flow rate of 1 mL/min (Takeoka et al., 1979).

In an effort to develop an HPLC analytical method to separate the tryptophan Amadori product from tryptophan and its other degradation products such as HMF and maltol, we were also able to find solvent systems that separate, for the first time, the pyranose, furanose, and open-chain forms of the tryptophan Amadori product, using a reversed-phase column.

EXPERIMENTAL PROCEDURES

Materials and Methods. Tryptophan and D-glucose were purchased from Aldrich Chemical Co. and used without further purification; all the solvents used were of HPLC grade (BDH). Mobile phases were degassed by application of vacuum with gentle agitation for 5 min. Samples were loaded via a Rheodyne injector with a 20-μL loop. A C-18 Ultrasphere, 5 μm, 2.0 × 150 mm column from Beckman was used in the analysis. The column was operated at ambient temperature. Repeated injections of 20 μL of the sample were needed to achieve stable peaks for the Amadori product. Water was obtained from a Milli-Q reagent grade water system (Millipore Corp.). The wavelength used to detect the Amadori product was 280 nm.

Instrumentation. The modular HPLC system used was a Beckman System Gold consisting of a variable-wavelength UV detector Model 166 and a 110B solvent delivery module controlled by a NEC lap-top computer, connected to a Shimadzu C-R6A integrator–printer.

Synthesis of the Amadori Product. Amadori product of tryptophan with D-glucose was synthesized according to the procedure of Sgarbieri et al. (1973).

RESULTS AND DISCUSSION

In an effort to develop solvent systems capable of separating the Amadori compound of tryptophan from its degradation products, it was found that by use of mobile phases buffered at acidic pHs (between pH 2.9 and 3.1) the Amadori compound can be resolved into its pyranose and furanose anomers and into the keto form. To our knowledge, there are no reports in the literature of such separations. The separation of the anomers of Amadori products by HPLC can be an alternative method of determining their relative ratios, to that of the nuclear magnetic resonance (NMR) studies. Tjan and Ouweland (1974) studies 220-MHz ^1H NMR spectra of the Amadori rearrangement products of some cyclic amines with glucose and concluded that these compounds exist in solution predominantly as an equilibrium mixture of the furanose

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Table I. Effect of pH on the Retention Times and Concentrations of Various Tautomers of Tryptophan Amadori Product

	t_R, min	pH[a]	% concentration
β-pyranose	3.1	2.8	45.2
	3.1	2.9	42.0
	3.3	3.1	38.9
		5.1[b]	64
α-furanose	3.3	2.8	27.4
	2.9	2.9	27.9
	3.5	3.1	27.2
		5.1[b]	21
β-furanose	2.9	2.8	12.5
	2.8	2.9	18.1
	2.8	3.1	19.8
		5.1[b]	15
α-pyranose	3.5	2.8	10.1
	3.2	2.9	11.3
	3.1	3.1	08.1
		5.1[b]	nd[c]
keto	2.8	2.8	4.7
	3.6	2.9	0.8
	3.7	3.1	5.4
		5.1[b]	nd[c]

[a] pH 2.8 CH_3CN:H_3PO_4 (0.01 M) (95:5). pH 2.9 CH_3CN:H_3PO_4 (0.01 M) (92:8). pH 3.1 CH_3CN:H_3PO_4 (0.01 M) (96:4). Flow rate 0.6 mL/min. [b] Determined by ^{13}C NMR (Röper et al., 1983). [c] nd, not determined.

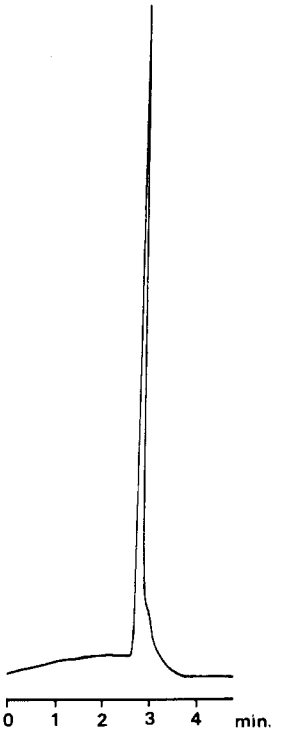

Figure 1. Chromatogram of tryptophan Amadori product. Solvent system: 1% H_3PO_4 (0.01 M) in CH_3CN (pH 5.2). Flow rate 0.6 mL/min.

and pyranose rings. Röper et al. (1983), however, studied in detail the high-resolution ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectra of 14 Amadori products of different amino acids with glucose including tryptophan, in D_2O (Table I shows the results for tryptophan Amadori product). The ^{13}C NMR spectra of these products show on the average 61% β-pyranose, 16% α-furanose, 15% β-furanose, 6% α-pyranose, and 2% keto form. Although reducing sugars, including Amadori products, exist in solution, as a mixture of two or more tautomeric forms, they often give only one peak when analyzed by HPLC.

