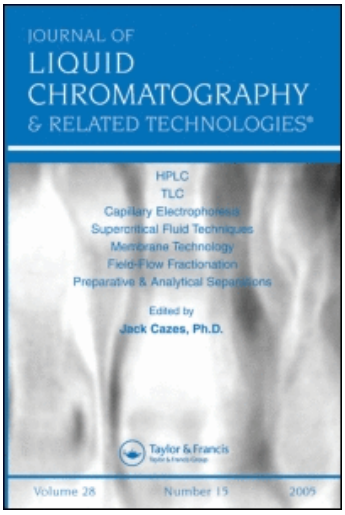

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Use of an Optimum Wavelength to Detect Glutamic Acid and its Metabolites in Human Serum by Reversed-Phase HPLC

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USE OF AN OPTIMUM WAVELENGTH TO DETECT GLUTAMIC ACID AND ITS METABOLITES IN HUMAN SERUM BY REVERSED-PHASE HPLC

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ABSTRACT

Dansylated glutamic acid, glutamine and γ-amino butyric acid (GABA) show maximum absorption at 221 nm. Using this wavelength, the detection limits for dansylated amino acids studied by reversed-phase HPLC are similar to those reported by fluorescence. This technique was used to look for the presence of glutamic acid and its metabolites in human serum. Glutamic acid and glutamine were present in significant amounts and their levels were 2.5 and 6.1 nmoles/ml respetively, while GABA was present in trace amounts, less than 0.3 nmoles/ml.

INTRODUCTION

Dansylated amino acids generally have been detected by absorption at 254 nm. However, as mentioned by Ross (1) in his review article, by using an ultraviolet (UV) wavelength of maximum absorption rather than the arbitrary selection of 254 nm, this technique would be as sensitive as spectrofluorometric detection. Hence, with the availability of variable wavelength UV monitors in conjunction with HPLC equipment, it is possible to utilize the wavelength of maximum absorption to detect dansylated amino acids.

Reversed-phase HPLC may also prove to be a valuable tool in the study of pathological states where abnormalities in amino acid metabolism are involved. Glutamic acid is one such example. Glutamic acid levels have

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been shown to rise in multiple sclerosis (2). GABA, which results from the decarboxylation of glutamic acid, is an inhibitory neurotransmitter in the central nervous system, and has been implicated in schizophrenia, Huntington's disease, Parkinson's disease, and epilepsy (3-6). The biological significance of GABA has been recently reviewed by Holdiness (7). Glutamine, the other major metabolite of glutamic acid, has been shown recently to be of therapeutic value in the treatment of peptic ulcers (8) and is one of the main storage forms of nitrogen in the body.

In view of the significance of these metabolites, we have developed a method for their determination by reversed-phase HPLC with UV absorption at an optimum wavelength.

EXPERIMENTAL

Instrumentation

The HPLC apparatus included a Beckman Model 332 gradient liquid chromatograph, a Beckman 210 sample injection valve with a 20 µl loop, and an LDC UV variable wavelength detector. The chromatographic column was an Altex Ultrasphere reversed-phase ODS-5 (250 x 4.6 mm). The mobile phase was a gradient of 100 mM potassium dihydrogen phosphate pH 2.1 with 0-50% acetonitrile. The flow rate was 1 ml/min at room temperature. A variable wavelength Spectrophotometer (Shimadzu UV-3000) recorder was used to determine the UV absorption spectra of dansylated amino acids. Reagents

Reagents

Dansylated amino acids were obtained from Sigma (St. Louis, MO, USA). Radiolabelled amino acids were obtained from New England Nuclear (Boston, MA, USA). Acetone and acetonitrile were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Potassium dihydrogen phosphate was obtained from Gallard-Schlesinger Chemical Corp. (Carle Place, NY, U.S.A.). Phosphoric acid was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Dansyl chloride for derivatization was prepared fresh daily by diluting the

stock solution (10% in acetone obtained from Pierce, Rockford, IL, U.S.A.) with acetone to yield a final concentration of 0.25%. Sodium bicarbonate buffer (100 mM, pH 9.5) was passed through a 0.45-μm filter (Millipore).

Dansylation

The amino acids were dansylated as described previously (9). For dansylation of amino acids in human serum, 20 μl of serum was mixed with 80 μl of acetonitrile. The precipitate was removed by centrifugation and supernatant was evaporated under a stream of nitrogen. To the residue was added 50 μl of bicarbonate buffer, pH 9.5, and 100 μl of dansyl chloride solution (0.25% in acetone) in a PTFE lined capped sample vial. After incubation at 70°C for 15 min in a water bath, the vial was removed, cooled in ice for about five min, and 20 μl injected into the column.

