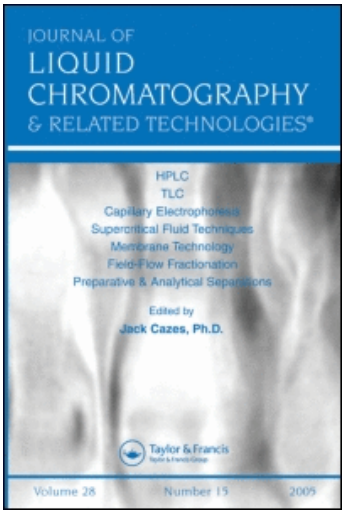

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Ion Exchanger Liquid Chromatography of N-Acetylaspartic Acid and Some N-Acetylaspartyl Peptides

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To cite this Article Lenda, Karel(1981) 'Ion Exchanger Liquid Chromatography of N-Acetylaspartic Acid and Some N-Acetylaspartyl Peptides', Journal of Liquid Chromatography & Related Technologies, 4: 5, 863 — 869
To link to this Article: DOI: 10.1080/01483918108059980
URL: http://dx.doi.org/10.1080/01483918108059980

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(5), 863-869 (1981)

ION EXCHANGER LIQUID CHROMATOGRAPHY OF N-ACETYLASPARTIC ACID AND SOME N-ACETYLASPARTYL PEPTIDES

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ABSTRACT

A high performance liquid chromatographic (HPLC) procedure for the rapid separation of N-acetylaspartic acid, N-acetyl-aspartyl-glutamic acid and N-acetylaspartyl-glutamyl-aspartic acid is described. The procedure utilizes a pellicular ion-exchange column support, and ionic strength gradient with mobile phase solutions buffered to pH 5.0 and a UV detector operated at 210 nm. Reproducibility and quantitative capabilities are also discussed. The method has been used for a tentative estimation of N-acetylaspartic acid and N-acetylaspartyl peptides in a rat brain synaptosomal extract.

INTRODUCTION

N-acetylaspartic acid (Ac-Asp) is present in brain in a concentration of 5-6 μmol/g of brain (wet weight) (1). Moreover, Ac-Asp is metabolically active (2-6). The formation of N-substituted acetylaspartyl peptides and acetylaspartyl-monoamine complexes has been shown in brain both in vitro and in vivo experiments (5-10). For determination of N-acetylaspartyl peptides formed in brain from N-acetyl (U-^{14}C) L Asp various chromatographic methods have been used, followed by sequence determination of the purified peptides (2-10).

Here is described a procedure for the direct determination of Ac-Asp, N-acetylaspartyl-glutamic acid (Ac-Asp-Glu) and N-acetyl-aspartyl-glutamyl-aspartic acid by HPLC.

EXPERIMENTAL

Apparatus

A Spectra-Physics high-performance liquid chromatograph model 8000, equipped with a Schoeffel 770 variable wavelength detector operated at 210 nm was used throughout this work. The samples were introduced by means of a 10 µl loop autoinjector (Valco model CV-6-UHPe-N60). A computing integrator system (Supergrator, CSI, Austin, TX, U.S.A.) was used for peak height measurement and data treatment.

The column (50 cm x 2 mm I.D., 316 stainless steel) was dry packed with Vydec SC Anion exchanger, mean particle size 37 um (The Separation Group, Hesperia, CA, U.S.A.).

Samples and reagents

N-acetylaspartic acid was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. N-acetylaspartyl-glutamic acid was prepared by Jan Böler, Rikshospitalet, Norway and N-acetylaspartyl-glutamyl-aspartic acid was a gift from Professors L. Moroder and E. Wunsch, Max-Planck Institut für Biochemie, Martinsried, W. Germany.

Ac-Asp and the peptides were dried in a vacuum over silica before use. Stock solutions containing 10 mg/ml were prepared by dissolving the compounds in 0.01 M HCl and they were stored at -20°C. Working solutions were freshly prepared for each experiment by diluting stock solutions with water. All other chemicals were of analytical reagent grade and were used without further purification.

