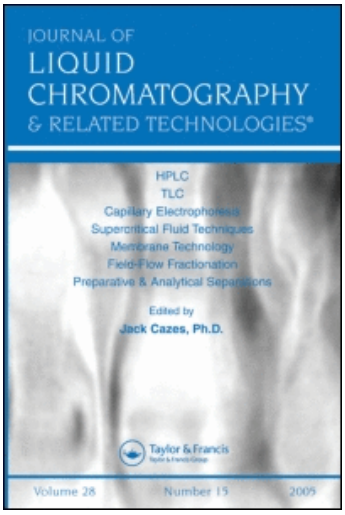

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DANSYLATION OF AMINO ACIDS AND BYPRODUCT FORMATION

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ABSTRACT

An ion pair reversed-phase HPLC method was developed for the determination of flourescent DNS-AA and by-products DNS-OH, DNS-NH_2, and DNS-$N(CH_3)_2$ which form during the dansylation reaction. The method permits the separation of the four fluorescent compounds and ultraviolet absorbing DNS-Cl in 25 min. Dansylation of amino acids was carried out under different reaction conditions and evaluated with regard to major products formed and not only the DNS-AA. Optimum reaction conditions can readily be obtained for the dansylation of amino acids using this method.

INTRODUCTION

Dansyl (5-dimethylaminonapthalene-1-sulfonyl) chloride reacts with amino acids to form fluorescent derivatives which can be detected at extremely low concentrations (1). The reason why this reagent is not widely used, however, is the formation of multiple derivatives of several amino acids and other fluorescent by-products (2). Some of the by-products known to form are DNS-OH[1] from the hydrolysis of DNS-Cl (3), DNS-NH_2[1] from the decomposition of DNS-amino acids (4), and DNS-$(CH_3)_2$[1] probably from the

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decomposition of DNS-CL[1] (5). Considerable effort has been spent on developing optimal reaction conditions for the formation of DNS-amino acids (2,3,6), but few reports have appeared in the literature on the effect of reaction parameters on the formation of the fluorescent by-products. Therefore, a method that permits the rapid determination of optimal reaction conditions for the dansylation of amino acids will be helpful.

In this paper we describe a reversed-phase ion pair HPLC[1] method for monitoring the formation of DNS-OH, DNS-NH_2, DNS-$N(CH_3)_2$ by-products, and DNS-AA directly during the dansylation reaction under different reaction conditions. Ultraviolet absorbing dansyl chloride is also monitored.

MATERIALS AND METHODS

Apparatus

Chromatographic separations were carried out on a Waters Associate (Milford, MA) HPLC system which included the following components: Model 6000A[2] solvent delivery system, Model U6K injector, 30 cm X 4 mM I.D. μ Bondapak C_{18} column, Model 420 fluorescent detector. For isocratic conditions the mobile phase consisted of 5 mM t-butylammonium hydroxide in 10 mM K_2HPO_4 adjusted to pH = 7.00 and 40% v/v acetonitrile. The flow rate was 1 ml/min. The filters used were 370 ± 110 nm excitation and 500 nm long pass emission.

Reagents

Amino acids, gold label (Calbiochem-Behring Corp., La Jolla, CA), dansyl chloride, dansylamide, dansylamino acids (Pierce Chemical Co., Rockford, IL), t-butylammonium hydroxide, 40% w/v (Aldrich Chemical Co., Inc., Milwaukee, WI) were used without further purification. Dansyl acid was prepared by hydrolyzing dansyl chloride with 20% KOH v/v. Completeness of hydrolysis was determined chromatographically by the absence of DNS-Cl absorbance at 250 nM. Dansyl dimethylamide was synthesized and purified according to a published procedure (7). Dansyl dimethylamide purity was determined chromatographically. Water used in all determinations was deionized and twice distilled, obtained from a MegaPure System (Corning, Corning, NY).

Reaction Conditions

The dansylation of leucine is given as a typical reaction. The ratio of DNS-Cl:leucine was 10:1 at pH = 9.

