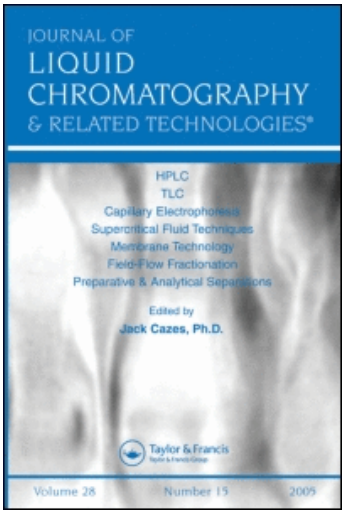

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HPLC Separation of Geometric Isomers of Carbamyl Peptides

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HPLC SEPARATION OF GEOMETRIC ISOMERS OF CARBAMYL PEPTIDES

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ABSTRACT

Carbamylated peptides that were studied showed much improved resolution on C-18 reversed phase HPLC columns compared to the parent peptides. A number of dipeptides were carbamylated with ethyl-, n-propyl- or isopropyl isocyanates. The three carbamyl derivatives of each dipeptide could easily be resolved. Carbamylated dipeptides with reversed amino acid sequences were also easily separated. Methionine-enkephalin, leucine-enkephalin, Angiotensins I, II and III and substance P were carbamylated with isocyanates derived from certain carcinostatic 2-chloroethyl nitrosoureas. 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), 1-(2-chloroethyl)-3-(cis-4-hydroxycyclohexyl)-1-nitrosourea (cis-4-hydroxy-CCNU) and 1-(2-chloroethyl)-3-(trans-4-hydroxycyclohexyl)-1-nitroso urea (trans-4-hydroxy-CCNU) gave carbamylated derivatives of each peptide and each mixture of derivatives from a single parent peptide could be resolved. Conditions were found in each case whereby baseline resolution of the corresponding cis-4- and trans-4-hydroxycyclohexylcarbamyl peptides was attained. Cyclohexylcarbamyl peptides were easily separated from the corresponding peptides from hydroxy-CCNUs. Potential applications are discussed.

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INTRODUCTION

The 2-chloroethyl nitrosoureas (CENUs) are important anticancer drugs and are useful in treating malignant tumors, especially brain tumors and Lymphomas (1-3).

We are interested in the role of carbamylation of protein by CENUs in the expression of their carcinostatic activity and/or toxicity to normal tissues. During the course of study we have found that peptides which have been carbamylated by closely related isocyanates can be resolved by HPLC. It is hoped that the results can be usefully applied to more general problems involving peptide separations.

MATERIALS AND METHODS

Materials

Leucine-enkephalin (Leu-Enk), methionine-enkephalin (Met-Enk), Angiotensins I, II, III (Ang), substance P and all dipeptides were from Sigma, St. Louis MO. Ethyl-, n-propyl- and isopropyl isocyanates were from Aldrich, Milwaukee, WI. CCNU was from Dr. Robert Engle, National Cancer Institute. Cis-4-hydroxy-CCNU and trans-4-hydroxy-CCNU were synthesized according to our previous method (4). HPLC solvents were from Fisher, Dallas TX. Triethylamine and formic acid were from Pierce, Rockford IL.

Preparation of Carbamylated Peptides from Isocyanates.

The peptide was treated with a slight molar excess (1.1) of the isocyanate in 0.01 M Tris-HCl, pH 7.7 for 3 hrs. Samples could be injected directly onto the HPLC column.

Preparation of Carbamylated Peptides from Nitrosoureas.

One ml of peptide (0.5 mM in 0.1 M Tris-HCl, pH 7.7) was treated with 0.05 ml of the 2-chloroethyl nitrosourea (35 mM in acetone) to give a peptide/CENU molar ratio of 3.5. The sample was incubated at room temperature for 20 hrs and could be injected directly onto the HPLC column.

HPLC Separations.

