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Technical Note

Reversed-Phase High-Performance Liquid Chromatographic Analysis of Oligoglycines (One to Six Amino Acid Residues)

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INTRODUCTION

Oligoglycines (one to six residues) have been used to study absorption mechanisms in the intestine (1) and hence are of interest for studies in other mucous membranes. They have been analyzed by ion-exchange chromatography; however, this required a 190-min run time (1). Metal ion-modified mobile phases have also been used in conjunction with a 600-NH amino column (2) but separation of glycine peptides larger than triglycine was inadequate for quantitiation. A number of reverse-phase systems have been used for di- and tripeptides but retention of di- and triglycines in these systems was minimal [capacity factors of 0–0.05 (3)].

This report describes a reversed-phase high-performance liquid chromatographic (HPLC) method for the rapid quantitation of oligoglycines (one to six residues).

EXPERIMENTAL

Materials

Glycine (Aldrich, Milwaukee, Wisc.), glycine oligopeptides (two to six residues), and L-alanine (Sigma Chemical Co., St. Louis, Mo.) were used as supplied. Sodium octanesulfonate (SOS) (Eastman Kodak, Rochester, N.Y.), phosphoric acid (Fisher Scientific, Springfield, N.J.), and acetonitrile (Baker, Phillipsburg, N.J.) were of HPLC grade. Double-distilled water was used throughout.

Chromatography

The chromatography system consisted of Beckman components: a 110B solvent delivery module, 20-μl loop injector, and 164 variable-wavelength detector set to 200 nm. Separations were performed on a Beckman Ultrasphere ODS column (15 cm × 4.6 mm; particle size, 5 μm) with a Hibar-LiChrocart LiChrosorb RP18 5-μm guard column. Except where noted otherwise, the mobile phase was 1–2 m*M* SOS in water adjusted to pH 2.5 with phosphoric acid. The flow rate was 2.0 ml/min, giving a typical back-pressure of 3000 psi at the ambient temperature conditions (typically 20–25°C).

Sample Preparation

Tissue homogenates were prepared by homogenizing porcine buccal mucosa in Krebs Ringer buffer, then centrifuging (2000*g* for 10 min) to remove cellular and nuclear debris (4). The supernatant was diluted with Krebs Ringer buffer to give typical protein concentrations of 1 mg/ml (Lowry method).

To each 200-μl sample of supernatant containing the various oligoglycines was added internal standard (20 μl of 250 m*M* alanine) followed by acetonitrile (800 μl) and phosphoric acid (25 μl). These were vortexed and centrifuged (1000 *g* for 10 min) and 20 μl of supernatant was injected for HPLC analysis.

Preliminary experiments showed that this concentration of acetonitrile was sufficient to remove interfering tissue homogenate peaks from the chromatogram with complete recovery of the glycines. This is in agreement with previous work (5).

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 1. The mobile phase is transparent at 200 nm, allowing detection at this wavelength, giving greater sensitivity than that at 210–220 nm, the wavelength usually used for peptides.

Heptafluorobutyric acid (HFBA) is often used for HPLC analysis of peptides. Although in preliminary work, reasonable separation was achieved with 0.05% HFBA in water (pH 2.6), the sensitivity was reduced because absorption by this mobile phase precludes its use below 215 nm. More importantly, injections of samples in solvents other than the mobile phase gave rise to induced vacancy peaks which interfered with the chromatogram (6,7).

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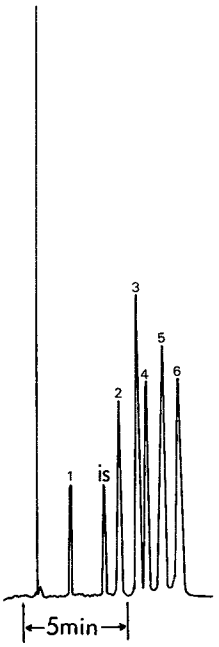

Fig. 1. Chromatogram of a sample containing (1) 15 m*M* glycine, (2) 0.75 m*M* diglycine, (3) 0.75 m*M* triglycine, (4) 0.45 m*M* tetraglycine, (5) 0.4 m*M* pentaglycine, (6) 0.35 m*M* hexaglycine, and (is) the internal standard, alanine. Absorbance units full scale = 0.1.

Separation was not achieved using mobile phases containing organic modifier only. Ion pairing with SOS was required. This is in contrast to oligoalanines (one to six residues), which have been separated using a pH 2.1 buffer and a C18 column (8). This reflects the very low hydrophobicity of glycine oligomers.

