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Note

Ion-exchange separation and pK_a determinations of the α- and β-isomers of N-acetyl-L-aspartyl-L-glutamic acid

Correlation of chromatographic properties with pK_a values

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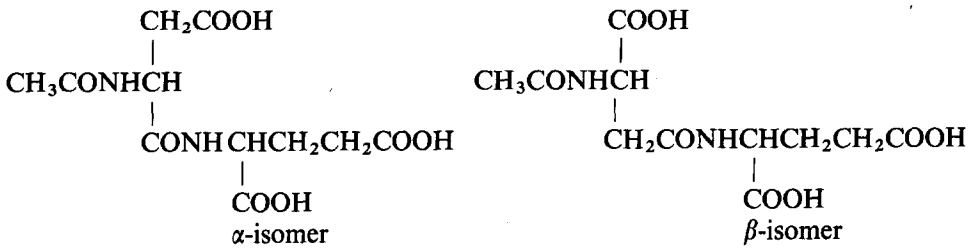
We have been interested in the individual properties of the α- and β-isomers of N-acetyl-L-aspartyl-L-glutamic acid (NA-Asp-Glu), which have the following formulae:



NA-Asp-Glu is an anti-allergic drug[1], and is used in nasal and eye therapy to treat inflammation due to conjunctivitis and rhinitis. Despite its peptide like appearance, NA-Asp-Glu must be regarded as a tricarboxylic acid (H_3A) rather than a dipeptide, because of the acetylation of the N-terminus and the chromatographic properties of the isomers should be those of weak organic acids.

Separations of carboxylic acids by high-performance liquid chromatography have been reviewed[2], and separations of natural occurring N-acetylaspartic acid and N-acetylaspartyl peptides by ion-exchange chromatography have also been studied[3–5].

Extensive studies have been reported on the relationship between the pK_a values and the chromatographic properties of ionogenic compounds and on the use of chromatography for the determination of pK_a values[6–11]. Classically, the retention of ionogenic solutes can be controlled by manipulation of the pH near their pK_a values, and the sigmoidal curves obtained may afford pK_a values from chromatographic results and *vice versa*. However, with a short-chain tricarboxylic acid such as NA-

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Asp-Glu, where the three pK_a values are expected to be very close, the sigmoidal obtained curves look like that of monoprotic acids and the pK_a values cannot be calculated[9].

Computer-assisted titration procedures are now widely used[12,13]. We report here the determination of the pK_a values of the α- and β-isomers of NA-Asp-Glu by conventional titration methods, and comparison of the experimental points with simulated curves obtained with an IBM PC computer and a software developed in our laboratory[14]. The separation of the two isomers is readily obtained by anion-exchange chromatography and we show that the resolution between the two peaks fit well with the net charge difference of the two analytes, calculated from pK_a data.

The agreement of these two independent experiments shows the pertinence of the computer-assisted titration procedure and the adequacy of ion-exchange chromatography for the separation of very similar ionogenic compounds.

EXPERIMENTAL

Reagents

N-Acetyl-α-L-aspartyl-L-glutamic acid (NA-αAsp-Glu) and N-acetyl-β-L-aspartyl-L-glutamic acid (NA-βAsp-Glu) were synthesized and purified by Transphyto (Clermont-Ferrand, France). Pyroglutamic acid (Pyro.Glu) and N-acetyl-L-aspartic acid (NA-Asp) were obtained from Serva (Heidelberg, F.R.G.) and ammonium dihydrogenphosphate, ammonia, orthophosphoric acid and hydrochloric acid from Prolabo (Paris, France). Water was deionized and quartz distilled (Quartex, Paris, France).

Titration curves

Titrations were performed at a ionic strength of 0.1 *M* (sodium chloride). A 20-ml volume of a $2 \cdot 10^{-3}$ *M* solution of NA-αAsp-Glu or NA-βAsp-Glu as their trianionic salts were neutralized with 0.125 *M* hydrochloric acid. pH was measured with a glass electrode (TB 10), a calomel reference electrode (C 10) and a pH meter (TS 80) (Tacussel, lyon, France). A delay of a few minutes was allowed after each addition of the hydrochloric acid, and about 50 points were obtained from pH *ca.* 8 to 2.23. Two different runs were carried out for each isomer to check for reproducibility.