Dipeptides and carbamyl derivatives were separated on a μBondapak C-18 reversed phase column (Waters Associates) using acetonitrile in pump B and 0.04 M triethylamine formate (TEAF), pH 3.15 in pump A at a flowrate of 1.5 ml/min. Effluent was monitored at 205 nm using a Hewlett Packard 1084B liquid chromatograph (5). Solvent percents are given in the text or figures and tables. Peptides carbamylated by CCNU, cis-4-hydroxy-CCNU and trans-4-hydroxy-CCNU were chromatographed using the same column and conditions but 0.04 M triethylamine phosphate (TEAP), pH 2.2 (6) was used in pump A instead of TEAF.

RESULTS

HPLC of Ethyl-, n-Propyl- and Isopropylcarbamyl Dipeptides.

Three carbamyl derivatives each of Ala-Ser and Ser-Ala were prepared by incubating the dipeptide with ethyl-, n-propyl- and isopropyl isocyanates, respectively. Derivatives were chromatographed on a reversed phase C-18 column and eluted with 2% acetonitrile in TEAF (0.04 M, pH 3.1). Figure 1 shows that all six carbamylated dipeptides are resolved and the underivatized Ala-Ser

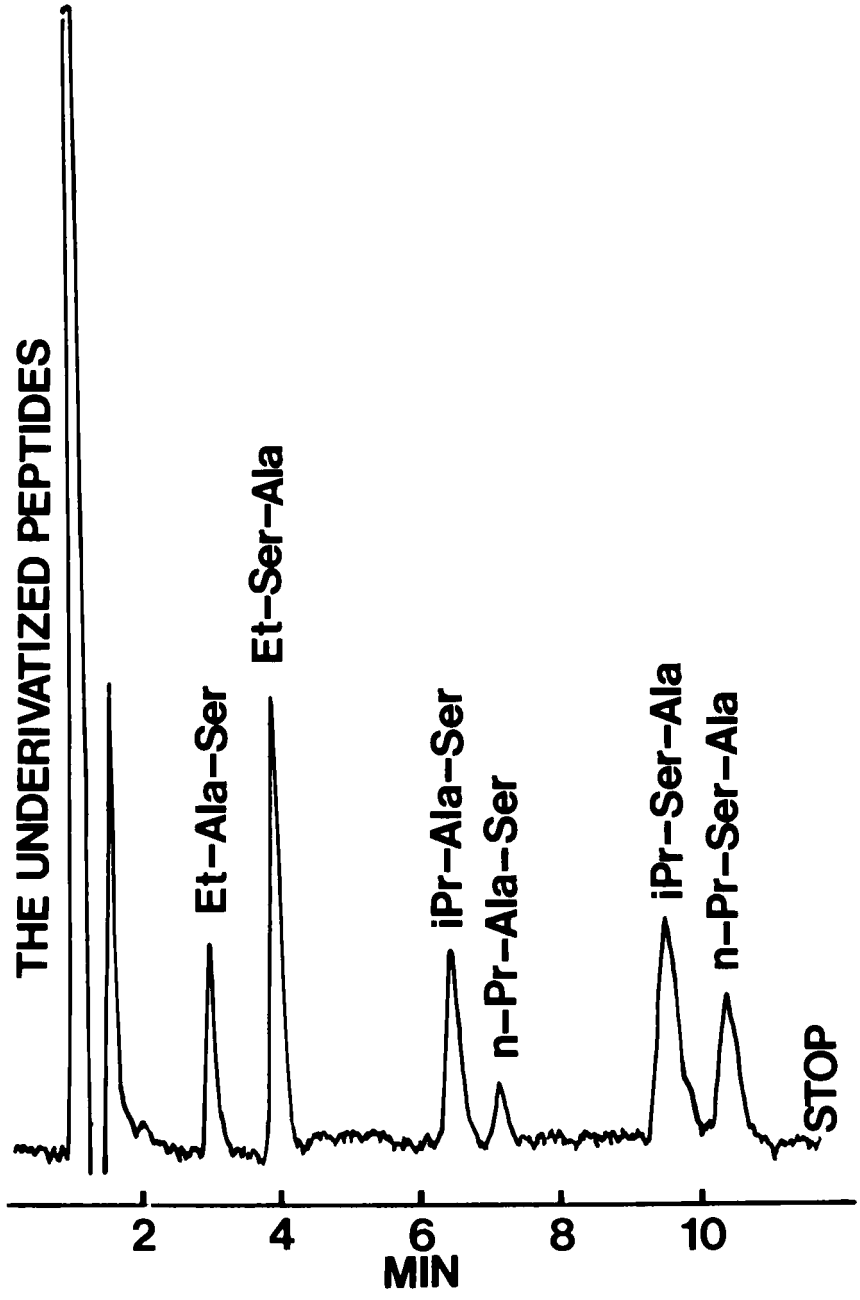

Figure 1 Effect of variation of carbamyl group on HPLC behavior of Ser-Ala and Ala-Ser. Pump A contained 0.04 M TEAF, pH 3.1 and pump B contained acetonitrile. 2% B was used.

TABLE 1

Comparison of Retention Times of Carbamyl Dipeptides and Carbamyl Glutathione.

Carbamyl Derivative	Organic Modifier [a]	Retention Time
Et-Ser-Ala	2% Acetonitrile	3.8
Et-Ala-Ser		2.9
Pr-Ser-Ala		9.9
Pr-Ala-Ser		6.8
iPr-Ser-Ala		9.1
iPr-Ala-Ser		6.2
Et-Ala-Thr		4.8
Pr-Ala-Thr		12.8
iPr-Ala-Thr		11.6
Et-Glutathione		10.2
Pr-Glutathione		26.8
iPr-Glutathione		22.9
iPr-Met-Lys	15% Acetonitrile	3.4
iPr-Tyr-Gly		4.0
iPr-Gly-Tyr		4.6
iPr-Tyr-Glu		4.9
Et-Val-Tyr	10% Acetonitrile	8.9
Et-Tyr-Val		11.6
Pr-Tyr-Val		26.5
Pr-Val-Tyr		20.4
