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Note

Analysis of natural and modified amino acids and hexosamines by reversed-phase high-performance liquid chromatography[a]

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Because of its speed and sensitivity, amino acid analysis by high-performance liquid chromatography (HPLC) of the phenylthiocarbamyl (PTC) derivatives of amino acids has become a widely used procedure. Based on methodology developed by Koop *et al.*[1], the method has been popularized as the "Pico-Tag" system by Waters Instruments[2] in which pretested reversed-phase HPLC columns are recommended. Similar resolution of amino acids can also be obtained using other high-efficiency reversed-phase supports[2–7]; it appears, however, that each support requires different chromatographic conditions to achieve adequate resolution and that these conditions must be adjusted for each individual column[4]. This process can be time consuming since there are many different variables that affect resolution including column temperature, flow-rate, composition of solvents and gradient shape. Systematic studies indicating how each of these variables affect the retention times of individual amino acids, such as that carried out by Ebert[5] on the effect of triethylamine concentration, are helpful in minimizing the amount of time required for adjusting chromatographic conditions since the effects of modifications can be predicted.

Amino acid analysis is frequently used to analyze compounds other than the standard amino acids present in simple proteins. For example, glycoproteins contain glucosamine and galactosamine which can be derivatized with phenylisothiocyanate (PITC) and which may interfere with quantification of other amino acids since they elute very close to Ser and Gly. Cheng[8] has published a procedure for analyzing the PTC derivatives of the hexosamines and hexosaminitols but this method uses a mixed bed ion-exchange step to separate amino acids from sugars and it is not clear whether the early eluting amino acids can be resolved from the hexosamines in this procedure. Other compounds of interest include rare amino acids and amino acid derivatives generated by chemical procedures employed in protein chemistry. Retention times for several of the less common amino acids have been determined by O'Hare *et al.*[4] but it is not clear whether these were resolved from the standard set of 17 amino acids. Tarr[9] has also indicated elution positions for some of these derivatives.

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This study investigates the effect of buffer pH on the retention times of the PTC derivatives of 26 amino acids and derivatives as well as galactosamine and glucosamine. For general use, the optimum pH was found to be 5.5, and at this pH hexosamines and amino acids can be resolved within 18 min. The data presented can also be used to predict optimal conditions for separating closely eluting peaks and as a guide for manipulating conditions to obtain optimal resolution with new columns.

EXPERIMENTAL

Materials

Spherisorb ODS-2 columns (150 × 4.6 mm I.D., 3 μm in particle size) were purchased from Alltech. An amino acid standard mix containing 2.5 μmol/ml of each of the 17 amino acids and 1.25 μmol/ml L-cystine was purchased from Beckman. Other amino acids and hexosamines were either from Sigma or from Fluka. HPLC-grade triethylamine (TEA), phosphoric acid and acetonitrile were purchased from Fisher Scientific. Sodium acetate (HPLC grade) was from Merck. Constant boiling hydrochloric acid and phenylisothiocyanate were from Pierce. Sheep submaxillary mucin was prepared as described[10]. Tubes for amino acid analysis were washed with chromic acid before use.

Methods

Hydrolysis. Samples of sheep submaxillary mucin (100 μg) and lysozyme (100 μg) were hydrolyzed for 20 h at 110°C in 1.0 ml of constant boiling, 6*M* hydrochloric acid[11,12]. Lower ratios of acid to glycoprotein causes destruction of both amino acids and hexosamines[12]. For hexosamine analysis, the glycoprotein was hydrolysed in 4 *M* hydrochloric acid for 4 h at 100°C[13]. α-Aminobutyric acid (25 nmol) was added as an internal standard. After completion of hydrolysis the samples were cooled and then immediately dried in a vacuum drier from Savant Instruments since storage of samples in hydrochloric acid caused losses of some of the amino acids. The last traces of hydrochloric acid were removed by adding 200 μl of water and redrying. This process was repeated at least two times. Samples containing particulate material were filtered through 0.22-μm filters (Gelman). Losses of amino acids occuring during hydrolysis were corrected either by hydrolyzing for 4, 8, 12, 16 and 24 h and extrapolating to zero time or by hydrolyzing the standard amino acid mix and then calculating molar response ratios for the amino acids in the hydrolyzed standard. Samples were hydrolyzed and processed in duplicate.