Simulated titration curves and their agreement with the experimental points were realized with the TOT software conceived by Rosset *et al.*[14], working on an IBM PC.

Chromatography

Chromatography was carried out using a Waters liquid chromatograph with an UV detector operating at 210 nm. The column was 15 × 0.46 cm I.D., filled with Partisil 10 SAX (Whatman, Clifton, NJ, U.S.A.), which is a strong anion-exchange silica-based material.

Mobile phases were aqueous solutions of ammonium dihydrogenphosphate of various concentration (0.05–0.1 *M*). The pH was adjusted by addition of ammonia or orthophosphoric acid and continuously checked with a glass electrode (TCBC 102/HS) (Tacussel), located on-line after the detector, in a 1-ml volume vessel.

Post-run calculations

Post-run calculations were performed with a Macintosh Plus (Apple, Cupertino, CA, U.S.A.) with TK!Solver (Software Arts, Wellesley, MA, U.S.A.) and Cricket Graph (Cricket Software, Philadelphia, PA, U.S.A.) software.

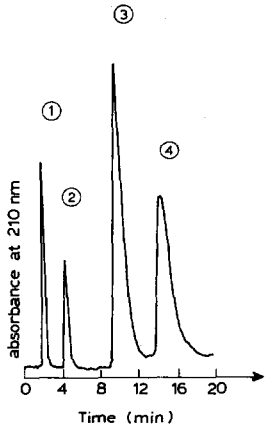
RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 1; the α- and β-isomers of NA-Asp-Glu are well separated from each other and from pyroglutamic acid and N-acetyl-aspartic acid, which may be present as impurities in commercial NA-Asp-Glu. The variation of the retention times with the inverse of the buffer concentration (Fig. 2) is typical of the variation of anionic species bearing one charge, showing that at this pH values (4), the α- and β-isomers may be regarded as being simple ions bearing one charge, despite their peptidic bonds and tricarboxylic nature.

The capacity factors and resolution (R_s) of the two peaks are reported in Table I; R_s passes through a maximum at pH 3.36.

Titration curves of the trianionic salts of the α- and β-isomers of NA-Asp-Glu are shown in Fig. 3; the points represent the experimental results and the curves were calculated on an IBM PC computer using the TOT software[14], giving the pK_a values of the solutes to within 0.05 unit (Table II).

The pK_a values of the α- and β-isomers are similar, but the differences are relevant and may be regarded as the causes of the chromatographic separation of the solutes. It can be seen that the pK_a values for the α-isomer are greater than the corresponding values for the β-isomer; the α-isomer seems less acidic than the β-isomer and is less retained.



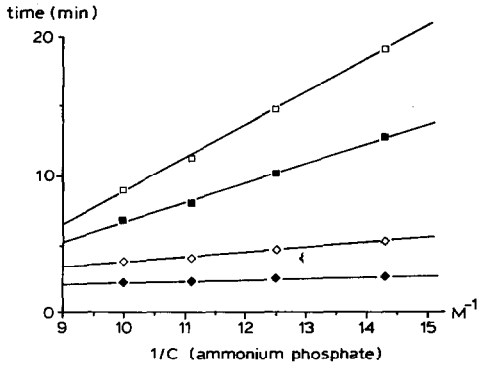


Fig. 1. Separation of α- and β-isomers of NA-Asp-Glu by anion-exchange chromatography. Column, 15 × 0.46 cm I.D., filled with Partisil SAX; mobile phase, 0.08 *M* ammonium phosphate (pH 4); flow-rate, 1.5 ml/min; detection, UV (210 nm). Peaks: 1 = Pyro-Glu; 2 = NA-Asp; 3 = NA-αAsp-Glu; 4 = NA-βAsp-Glu.

Fig. 2. Variation of retention time of solutes *versus* concentration of buffer. Column, 15 × 0.46 cm I.D., filled with Partisil SAX; mobile phases, aqueous solutions of various concentrations of ammonium phosphate (pH 4); flow-rate, 1.5 ml/min; detection, UV (210 nm). ◆, Pyro-Glu; ◇, NA-Asp; ■, NA-αAsp-Glu; □, NA-βAsp-Glu.