iPr-Val-Tyr		18.7
iPr-Tyr-Val		25.1

a Solvent contains the indicated acetonitrile in 0.04 M triethyl amine formate buffer, pH 3.1.

and Ser-Ala elute at the solvent front under these conditions. For both the Ser-Ala and the Ala-Ser series the elution order is ethyl-, isopropyl- and n-propyl carbamyl peptide, as would be expected based on the relative hydrophobicities of the added groups. It is interesting that both the peptide sequence and the nature of the carbamyl group has a substantial effect on retention time. Under

the same chromatography conditions the cyclohexylcarbamyl derivatives of Ser-Ala and Ala-Ser had retention times in excess of 40 min. Figure 2 shows the large effect of carbamylation on Tyr-Val and Val-Tyr. These dipeptides, being more hydrophobic, required a higher amount of acetonitrile (10% versus 2%) in the elution solvent than the corresponding Ser-Ala and Ala-Ser derivatives. Again all Val-Tyr derivatives elute before Tyr-Val derivatives and in the order ethyl-, isopropyl- and n-propyl carbamyl peptide. Table 1 summarizes the retention times of a series of carbamylated dipeptides and glutathione. The general trends are 1) retention times for a peptide derivatized with different isocyanates increase in the order ethyl-, isopropyl-, n-propylcarbamyl peptide, 2) carbamyl derivatives of dipeptides with reversed sequences were easily separable, 3) increased retention times in the dipeptide series containing the same carbamyl group were related to increased hydrophobicity of the aminoacid side chains in the peptide, 4) all carbamyl derivatives were greatly separated from the parent peptide, which usually eluted from the column near the solvent front under the conditions used.

HPLC of Carbamylated Peptides Derived from CCNU, cis-4-Hydroxy-CCNU and trans-4-Hydroxy-CCNU.