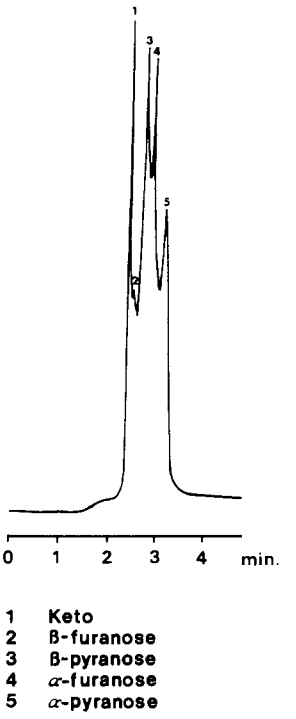

Figure 2. Chromatogram of equilibrated tryptophan Amadori product. Solvent system: 5% H_3PO_4 (0.01 M) in CH_3CN (pH 2.8). Flow rate 0.6 mL/min. (1) Keto form; (2) β-furanose; (3) β-pyranose; (4) α-furanose; (5) α-pyranose.

Table II. Percentage Changes in the Concentration of Various Anomers of Tryptophan Amadori Product as a Function of pH[a]

change in pH	β-p	α-f	α-p	β-f	keto
from 2.8 to 3.1	−6.3	−0.2	−2.0	+7.3	+0.7
from 2.9 to 3.1	−3.1	−0.7	−3.3	+1.7	+4.6

[a] β-p, β-pyranose; α-f, α-furanose; −, decrease; +, increase.

There are, however, instances, where the separation of sugar anomers is encountered. Partial or complete separation of anomers of reducing sugars has been achieved by reversed-phase chromatography on alkyl-bonded silica stationary phases, among others (Cheetham et al., 1981; Verhaar et al., 1984). Anomers can be separated because their mutarotation rate is slow, relative to the speed of chromatography. Pyranose anomers of some sugars can be separated at room temperature (Goulding, 1975); however, when the temperature is lowered to 0–4 °C, nearly all of the β-pyranose anomers of the common aldohexoses and aldopentoses can be separated also (Honda et al., 1984). Furanose anomers interconvert at higher rates, and temperatures of −25 to −45 °C are needed along with special solvents to separate the α- and β-furanose anomers of L-fucose (Moriyasu et al., 1984a) and D-galactose (Moriyasu et al., 1984b). A recently developed (Alltech-Applied Science) silica-based polyol column separates the anomeric forms of sugars at room temperature.

Freshly prepared aqueous solutions of the tryptophan Amadori product, when chromatographed on a C-18 Ultrasphere, 5 μm, 2.0 × 150 mm column, using 1% H_3PO_4 (0.01 M) in CH_3CN, produced a single peak (Figure 1); when the solvent system was changed to 5% H_3PO_4 (0.01 M) in CH_3CN, the single peak was separated into five peaks. Repeated injections of the sample at different intervals of time after the preparation of the fresh solution, using 5% H_3PO_4 (0.01 M) in CH_3CN, produced significantly different peak areas (relative concentrations) of the five resolved peaks. This observation led to the

conclusion that the five observed peaks represent the different tautomers of the Amadori product; consequently, the solution of the Amadori product was equilibrated for 24 h prior to its chromatography, using 1% H_3PO_4 (0.01 M) in CH_3CN. A single peak was produced with retention time identical with that of the unequilibrated sample; however, changing the concentration of the H_3PO_4 from 1% to 5% resolved the single peak into five peaks, and repeated injections of the equilibrated sample produced no changes in the relative concentrations of the five resolved peaks (Figure 2), confirming the conclusion that the five resolved peaks represented different tautomers.

The assignments of the peaks (Table I) were based on their relative concentrations as determined by ^{13}C NMR studies (Röper et al. 1983).

Effect of pH. The ^{13}C NMR studies (Röper et al., 1983) concluded that there is no significant change in the ratios of the various forms with change in pH of the solvent; however, we observed slight changes in the relative concentrations of each form as a function of pH (see Table II). According to our results, increasing the pH resulted in a slight increase in the concentrations of β-furanose and the keto forms at the expense of β-pyranose, α-furanose, and α-pyranose forms.

There is a definite advantage in using HPLC over ^{13}C NMR to determine the ratios of various anomers. The signal-to-noise ratio in the latter case did not allow an accurate determination of the α-pyranose and the keto forms; hence, their contribution to the total concentration has been incorporated into the remaining forms.

Conclusion. The separation of the different anomers of the tryptophan Amadori product can be achieved at room temperature at low pH values by using acetonitrile–phosphoric acid mixtures and reversed-phase columns.

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Registry No. Tryptophan Amadori product, α-pyranose tautomer, 87332-31-8; tryptophan Amadori product, α-furanose tautomer, 87251-66-9; tryptophan Amadori product, β-furanose tautomer, 87251-86-3; tryptophan Amadori product, β-pyranose tautomer, 81812-31-9; tryptophan Amadori product, keto tautomer, 25020-15-9.