DNS-Cl, 0.5 ml (4.3 mg/ml acetonitrile, 0.016 M) was added to 0.5 ml leucine (0.21 mg/ml, 0.0016 M), dissolved in buffer (0.1 M Na_2CO_3), adjusted to pH 9 with 6 N HCl, then stored in the dark. Periodically, 0.1 ml samples were withdrawn and diluted to 2 ml with mobile phase and 20 µl were injected on the column.

Calculations

The amount of DNS-AA and DNS-NH_2 found in the reaction mixture was determined by their fluorescent response and expressed as the percentage of the initial concentration of amino acid. The amount of DNS-$N(CH_3)_2$ and DNS-OH found was also determined by their fluorescent response and expressed as the percentage of the initial concentration of DNS-Cl. Chromatographically DNS-Cl was 98% pure based on the presence of DNS-OH as an impurity.

DISCUSSION

To monitor the formation of DNS-OH, DNS-NH_2, DNS-$N(CH_3)_2$, and DNS-AA during the dansylation of amino acids, a reversed-phase ion pair HPLC method was developed. Separation of these fluorescent derivatives required the addition of tetrabutylammonium phosphate to the mobile phase to prevent DNS-OH from eluting with the Vo. A typical separation of fluorescent products for the dansylation of leucine was accomplished in 10 min under isocratic conditions. Dansyldimethylamide 4, which results from the decomposition of DNS-Cl, can elute with the other fluorescent components within 25 min by gradient elution (Fig. 1). Dansyl chloride 5 does not fluoresce but its ultraviolet absorbance can be monitored at 250 nm and included in the chromatograph (Fig. 1). Dansyl dimethylamide results from the decomposition of DNS-Cl since it was present in the blank at the same concentration that it was found in the DNS-AA reaction mixture but not as an impurity in DNS-Cl. The formation of DNS-$N(CH_3)_2$ is a function of pH but not temperature. After 30 min at pH = 8, 0.07% DNS-$N(CH_3)_2$ was found compared to 1.4% at pH = 10.

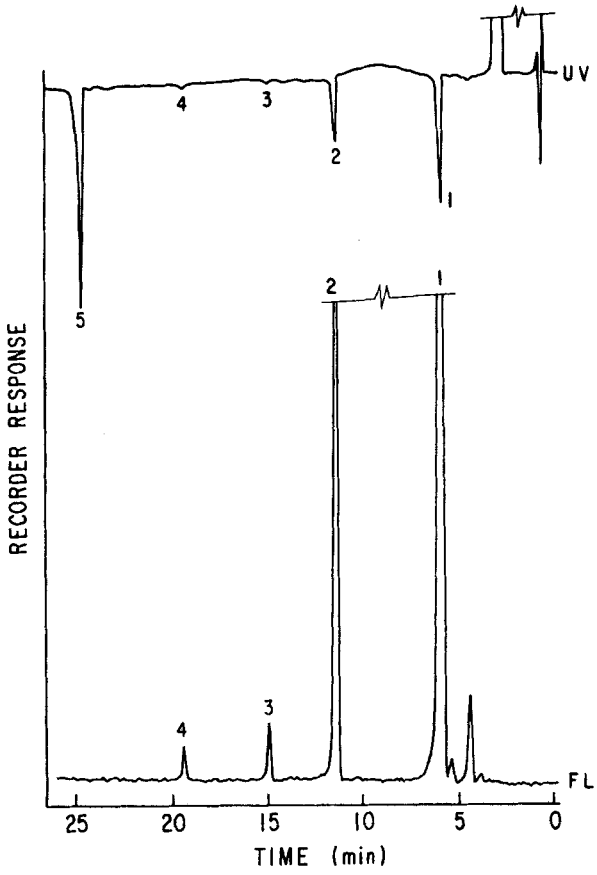

Fig. 1. Gradient elution of dansyl derivatives solvent: A = 5mM tetrabutylammonium phosphate; B = CH_3CN; gradient 40%-70% B, curve 6 (linear), 25 min. Peaks 1, DNS-OH; 2, DNS-leu; 3, DNS-NH_2; 4, DNS-$N(CH_3)_2$; 5, DNS-Cl.