The CENUs used spontaneously decompose in buffer to form cyclohexyl isocyanate or a corresponding 4-hydroxycyclohexyl isocyanate. These CENUs carbamylate proteins in vivo (7) and the reaction accounts for some of the antitumor properties these compounds possess. It is possible that particular isocyanates, derived either

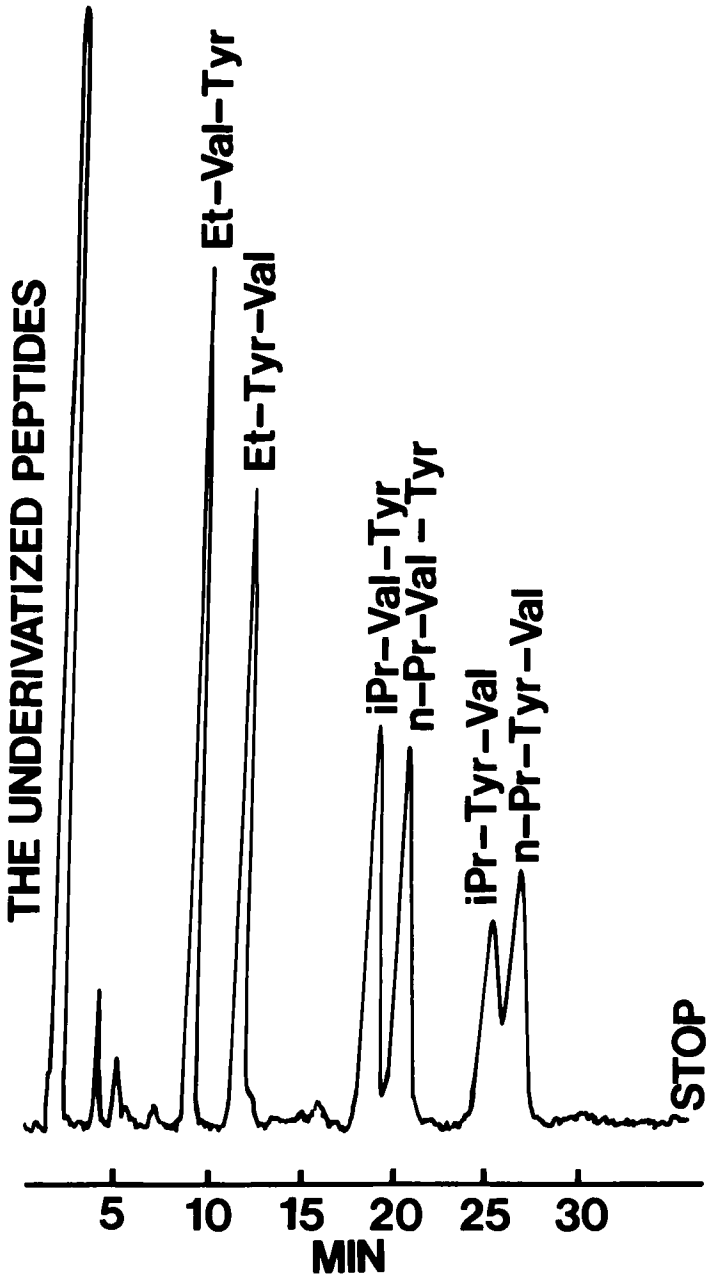

Figure 2 Effect of variation of carbamyl group on HPLC behavior of Val-Tyr and Tyr-Val. See figure 1 for chromatography conditions.

from the parent drug or its metabolites, are directed toward particular proteins/peptides in the cell. In order to study potential selectivity of nitrosourea-derived isocyanates toward particular proteins, it was necessary to determine whether the different carbamyl derivatives of a particular model peptide could be separated by HPLC.

Leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu) or methionine-enkephalin (Tyr-Gly-Gly-Phe-Met) were derivatized using CCNU or cis-4-hydroxy-CCNU or trans-4-hydroxy-CCNU as described in Methods. Figure 3 gives the results of the chromatographic behavior of the underivatized Leu-Enk and Met-Enk along with cyclohexyl-, cis-4-hydroxycyclohexyl- and trans-4-hydroxycyclohexyl carbamyl derivatives. In each case log k' is a linear function of the amount of acetonitrile in TEAP buffer. A mixture of the two parent pentapeptides and the six carbamyl derivatives can readily be separated. The trans-4- isomer elutes before the cis-4- isomer in each case and gives baseline separation. The cyclohexyl-Leu-Enk and cyclohexyl-Met-Enk both elute considerably later than cis-4- and trans-4-isomers of Leu-Enk and Met-Enk. The k' (figure 3) is the number of void volumes a sample is retained and is linearly related to retention time, corrected for void time. It can be seen that for any isocratic condition selected, by following any vertical line to the intersections of curves, there is good resolution of these compounds. However, it is not practical to select a single isocratic condition to separate all of them because either the parent peptides elute too early for good resolution or the cyclohexyl-

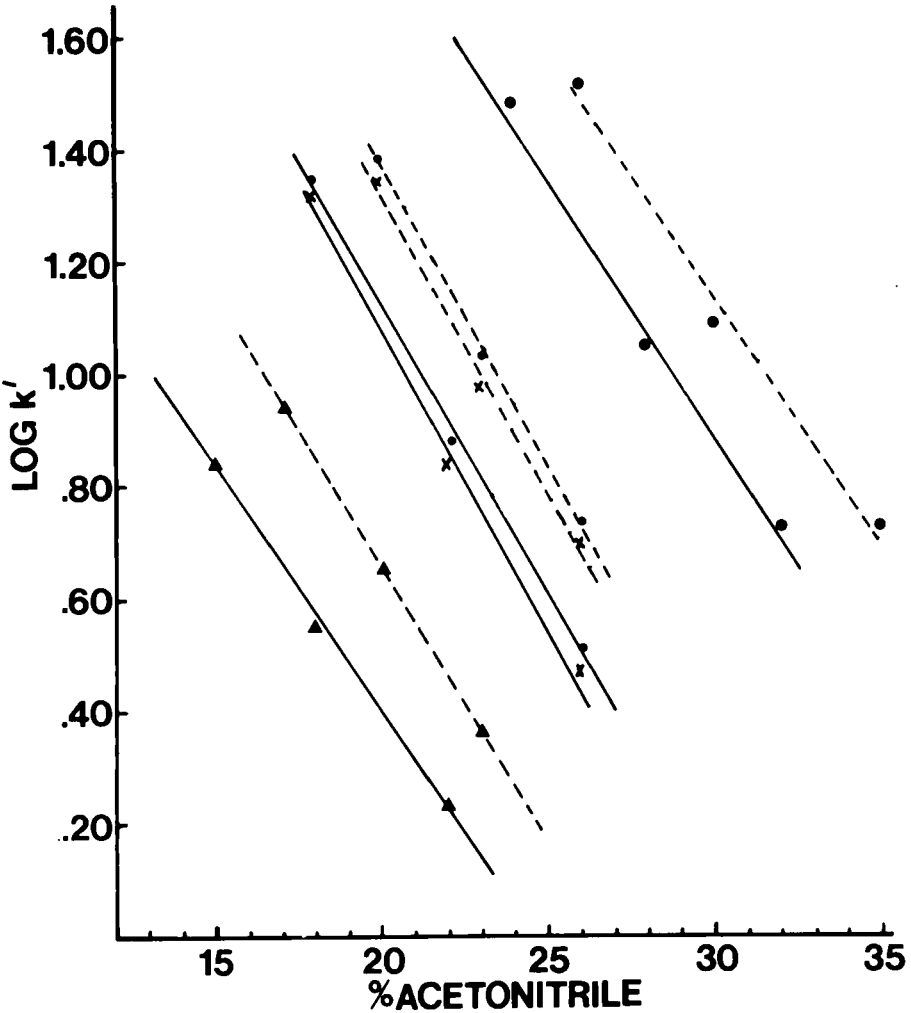

Figure 3 Effect of percent organic modifier on log k' of carbamyl Met-Enk and Leu-Enk. $k' = (k_R - k_o)/k_o$ where k_R = retention time and k_o = void time. Pump A contained 0.04 M TEAP, pH 2.2 and pump B contained acetonitrile. Solid lines - Met-Enk derivatives. Dashed lines - Leu-Enk derivatives. ▲ , underivatized; ● , cyclohexyl derivatives; ● , cis-4-hydroxycyclohexyl derivatives; X trans-4-hydroxycyclohexyl derivatives