Mobile phase

Mobile phase A contained 0.1 M potassium dihydrogen phosphate. Mobile phase B contained 0.1 M potassium dihydrogen phosphate to

which potassium chloride was added to give a final ionic strength of 0.5. The mobile phase solutions were adjusted to pH 3.0 ; 5.0 and 7.0, respectively by addition of phosphoric acid or potassium hydroxide. The solutions were passed through a 0.45 μm filter (Millipore Corp., Belford, MA, U.S.A.) and saturated with helium during the experiments. The fraction of B mobile phase increased linear by 2 ; 4 ; 6 or 8 % per min, from a starting value of 0 % at a flow rate of ml/min at room temperature (22°C).

RESULTS & DISCUSSION

Several gradient elution programs using different pH values were attempted to define optimal conditions (Table 1). The capacity factor k' was calculated as the ratio of the corrected (to

TABLE 1

Capacity Factors (k') and Peak Asymmetry (AFL) Isocratic and Gradient (2 %; 4 %; 6 %: and 8 % B Mobile Phase per min, resp.). Elution at pH 3.0 ; 5.0 and 7.0, resp., n=3 at each run. Sample: Ac-Asp (51 nmol), Ac-Asp-Glu (27 nmol) and Ac-Asp-Glu-Asp (19 nmol).

pH	gradient % B/min	Ac-Asp k'	Ac-Asp AFL	Ac-Asp-Glu k'	Ac-Asp-Glu AFL	Ac-Asp-Glu-Asp k'	Ac-Asp-Glu-Asp AFL
	-	1.88	1.00	7.32	1.00	+	+
	2	1.83	1.16	7.24	2.30	9.77	1.24
3.0	4	1.77	1.06	6.92	2.03	8.49	1.13
	6	1.49	1.06	6.30	1.75	7.74	1.10
	8	1.37	1.05	5.20	1.22	7.08	1.11
	-	19.80	1.00	+	+	+	+
	2	9.51	1.30	27.84	1.31	38.68	1.11
5.0	4	7.99	1.35	18.67	1.27	25.27	1.26
	6	7.61	1.20	15.30	1.21	19.73	1.05
	8	7.18	1.20	13.82	1.35	17.61	1.09
	-	1.12	1.00	+	+	+	+
	2	1.08	1.26	12.00	1.03	27.97	1.04
7.0	4	1.07	1.40	10.68	1.38	19.83	1.21
	6	1.05	1.28	9.51	1.24	16.13	1.22
	8	1.03	1.22	8.97	1.31	14.14	1.26

+Value not listed due to broad peaks.

the void volume) retention times t'_R to the retention time of the unretained samples. Peak asymmetry (ASF) was defined as a perpendicular dropped from the peak maximum to the baseline and the ratio of the larger to the smaller baseline segments between the tangents drawn through the points of inflection is calculated (11).

Best results were obtained using a mobile phase at pH 5.0 and a linear gradient of B increasing by 6 % per min. Thus, Ac-Asp is clearly separated from the void volume and the ASF for the peptides were < 1.20. The base line drift, expressed as a ratio of Ac-Asp-Glu-Asp peak height (10 nmol) to the base line deviation at the retention time, is 1.37 $\pm$ 2.1 % (n=5) under the employed conditions.

A sufficiently high separation efficiency is obtained using a pellicular ion-exchanger, which has the advantage of a very low pressure drop ($\sim$ 55 bar by 2 ml/min flow rate) over the column. The rapid mass transport using pellicular support is advantagenous to achieve a rapid reequilibration of the column after the run.

A typical separation of the components under study is shown in Fig. 1.