Rate of formation of DNS-AA was a function of excess DNS-Cl. At equimolar concentrations of DNS-Cl and leucine, a maximum yield of 81% DNS-leu was obtained after 2 h (Fig. 2). During the same reaction period 18% of DNS-OH was formed from the hydrolysis of DNS-Cl and less than 2% DNS-NH_2 resulted from the decomposition of the DNS-leu product. Quantitative yields of DNS-leu were obtained after 20 min and 5 min when the ratio of DNS-Cl:leucine

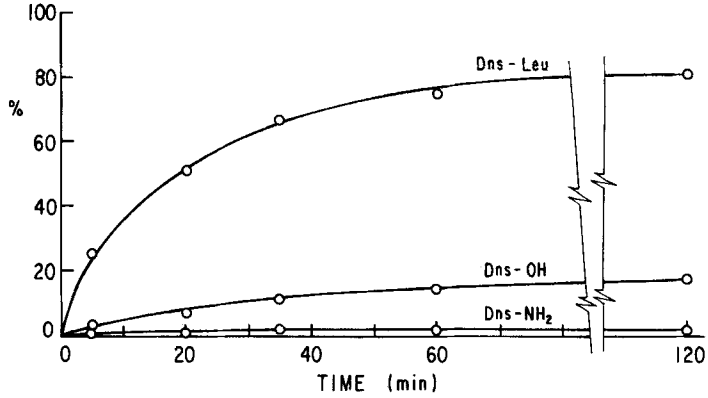

Fig. 2. Dansylation of leucine. Conditions, DNS-Cl:leu, 1:1, pH = 8, 25°C.

was increased to 10:1 and 20:1, respectively. Increasing the DNS-Cl:leucine ratio, however, resulted in a significant increase in the amount of DNS-NH_2. A 20-fold increase in DNS-Cl increased the DNS-NH_2 concentration 40 times after 2 h (Fig. 3).

Dansylation of amino acids is usually carried out under alkaline conditions. We have found that dansylation of leucine can proceed at pH = 7; a maximum yield of 95% was reached after 50 min (Fig. 4). At more alkaline conditions, pH 9 and 10, quantitative yields of DNS-leu were obtained in 5 min. The amount of DNS-OH formed during the reaction was essentially constant for pH 7, 8, and 9, however, at pH = 10, 51% DNS-OH was found after 60 min (Fig. 4). The amount of DNS-NH_2 formed was constant over the pH range 7-10.

Elevated reaction temperatures had an adverse effect on the formation of dansylated amino acids. A maximum yield of 66% DNS-leu was obtained after 5 min at 35°C and 60°C. Reported attempts to accelerate the dansylation of amino acids with elevated temperatures also resulted in lower yields of dansylated amino acids (6). By quenching the reaction with $EtNH_2$ poor yields were presumed to be due to rapid hydrolysis of DNS-Cl at the higher temperature, as evidenced by the very low chromatographic peak observed for DNS-NHEt (6). We found, by following the formation of dansyl product as well as dansyl by-products directly during the reaction, that the poor

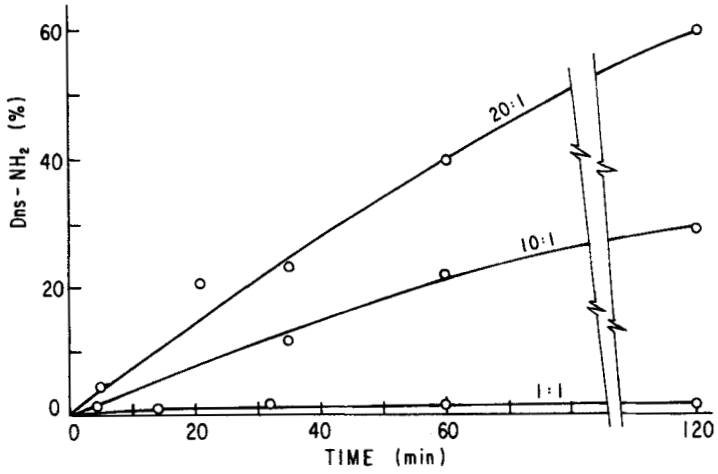

Fig. 3. Effect of DNS-Cl concentrations on DNS-NH_2 formation. Conditions, DNS-Cl:leu, 1:1, 10:1, 20:1, pH = 8, 25°C.