TABLE 2

Comparison of HPLC of Nitrosourea-Derived Carbamyl Angiotensin III with Carbamyl Met-Enk

Carbamyl Group	Retention Times[a]	
	Angiotensin III[b]	Met-Enk[b]
None	13.9	11.7
trans-4-hydroxycyclohexyl	39.7	44.5
cis-4-hydroxycyclohexyl	45.9	45.9
cyclohexyl	64.4	67.1

a Gradient elution was as follows: Pump A contained 0.04 M TEAP, pH 2.2 and pump B contained acetonitrile. 15% B for 15 min then gradient 15 to 25% B (0.25%/min), 25% B from 55 min to 75 min.

b Angiotensin III - Arg-Val-Tyr-Ile-His-Pro-Phe
Met-Enk - Tyr-Gly-Gly-Phe-Met

Leu-Enk and cyclohexyl-Met-Enk are retained too long for convenient analysis. The trans-4 isomer elutes before the cis-4 isomer in each case and good resolution can be attained. Table 2 gives an example comparison of retention times of carbamylated Angiotensin III (X-Arg-Val-Tyr-Ile-His-Pro-Phe) and carbamylated Met-Enk (Tyr-Gly-Gly-Phe-Met). The question arises, what is the limit of peptide size that can be carbamylated by the nitrosoureas and still be resolved by HPLC? Carbamyl derivatives of angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-Leu), a nonapeptide shows similar resolution (figure 4). The cyclohexyl-Ang I deviates from linearity in the graph but there is still excellent separation of cis-4-

