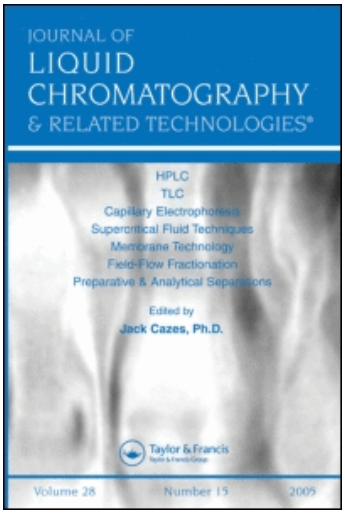

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HPLC MEASUREMENT OF DANSYL-THYROXINE IN FEMTOMOLE RANGE

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ABSTRACT

The formation of fluorophores by the action of Dansyl Chloride (1-dimethylaminonaphthalene-5-Sulfonyl Chloride) with iodoamino acids has provided a highly sensitive method for the detection of these compounds in the femtomole range. A relatively simple system is described for the reaction, chromatographic separation and characterization of individual iodoamino acids.

Dansyl chloride was found to react specifically with the primary amino group. The spectral characteristics of dansyl-thyroxine showed an excitation of 295 nm and an emission of 510 nm. The fluorescence of Dansyl-T4 in the femtomole range was linear with a coefficient of determination of 0.99.

INTRODUCTION

Several techniques have been reported for the separation of iodoamino acids by Gas and Liquid Chromatography. Using gas chromatography, Petersen et al. (1), were able to show that iodoamino acids were detected in the picogram levels. Although this method showed an application to biological samples, it has not been widely accepted, perhaps, due to the time consuming steps of sample clean-up and derivatization in non-aqueous medium.

In the last several years, high performance liquid chromatography (HPLC) has been introduced for the separation of iodoamino acids, and although several investigators (3,4,5) were able to show excellent separations, the lack of sensitivity has been a major difficulty in its application to biological samples.

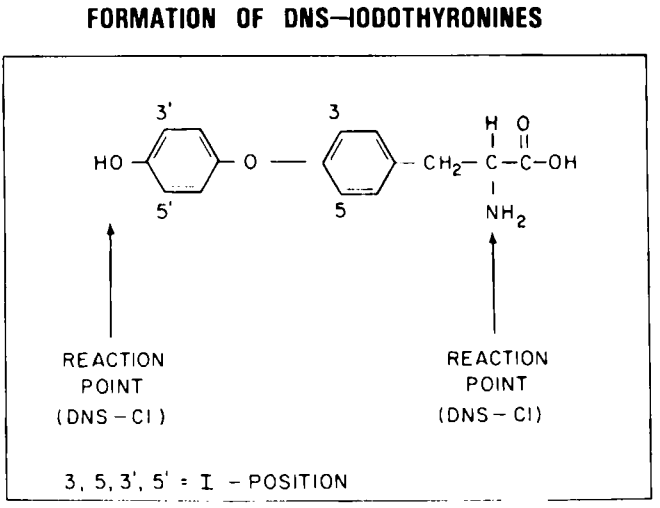

Figure 1

Derivative formation of Iodoamino acids with 1-dimethylaminonaphthalene-5-Sulfonyl Chloride (Dansyl-CL).

Recently, a novel approach to post-column derivatization was presented by Nachtmann et al. (2). Their method is based on the redox reaction between cerium IV and arsenic (III) that is catalyzed by trace amounts of iodine. Although this approach holds promise, preliminary studies in our laboratory concerning iodoamino acids demonstrated that it had insufficient sensitivity when applied to biological samples (6). Therefore, because dansyl chloride has been extensively discussed as an ideal derivatizing agent (7,8), we next studied dansyl reactions since its amine derivatives have previously been characterized and easily measured at the femtomole range for amino acids, polyamines and catechols (9-12).

In the present study, it will be shown that dansyl chloride will react with iodoamino acids as shown in Figure 1, and will greatly enhance the sensitivity.

MATERIALS

Equipment

The liquid chromatography system used consisted of two model 6000-A solvent delivery pumps, a model U6K sample injector, a model 440 ultraviolet detector, and a model 660 solvent programmer (Waters Associates, Inc., Milford, MA 01757). An Amineo Fluoro-Monitor™ was used with a primary filter Corning 7-51 (Excitation 340 nm) and a secondary filter Wratten No. 8 (Emission 480 nm) and a two-channel omniscribe recorder (Houston Instruments, Austin, Texas, USA). Retention times and peak areas were obtained with an HP 3381-A electronic integrator (Hewlett-Packard, Avondale, PA., USA).