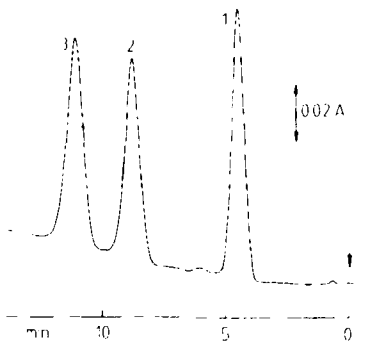

FIGURE 1

Chromatogram (x) of a test mixture of Ac-Asp (1), Ac-Asp-Glu (2) and Ac-Asp-Glu-Asp (3), (102; 54; 37 nmol, resp.). Flow rate 2 ml/min, gradient (y) 6 % B mobile phase/min.

No impurities in the peptides have been observed using this technique. A minor impurity in the Ac-Asp preparate is eluted with a retention time of 6.03 min (pH = 5.0, 6 % B mobile phase per min).

Linearity of peak height as a function of the amount determined is found in the range 600 pmol - 220 nmol of Ac-Asp ; 900 pmol - 250 nmol of Ac-Asp-Glu and Ac-Asp-Glu-Asp with correlation factors 0.99294 for Ac-Asp ; 0.99370 for Ac-Asp-Glu and 0.99941 for Ac-Asp-Glu-Asp (n=8). The detection limit expressed as 3x signal to noise ratio is 500 pmol for Ac-Asp and 800 pmol for Ac-Asp-Glu and Ac-Asp-Glu-Asp.

It should be noted that with this procedure the day to day variation of the retention times expressed as coefficient of variation, is < 2 %. The coefficient of variation for the factors in external standard calibration procedure is < 5 % during weekly runs.

To demonstrate the applicability of this method the content from rat brain synaptosomes was analysed. The proteins from 300 µl of synaptosomal extract (12) (20 mg brain tissue per ml) were precipitated with ice cold ethanol (70 % final concentration). 600 µl of supernatant, after centrifugation at 4000 rpm for 4 min, was evaportated to dryness and reconstituted in 50 µl of the mobile phase A. 10 µl was introduced on the column. The resulting chromatogram is shown in Fig. 2.

The retention times for peaks A, B and C, respectively, in Fig. 2 have the same retention times as the compounds 1, 2 and 3, respectively, in Fig. 1. When the pure compounds were mixed with the synaptosomal extract, no new peaks were obtained. Further proof of identity is under progress.

In conclusion the described method is especially attractive for the rapid and complete separation of Ac-Asp, Ac-Asp-Glu and Ac-Asp-Glu-Asp in 15 min. The method gives reproducible results and no radioactive and derivatized samples are needed for detection. The procedure is well suited for a control of sample

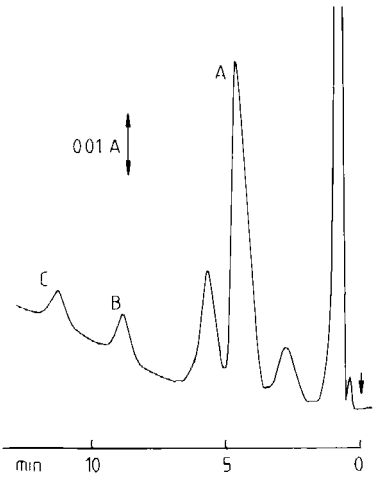

FIGURE 2

Analyses of rat brain synaptosomes. Chromatographic conditions as in Fig. 1.

purity. The application of the methodology to biological materials is under study.

ACKNOWLEDGEMENT

The author is grateful to Dr. E. Kvamme and Dr. G. Svenneby for valuable discussions, to the A. Jahre foundation for financial support and to the Royal Norwegian Council for Scientific and Industrial Research for a postdoctoral fellowship. The kind gifts of Ac-Asp-Glu from J. Böler of the Institute of Pediatric Research, Rikshospitalet, Oslo, Norway and Ac-Asp-Glu-Asp from Professors L. Moroder and E. Wünsch, Max Planck Institut für Biochemie, Martinsried, West Germany are appreciated.

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