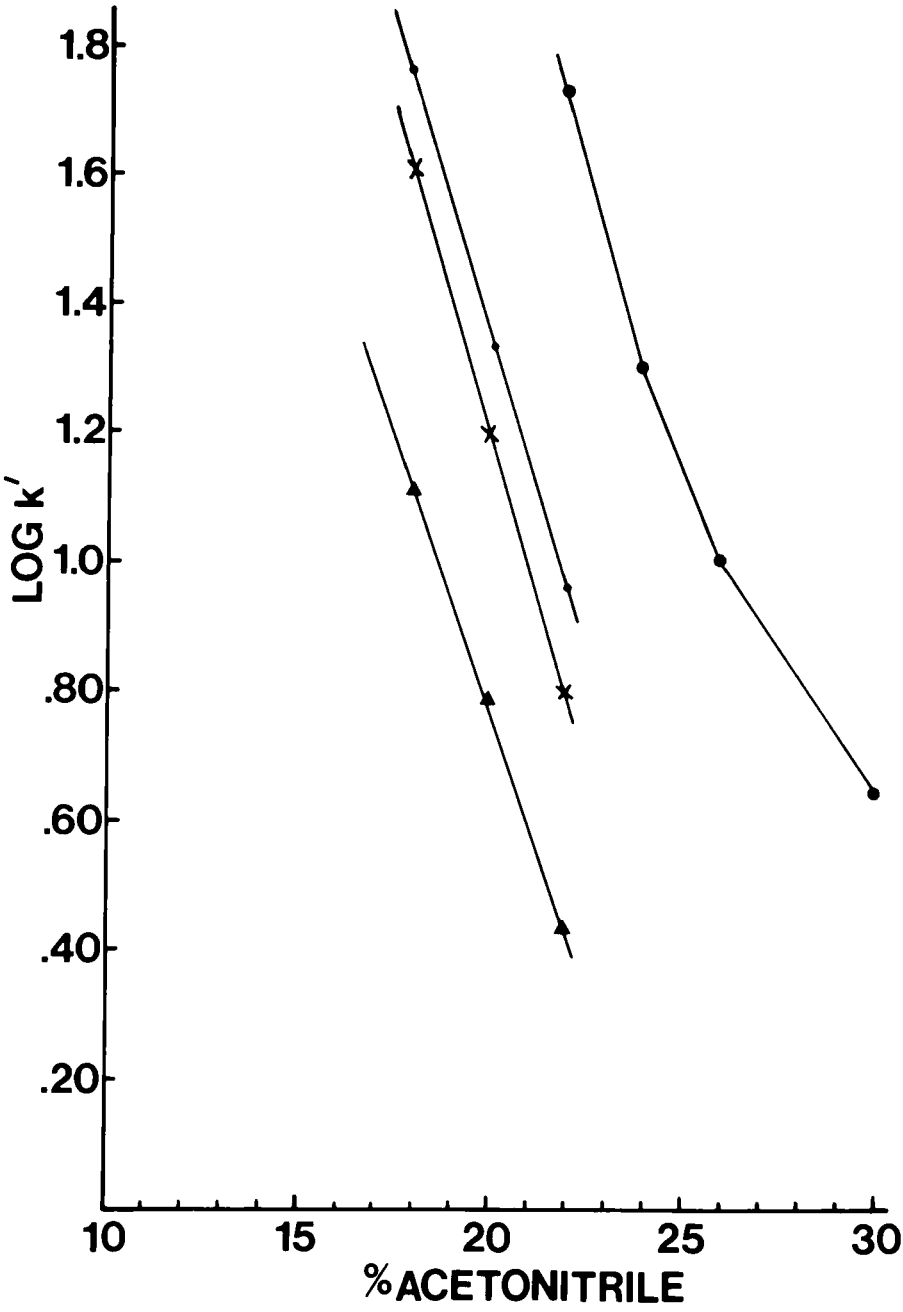

Figure 4 Effect of percent organic modifier on cyclohexyl-, cis-4-hydroxycyclohexyl- and trans-4-hydroxycyclohexyl carbamyl angiotensin I. See figure 3 for definition of k' and chromatography conditions. ▲ , untreated Ang; ● , cyclohexyl Ang; • , cis-4-hydroxycyclohexyl Ang; X , trans-4-hydroxycyclohexyl Ang.