Reagents

All thyronine reference material was purchased from Henning Berlin GM13H (Komturstr. 19-20, D1000 Berlin 42, Germany), except Thyroxine

(T4) and 3,3',5-triiodothyronine (T3) were purchased from Sigma (St. Louis, MO., USA) and 3,3',5'-triiodothyronine (rT3) was purchased from CalBiochem, San Diego, CA. Standards were prepared by dissolving in 1% methanol-NaOH (99:1,v/v) at a concentration of 1 mg/ml.

METHODS

Sodium Carbonate Buffer

A 0.5 mole/liter sodium bicarbonate was adjusted to pH 8.99 $\pm$ 0.02 with 0.5 mole/liter sodium carbonate. Dansyl chloride was prepared at a concentration of 2 mg/ml in acetone. It was purchased from Pierce Chemicals (Rockford, IL., USA 61105).

Preparation of Dansyl Reactions

Specific nanogram amounts of each iodoamino acid were added to a series of glass test tubes (10 mm x 15 cm) and brought to dryness at 55°C with a stream of nitrogen. Two hundred microliters of sodium carbonate buffer was added to each test tube followed by an equal volume of dansyl chloride; the tubes were covered with parafilm and reacted at 55°C for one hour. The dansyl derivatives were then extracted with two volumes of nHeptane. Specific volumes of the aqueous solutions were injected on column.

Chromatographic Conditions

Separations were obtained on microparticulate, chemically bonded, reversed-phase columns (300 x 3.9 mm ID, uBondapack C_{18}, Waters Assoc.). A two solvent system was used. Pump A - a 1% glacial acetic acid in distilled water, Pump B - HPLC grade acetonitrile (ACN). A 60 min. convex gradient program beginning at 15% ACN and ending with 50% ACN at a flow rate of 1.5 ml/min was used.

RESULTS AND DISCUSSION

Optimization Of The Dansyl Reaction

It was found that the derivatization of most iodoamino acids is optimal at pH 9.5 to 10.5; at higher pH values the reagent is hydrolyzed too rapidly. At pH values lower than 8 the protonated form of the amino group predominates, therefore, preventing the formation of dansyl derivatives. Dansylation by-products, the result of excessive use of dansyl reagent, are minimized by keeping the sample volumes small, limiting the concentration of dansyl chloride to 1.5-2.0 mg/ml and extracting the reaction mixture with nHeptane. A typical reaction of T4 is shown in Figure 2. In the figure, chromatogram (a) shows the HPLC elution pattern obtained after 20 ug of T4 was diluted to 420 ul with sodium carbonate buffer/acetone (50/50 v/v) and 5 ul was injected on column. Chromatogram (b) shows the result of a 20 ug T4 level reacted as described in methods and a 5 ul sample injected on column. Chromatogram (c) is the corresponding fluorescence to the dansyl T4 shown in (b).

Reaction In Site Identification

Dansyl chloride will react specifically with primary amino and phenol groups. However, when these two groups are present on the same compound mono and didansyl derivatives are formed. These derivatives are easily separated by reverse phase liquid chromatography. In the case of iodoamino acids, it was found that if iodide is present at either position 3' and 5' or both then dansyl chloride will not react with the phenol group (4'-OH position). Four specific iodocompounds were chosen and studied for the confirmation of the reaction site (see Figure 3).

Confirmation was achieved with the reaction and chromatography as described in the methods section (see Figure 2). The chromatography was