Derivatization with PITC. The derivatization procedure employed was that of Bidlingmeyer *et al.*[2]. To the dried sample was added 10 μl of ethanol–water–TEA (2:2:1); the sample was then dried under a stream of nitrogen. Derivatizing reagent, 20 μl ethanol–water–TEA–PITC (7:1:1:1), was added and the sample incubated at room temperature for 30 min in the dark. Each sample was then dried thoroughly under nitrogen, immediately redissolved in 250 μl sample diluent buffer (see below) and aliquots analyzed by HPLC. Solubilized samples could be stored at −20°C for up to 24 h whereas dried samples could be stored for at least one week. When samples were solubilized and left at room temperature, losses of aspartic acid and generation of artifactual peaks were observed. Sample diluent buffer was prepared by dissolving 6.32 g of dibasic sodium phosphate in 100 ml of 25% aqueous acetonitrile, adjusting

the pH to 7.0, and filtering through 0.22-μm Millipore filters. Alternatively, a 2:1 mixture of solvents A and B (see below) was used as the sample diluent buffer.

Chromatography. The instruments employed were a Varian Model 5000 liquid chromatograph, a Varian Model 2050 variable-wavelength detector, and a Shimadzu C-R3A integrator. The flow-rate was 1.4 ml/min and the column temperature was maintained at 39°C using a circulating water bath and column jacket.

The gradient consisted of a linear gradient from 6% solvent B to 25% B for the first 5.5 min, isocratic elution at 25% B for 1.5 min, a second linear gradient reaching 30% B at 8 min, a linear gradient to 35% B at 10 min, and another linear gradient reaching 51% B at 20 min. This was followed by a washing cycle consisting of a linear gradient to 100% B at 21 min followed by 4 min at 100% B. The column was reequilibrated to initial conditions by eluting with 6% B for 5–7 min prior to injecting the next sample. In most experiments, solvent A contained 0.14 *M* sodium acetate and 0.08% TEA with the pH adjusted to 5.5 with phosphoric acid. In experiments testing the effect of pH on retention times, the pH was adjusted to various values ranging from 3.5 to 7.5 using phosphoric acid. Solvent B consisted of 60% aqueous acetonitrile.

The effect of pH on retention times was studied by coinjecting PTC amino acids from the standard amino acid mixture together with one of the following mixtures: (1) cysteic acid, carboxymethylcysteine, aminoethylcysteine, glucosamine and galactosamine, (2) homoserine, methionine sulfone, methionine sulfoxide and hydroxyproline, or (3) norvaline, norleucine and α-aminobutyric acid.

RESULTS AND DISCUSSION

The separation of the standard 17 PTC amino acids as well as the PTC derivatives of galactosamine and glucosamine on a Spherisorb 3-μm ODS-2 column using a pH 5.5 buffer is shown in Fig. 1. The resolution of the amino acids is similar to that achieved with other systems that have been described[2–7]. The effect of decreasing the concentration of sodium acetate from 0.14 to 0.01 *M* is shown in Fig. 2. Under these conditions, the retention times are decreased, Lys elutes as a broad peak, and the relative retention times of both His and Arg are increased, causing the coelution of Ala and Arg. The shape of the gradient is critical to the ability of the system to resolve closely eluting amino acids; shallow gradients give better resolution at the expense of increased analysis time. The gradient used in these studies was chosen to maximize resolution of the hexosamines from the other early peaks without inordinately increasing total analysis time. These conditions were found to be the best compromise between resolution and speed. Other parameters such as TEA concentration, which has been studied by Ebert[5], and flow-rate were not investigated in detail.