RESULTS AND DISCUSSION

Figure 1 shows the UV absorption spectrum of dansylated glutamic acid. It is evident that the maximum absorption is at 221 nm. Absorption at 254 nm which is generally used for detection of dansylated amino acids is not significant. Absorption at 280 nm is more than at 254 nm but it is about one seventh that seen at 221 nm. Other dansylated amino acids showed an identical absorption spectra. The presence of acetonitrile used in the mobile phase does not affect the UV absorption maxima of dansylated amino acids.

Figure 2 shows the separation and detection of dansylated glutamic acid, glutamine and GABA at 221 nm . Thirteen amino acids were tested and found not to interfere with the elution of glutamic acid and its metabolites. The amino acids were arginine, lysine, serine, aspartate, threonine, glycine, alanine, valine, tyrosine, methionine, phenylalanine, isoleucine and leucine. 10-20 picomoloes of each amino acid could be detected easily and is well above background at 0.02 absorption units full scale (AUFS). The reported detection levels for dansylated amino acids by fluorescence are the same (10).

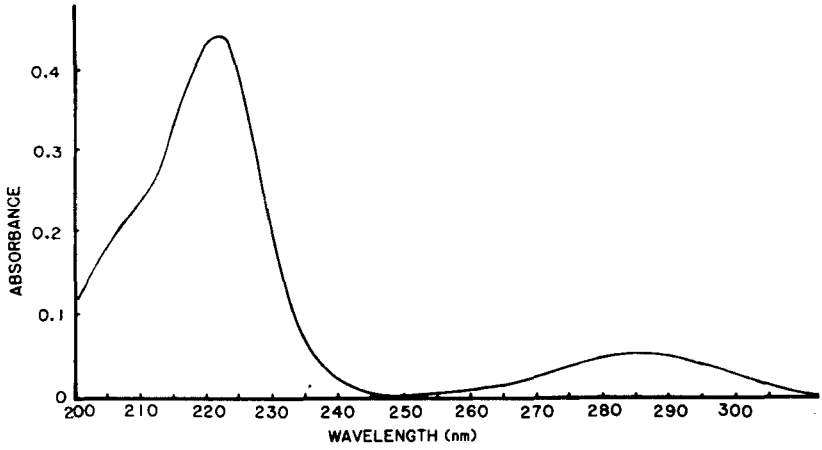

Figure 1. UV absorption spectrum of dansylated glutamic acid. The dansylated amino acid (13 nmoles) was dissolved in 0.1 M phosphate buffer, pH 2.1 and scanned with a chart speed of 2 cm/min at 1.0 AUFS.

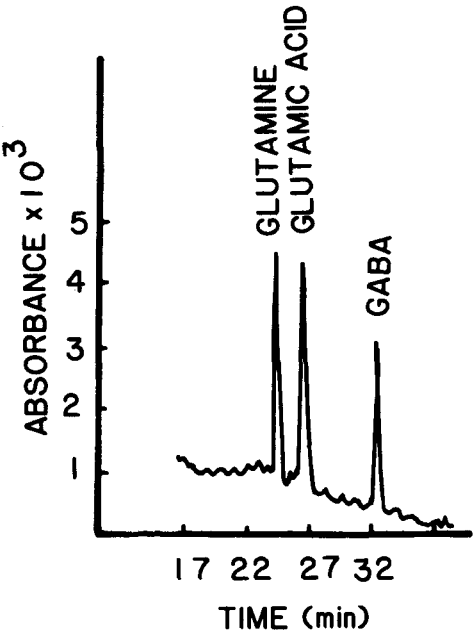

Figure 2. Chromatogram showing the separation of dansylated glutamic acid, glutamine and GABA. The UV detection of dansylated acids (17 pmoles) was at 221 nm (0.02 AUFS). 0.1 M sodium phosphate buffer, pH 2.1 (A) and acetonitrile (B) were used for elution. The composition of the gradient was as follows: 100% A, 3 minμ 0% to 10% B, 5 minμ 10% to 40% B, 30 minμ 50% B,10 minμ 50% to 0% B, 5 min.

In order to see if the procedure would be useful in biological samples, amino acids in serum were dansylated and chromatographed with detection at 221 nm (0.5 AUFS). GABA was present in trace amounts and constituted less than 0.3 nmoles/min, while glutamic acid and glutamine were present at the levels of 2.5 and 6.1 nmoles/ml respectively. The position of these amino acids was confirmed by the use of internal standards. Other amino acids were also present and resolved well from glutamic acid and its metabolites. As studied with radiolabelled amino acids, dansylation of each amino acid was more than 90% and there was no evidence of side product formation (data not shown).

In summary, we have described the usefulness of reversed-phase HPLC coupled with UV absorption at an optimum wavelength for the detection of dansylated amino acids in biological samples. The determination at 221 nm is is at least 20 times more sensitive than determination at 254 nm.

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