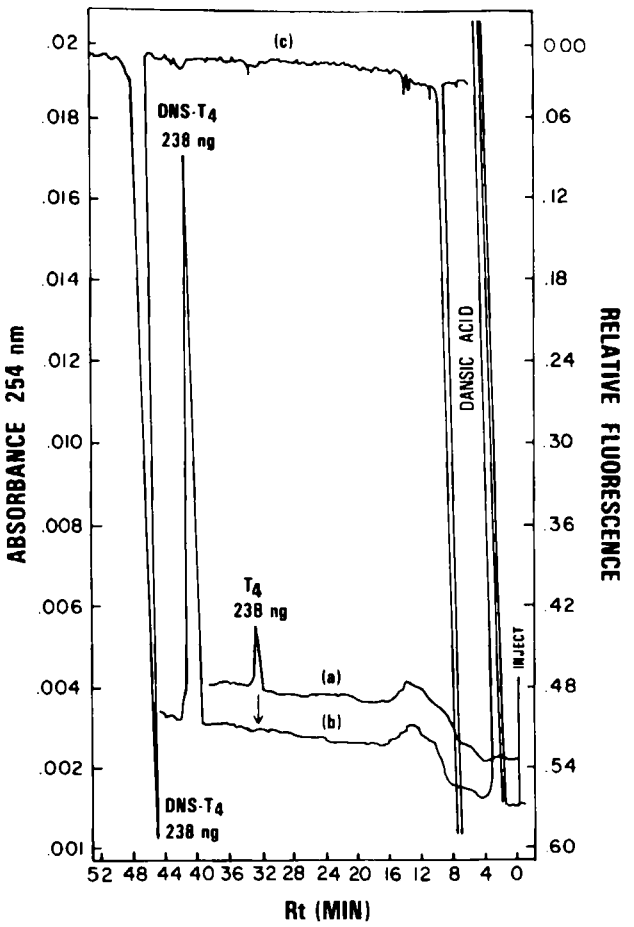

Figure 2

Dansylation of T4 (3,3',5,5' tetraiodothyronine). Chromatogram (a) reflects a 238 ng T4 non-derivatized compared to a dansyl derivative T4 (b). The absorbance response was compared to fluorescence as shown in (b) versus (c). The samples were applied to a reverse phase system using a convex gradient (No. 5) of changing solvent. A 60 minute convex gradient program (No. 5) beginning at ACN/1% Glacial (15:85, v/v) and ending with ACN/1% Glacial (50:50, v/v) at a flow rate of 1.5 mil/min.

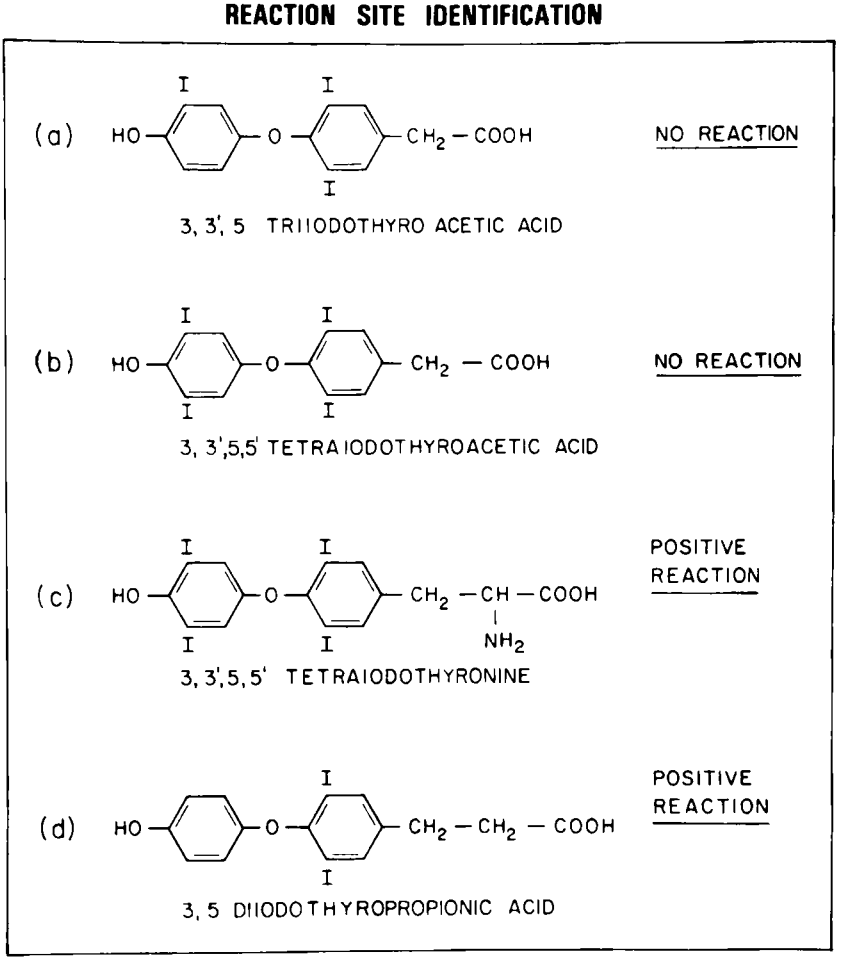

Figure 3

Reaction Site Identification - Group site reactions were identified after each standard was dansylated and chromatographed.

specific for the characterization of the reagents (dansyl chloride and iodoamino acid) and product formation. Because the product fluoresces, it was verified by post column detection with the Fluoro-Monitor. As shown in Figure 2, a decrease of the non-dansylated iodoamino acid peak with a corresponding formation of a new peak verified by fluorescence was classified as a positive reaction (see Figure 3). In the figure, the triiodo and tetraiodo-thyroacetic acid did not react with dansyl chloride, presumably due to steric hindrance from the iodide groups at positions 3' and 5', Dansyl reacted with the primary amino group in all cases. The results of the study are summarized in Figure 4.