The compounds that must be resolved in this system include neutral species, zwitterions and species containing one or two negatively charged groups. Thus, it seemed logical to investigate the effect of buffer pH on resolution, particularly since the practical pH range for HPLC on silica supports is in the vicinity of the pK_a values for carboxyl groups. The effect of pH on retention times for the 28 compounds investigated is shown in Fig. 3. Almost all of these compounds demonstrate increased retention times with decreased pH; this presumably reflects the partial titration of their free carboxyl groups. The greater sensitivity to pH shown by aspartic acid,

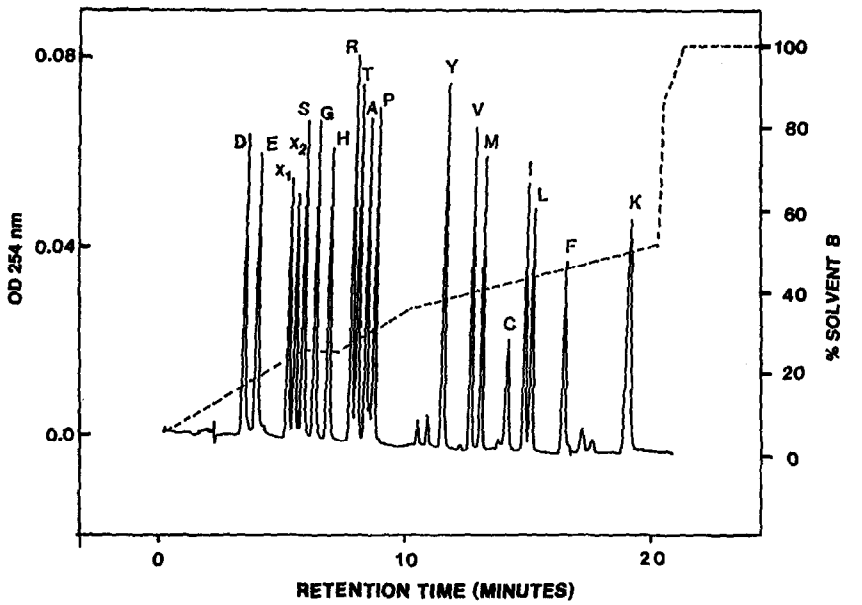

Fig. 1. Separation of PTC amino acids from PTC hexosamines at pH 5.5. Glucosamine and galactosamine were added to a standard mixture of amino acids. The mixture was derivatized with phenylisothiocyanate and separated by reversed-phase HPLC as described in *Methods*. Amino acids are identified by the single letter code; galactosamine is indicated by (X_1) and glucosamine by (X_2).

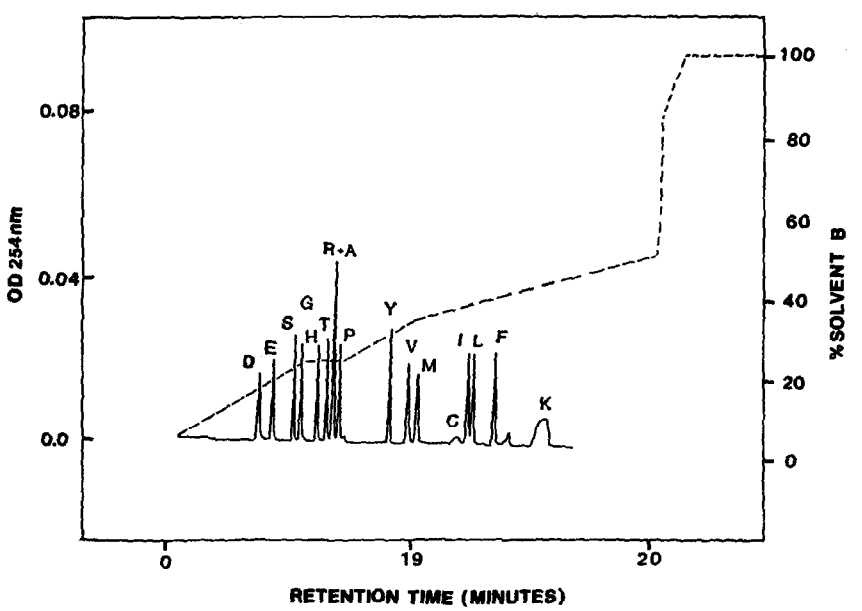

Fig. 2. Effect of the ionic strength of buffer A on separation of PTC amino acids. Conditions used were the same as in Fig. 1 except that buffer A contained 0.01 *M* rather than 0.14 *M* sodium acetate.