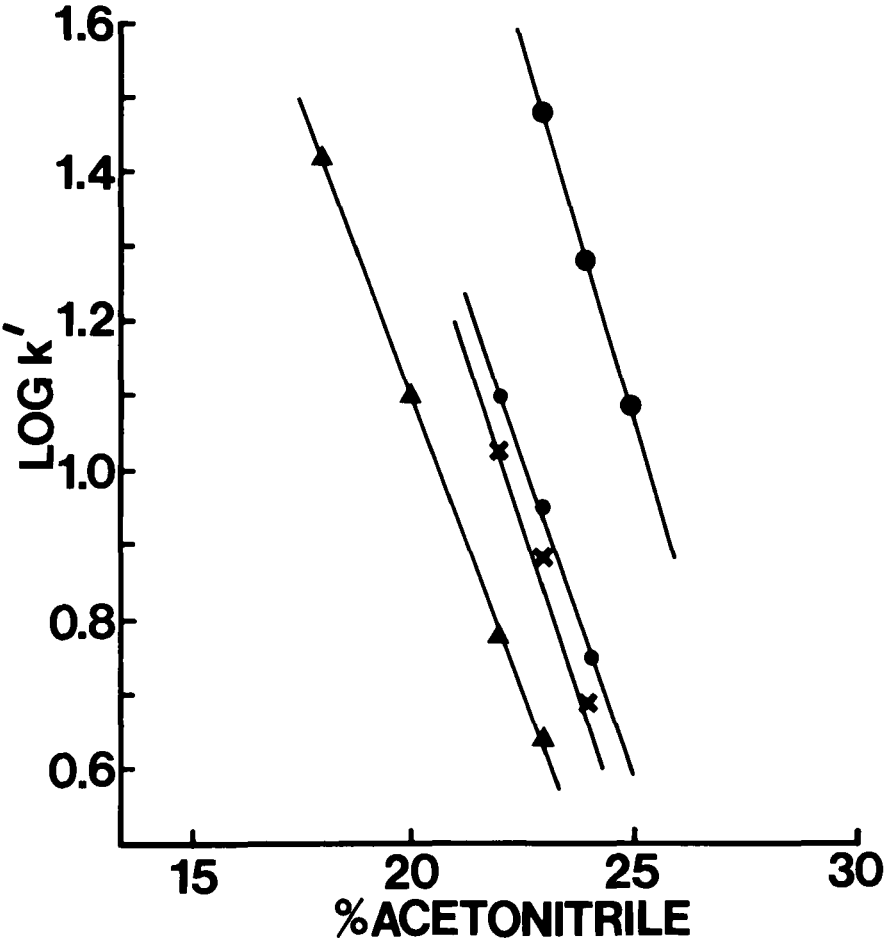

Figure 5 Effect of percent organic modifier on cyclohexyl-, cis-4-hydroxycyclohexyl- and trans-4-hydroxycyclohexyl carbamyl substance P. See figure 3 for definition of k' and for chromatography conditions. ▲ , untreated; ● , cyclohexyl substance P; ● , cis-4-hydroxycyclohexyl substance P; X , trans-4-hydroxycyclohexyl substance P.

hydroxycyclohexyl carbamyl Ang I from the corresponding trans-4 isomer. Figure 5 shows the chromatographic behavior of carbamyl derivatives of substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met) an undecapeptide. As can be seen the derivatives are all separable. Preliminary efforts to carbamylate β-endorphin (31 aminoacids) using nitrosoureas have been inconclusive.

DISCUSSION

There are at least two relatively general applications of the methods presented: 1) Carbamylation of small peptides dramatically increases resolution. A number of isocyanates are commercially available and may be useful to form more easily separable mixtures. Interestingly, at least for dipeptides, the method was very sensitive to aminoacid sequence as well as amonoacid composition. There remains the problem of quantitative yields, however. We have not studied reaction yields. It is assumed that carbamylation occurs at the amino-terminus. Certain peptides, particularly those with Ser, Thr, Cys, and Tyr may undergo carbamylation on the aminoacid side chain. We had no evidence of such multiple reactions in our studies. 2) Situations in which the same peptide is derivatized at the same site by different agents as may occur in biological studies may apply. Our particular interest is in the latter application.

CCNU is rapidly converted to a series of ring-hydroxylated nitrosoureas *in vitro* (8) and *in vivo* (9). The parent CCNU and the hydroxy-CCNUs are precursors to cyclohexyl and hydroxycyclohexyl isocyanates which carbamylate proteins (7). The role of

carbamylation in the expression of antitumor activity or toxicity to normal tissues is still not very clear. A number of DNA polymerases (10, 11), DNA ligase (12), glutathione reductase (13), Serine proteases (14, 15) and tubulin polymerization (16) have been shown to be inhibited by carbamylation by nitrosoureas or isocyanates.

The question of whether carbamylation by CENUs and their metabolites is selective to certain enzymes is an important one because nitrosoureas such as CCNU and Methyl-CCNU are converted to antitumor-active metabolites (8, 17, 18). If it is found that the individual metabolites target different proteins, it could have important applications in chemotherapy and drug design.

In order to determine whether such selectivity of carbamylation exists, it was required that analytical methodology be developed that would permit separation of peptides with the same sequence but with different carbamyl groups. These studies offer hope that if peptides with up to 11 aminoacids are carbamylated by CCNU or the 4-hydroxy-CCNUs, one may be able to separate them by HPLC. This limit may be extended if one need only separate cyclohexyl carbamyl peptides from mixtures of geometric and positional isomers of hydroxycyclohexyl carbamyl peptides as might occur in the cell.

ACKNOWLEDGMENTS

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