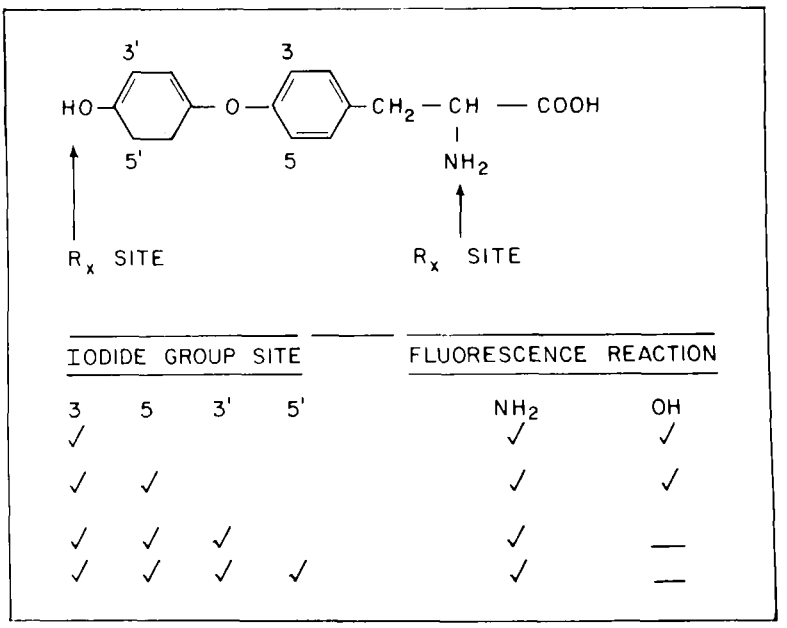

Figure 4

Derivative formation with 1-dimethylaminonaphtalene-5-Sulfonyl Chloride (Dansyl-Cl).

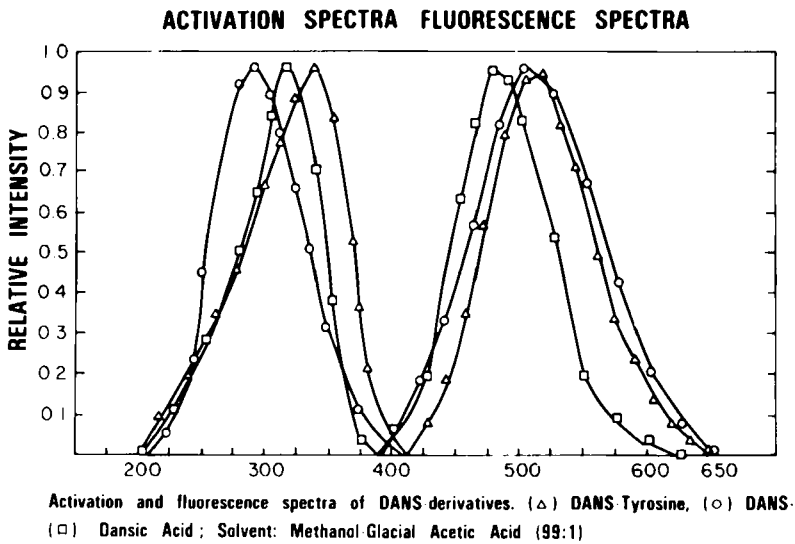

Figure 5

Activation and fluorescence of Dansyl-derivatives. (Δ) Dansyl-Thyrosine, (o) Dansyl-T4, (□) Dansic Acid; Solvent: Methanol-Glacial Acetic Acid (99:1).

The spectral properties of three dansyl derivatives were compared. The spectras for T4, tyrosine and dansic acid are shown in Figure 5. It was noted that the fluorescence excitation of dansyl T4 was shorter than tyrosine or dansic acid. Because spectral properties of dansyl derivatives are profoundly changed by solvents, absorption to active surfaces and pH, the results shown in the figure are in agreement with Siler's (13) results concerning amino, phenol and amino-phenol derivatives. The interesting point is that fluorescence excitation and emission maxima of compounds with primary amino groups are at shorter wavelengths than those with phenol or amino-phenol groups.

Linearity And Sensitivity

Thyroxine (T4) was reacted and chromatographed as described in methods. The derivative was found to be stable for several days and the concentration versus fluorescence response was linear in the picomole as well as femtomole range. Concentrations between 10 picomoles to 100 femtomoles of Dansyl T4 were directly compared to relative fluorescence response. The regression equation of the aqueous standards was $y = 7.5 x + 0.08$ with a coefficient of determination of $R^2 = 0.99$.

CONCLUSIONS

The use of pre-column derivatization will improve the sensitivity of the iodoamino acids with measurements in the picomole to femtomole range. The proposed method seems to be promising for the selective detection of iodoamino acids by fluorescence a more sensitive technique when compared to ultraviolet detection.

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FOOTNOTES

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