The similarity in structure and pK_a values (9) of the glycine peptides necessitated careful adjustment of the conditions for separation. These conditions may have to be manipulated for different columns so the following are noted. (i) Increasing the SOS concentration increased the capacity factors (k') of all peptides but also lead to peak spreading; 1–2 m*M* was optimal. (ii) Raising the pH from 2.5 to 3.0 reduced the k' values and resulted in poor separation. This implies that ionic interactions occur between the SOS and the cationic forms of the glycines, and not with the zwitterionic forms. The pK_a values of the carboxylic acid groups of the glycines are in the range 3–3.3 (9). (iii) Increasing the ionic strength with sodium chloride (0.025 *M*) or monobasic potassium phosphate (0.01 *M*) reduced the k' values, presumably due to reduced interactions between the SOS and the cationic glycines. Minimum ionic strength was required for separation. (iv) The addition of acetonitrile as low as 10% caused the peptides to elute near the solvent front without separation. These observations are explicable in terms of ion-pair reversed-phase HPLC theory (10).

When samples of the peptides in Krebs Ringer buffer (pH 7.4) were injected, peak splitting occurred. Presumably, the pH of the mobile phase was temporarily modified in a localized region at the top of the column. This would shift the ionic equilibrium, changing the ratio of cationic to zwitterionic forms of the peptides and so altering the interactions with the SOS. This problem was overcome by acidifying the samples with phosphoric acid.

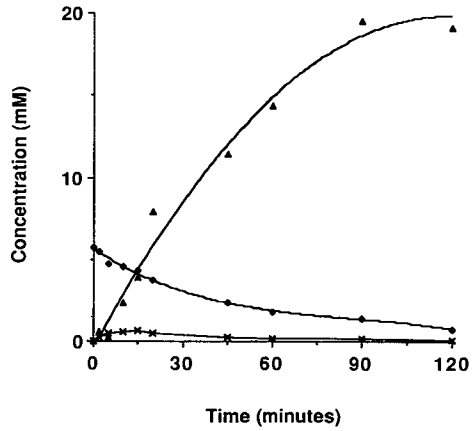

Fig. 2. Hydrolysis of triglycine in porcine oral mucosal tissue homogenates. (♦) Triglycine; (×) diglycine; (▲) glycine.

Calibration samples were prepared in supernatants from tissue homogenates. Standards containing mixtures of the glycines were made in triplicate at six concentrations in the ranges glycine (0.375–6.0 m*M*), di- and triglycine (0.05–4.0 m*M*), and tetra-, penta-, and hexaglycine (0.025–2.0 m*M*). Results were analyzed by linear regression, and the following equations obtained: glycine, PAR = $0.039\pm0.0030 \cdot C + 0.018\pm0.008$; diglycine, PAR = $2.35\pm0.026 \cdot C - 0.05\pm 0.043$; triglycine, PAR = $4.58\pm0.058 \cdot C - 0.1\pm0.10$; tetraglycine, PAR = $6.82\pm0.093 \cdot C - 0.07\pm0.08$; pentaglycine, PAR = $10.0\pm0.10 \cdot C - 0.14\pm0.088$; and hexaglycine, PAR = $10.5\pm0.15 \cdot C - 0.1\pm0.13$. PAR is the peak area ratio of oligoglycine to alanine and C is the millimolar concentration of the oligoglycine. Values shown are the least-squares estimates plus or minus the standard errors of the estimates. The linear equations were adequate in all cases as judged by statistical comparison of the lack of fit and pure error mean squares (11). In all cases the intercepts were not significantly ($P > 0.05$) different from zero. The coefficients of variation were typically 20% for glycine and 7% for the oligoglycines. The increasing slope in going from glycine to hexaglycine is a reflection of the increasing number of carbonyl groups in peptide bonds. The small slope for glycine indicates that the free carbonyl group makes only a small contribution to the absorbance at 200 nm and this is why it could not be determined with the same precision as the oligoglycines. Typical detection limits ranged from 0.01 to 0.015 m*M* for hexa-, penta-, and tetraglycine and from 0.02 to 0.03 m*M* for tri- and diglycine and were 0.75 m*M* for glycine.

The utility of the method is shown for the metabolism of triglycine in supernatants from porcine oral mucosal tissue homogenates (Fig. 2). These data suggest metabolism of triglycine to diglycine and glycine and a parallel breakdown of diglycine to glycine. The sum of the glycine residues is approximately constant as expected.

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