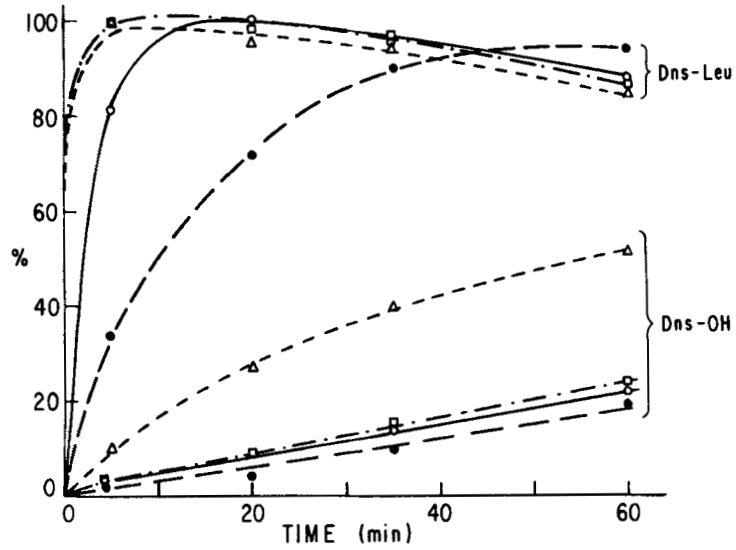

Fig. 4. Effect of pH on dansylation of leucine. Conditions, pH = 7(·); 8(0); 9(□); 10(Δ).

yields resulted from a combination of rapid hydrolysis of DNS-Cl and decomposition of DNS-leu to DNS-NH_2. At 60°C, 68% DNS-OH and 38% DNS-NH_2 were found after 20 min.

The amount of DNS-NH_2 formed during the dansylation of amino acids depends on the amino acid as well as the excess of DNS-Cl. Between 12%-16% DNS-NH_2 was formed for leucine, cysteine, and phenylalanine after 1 h with a 10-fold excess of DNS-Cl at pH = 8 and 25°C. However, for proline and asparagine, less than 0.5% DNS-NH_2 was found under the same reaction conditions and time period. Neadle and Pollitt (4) found 11%-15% DNS-NH_2 formed for the dansylation of α-alanine, valine, leucine, and norleucine using 10% excess of DNS-Cl. A method has been reported for the dansylation of amino acids which provides high yields independent of the ratio of DNS-Cl to amino acids over a 1,000-fold range (6). Using this procedure for the dansylation of leucine (40 mM Li_2CO_3, pH = 9.5, 25°C, and 10-fold excess DNS-Cl), we found that near quantitative yield was reached after 10 min but DNS-leu decomposed and 25% DNS-NH_2 formed after 1 h.

In conclusion, we have developed a reversed-phase HPLC method for following the formation of DNS-AA product and by-products directly. Using this method we improved the understanding of the dansylation reaction and were better able to arrive at optimum conditions for the dansylation of amino acids. Dansylation of amino acids was carried out under different reaction conditions and evaluated with regard to major products formed and not only the DNS-AA. Similar advantages should be obtained for the dansylation of N-terminal amino groups of small peptides hydrolysates using this method.

ACKNOWLEDGMENTS

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FOOTNOTES

[1] Abbreviations used: HPLC, high performance liquid chromatography; DNS-Cl, -OH, -NH_2, -$N(CH_3)_2$, -AA; dansyl-chloride, -acid, -amide, -dimethylamide, -amino acid; Vo, void volume.

[2] Reference to brand or firm name does not constitute endorsement by the

U.S. Department of Agriculture over others of a similar nature not mentioned.

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