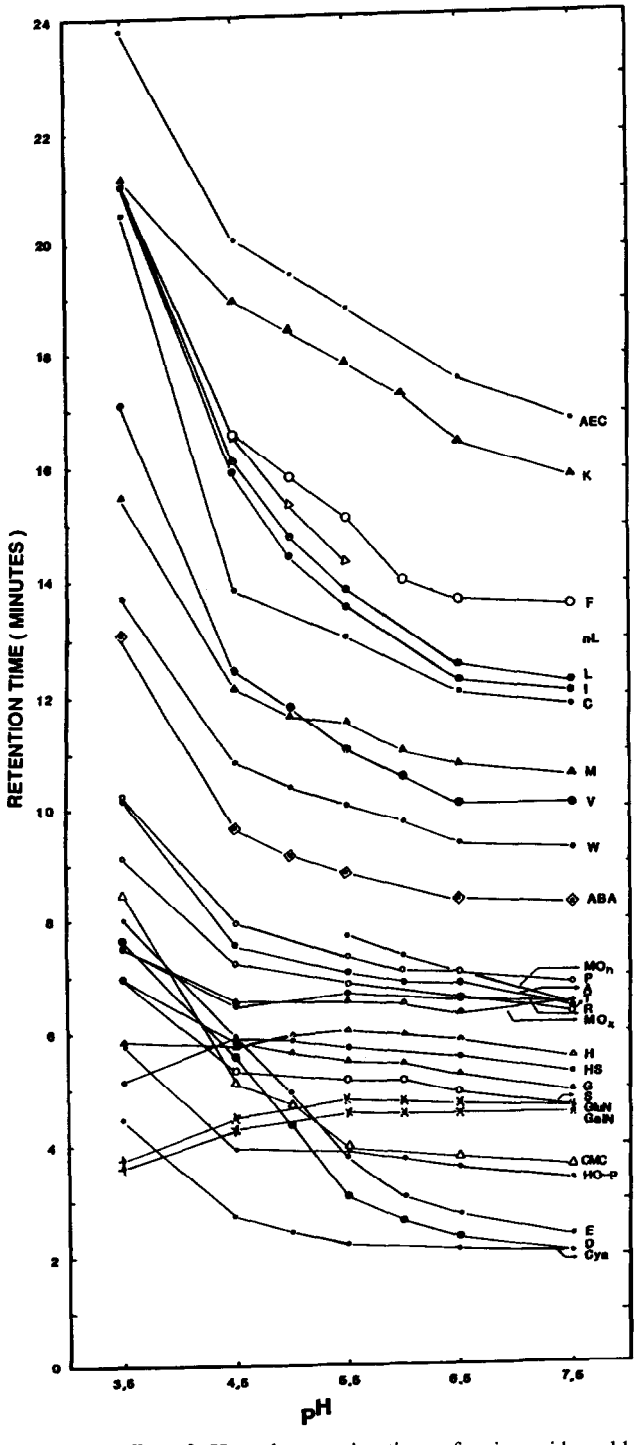

Fig. 3. The effect of pH on the retention times of amino acids and hexosamines. Mixtures of PTC amino acids and hexosamines were separated by HPLC using the conditions described in *Methods* except that buffer A was adjusted to different pH values ranging from 3.5 to 7.5. Amino acids are identified by the one letter code. Other compounds tested included the following: galactosamine, GalN; glucosamine, GlcN; homoserine, H-S; norleucine, nL; norvaline, nV; α-aminobutyric acid, ABA; aminoethyl cysteine, AEC; cysteic acid, Cya; carboxymethyl cysteine, CMC; hydroxyproline, HO-P; methionine sulfoxide, MO_x; methionine sulfone, MO_n.

carboxymethylcysteine and glutamic acid is consistent with the fact that they contain two carboxyl groups while the lack of increased sensitivity of cysteic acid to pH is consistent with the much lower pK_a value of sulfonates. The retention times of the PTC derivatives of the hexosamines, which are uncharged, are not greatly affected by pH and in fact decrease at lower pH values.

It is clear from Fig. 3 that α-aminobutyric acid represents a good choice for an internal standard since it is well separated from the other peaks at all pH values. Norleucine elutes in a crowded region of the chromatogram while norvaline coelutes with methionine at pH 5.5.

Amino acid analysis has frequently been used to quantify other compounds containing free amino groups in addition to the standard amino acids commonly found in proteins. These include compounds that occur naturally in specific proteins such as galactosamine and glucosamine in glycoproteins, hydroxyproline in collagen and methionine sulfoxide and sulfone which are formed from methionine under oxidizing conditions. Modified amino acids such as homoserine and aminoethyl cysteine are generated by standard procedures used in protein chemistry while the cysteine content of proteins is normally measured by making derivatives of cysteine such as carboxymethylcysteine or cysteic acid. Separation of these compounds in the chromatographic system is necessary both for their determination and also to prevent them from interfering with the analysis of the standard amino acids. An examination of Fig. 3 demonstrates that all peaks are resolved at pH 5.5 with the exception of hydroxyproline which coelutes with carboxymethylcysteine. These can, however, be resolved at pH 6.5. Similarly, phenylthiourea (from the reaction of PITC with ammo-

TABLE I

AMINO ACID ANALYSIS OF SHEEP SUBMAXILLARY MUCIN AND LYSOZYME

Values are given in terms of mol per thousand mol of amino acids. The literature data for lysozyme were taken from ref. 14 and the data for sheep submaxillary mucin from ref. 15.

Amino acid	*Sheep submaxillary mucin*		*Lysozyme*	
	Observed	*Literature*	*Observed*	*Literature*
Asx	20	22	185	183
Glx	46	63	39	43
N-Acetylgalactosamine	334	312	0	0
Ser	198	179	92	87
Gly	212	182	106	104
His	2	2	8	8
Arg	33	37	95	96
Thr	142	142	61	61
Ala	132	135	98	104
Pro	104	100	18	18
Tyr	4	2	32	26
Val	46	65	45	53
Met	0	0	11	18
Ile	9	16	51	51
Leu	31	35	75	76
Phe	16	16	26	26
Lys	6	7	59	60

nia) elutes as a very broad peak in the vicinity of the Ala and Pro peaks (data not shown) and may interfere with their quantification using this system. This peak can be suppressed by repeating the ethanol–water–TEA additions and drying steps prior to derivatization. Alternatively, other gradients are capable of separating phenylthiourea from the PTC amino acids[9].

The application of this methodology to the amino acid analysis of lysozyme and sheep submaxillary mucin is shown in Table I. Measured values for amino acid composition match literature values[14,15] reasonably well. Furthermore, the yield of amino acids recovered after hydrolysis of lysozyme accounted for 95.3% of the dry weight of the sample as calculated from the amount of internal standard added. Similarly, 94.1% of the dry weight of sheep submaxillary mucin was recovered as amino acids (40.8%), N-acetylgalactosamine (25.7%) and sialic acid (33.5%) using the known ratio of sialic acid to N-acetylgalactosamine of 0.91[16] to calculate amounts of sialic acid.

A number of manufacturers produce highly efficient reversed-phase HPLC columns capable of separating PTC amino acids[2–7]. Conditions for achieving adequate resolution appear to be somewhat different for each brand of stationary phase; appropriate conditions vary somewhat with different columns from the same producer and may also change as a column ages. Achieving separation by trial and error is tedious; this work on relating the buffer pH to retention times and that of Ebert[5] correlating TEA concentrations to separation provide a rational framework for predicting appropriate changes in elution conditions required to gain the desired separations.

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