TABLE I

CAPACITY FACTORS (k')* OF THE α- AND β-ISOMERS OF NA-Asp-Glu AND RESOLUTION (R_s) BETWEEN THE TWO PEAKS AS A FUNCTION OF pH

pH	*k'*		R_s**
	NA-αAsp-Glu	*NA-βAsp-Glu*	
2.73	0.05	0.25	1.005
3.03	0.31	0.67	1.429
3.12	0.41	0.92	1.773
3.36	0.91	1.78	2.090
3.50	1.25	2.25	2.037
3.75	1.79	3.01	2.021
3.98	2.60	3.87	1.720
4.21	3.71	4.81	1.244
4.28	4.00	5.02	1.138
4.33	4.32	5.55	1.244
4.44	4.83	5.56	0.741
4.90	7.00	7.00	0

* Calculated using $k' = (t_R - t_0)/t_0$.

** Calculated using $R_s = 0.25\sqrt{N}\{[(\alpha - 1)/\alpha] \cdot [(k'_B/(1 + k'_B)]\}$, with $N = 700$, determined on the β-isomer peak.

The resolution between the α- and β-isomers must depend on the pK_a values also, as it must be correlated with the net charge difference of the solutes for a given pH. It is easy to show that the net charge, z, of a tricarboxylic acid, H_3A, is a function of the pH value:

$$z = \frac{\sum_{i=1}^{3}\left(i \prod_{j=0}^{i-1}[K_{(3-j)}] \cdot 10^{ipH}\right)}{1 + \sum_{i=1}^{3}\left(\prod_{j=0}^{i-1}[K_{(3-j)}] \cdot 10^{ipH}\right)}$$

We can calculate the net charge difference, $|z(\beta) - z(\alpha)|$, of the solutes for various pH values and compare the calculated resulting curve with the variations of

TABLE II

IONIZATION CONSTANTS OF NA-αAsp-Glu AND NA-βAsp-Glu

Determined at an ionic strength of 0.1 *M* by agreement of simulated titration curves with experimental points.

pK	*NA-αAsp-Glu*	*NA-βAsp-Glu*	*Ionic form*
pK_3	3.75	3.40	H_3A/H_2A^-
pK_2	4.15	3.95	H_2A^-/HA^{2-}
pK_1	5.20	5.00	HA^{2-}/A^{3-}

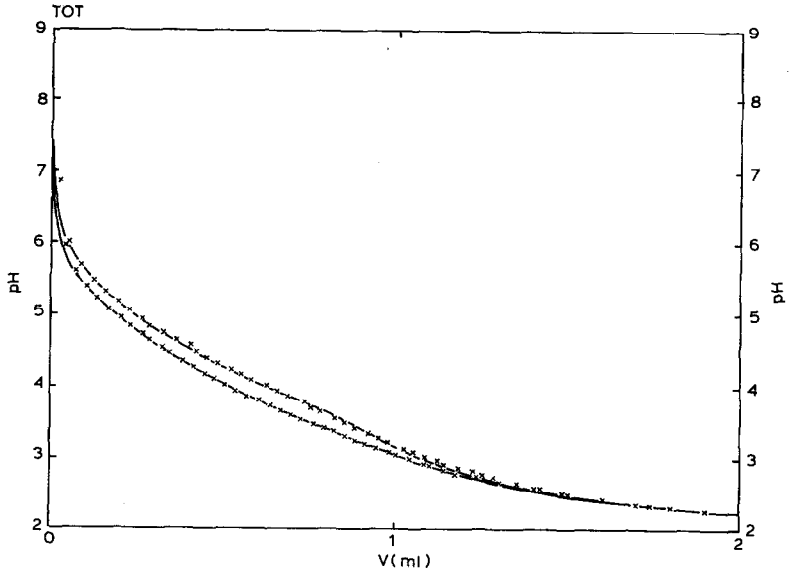


Fig. 3. Experimental points and simulated curves of the titration of NA-αAsp-Glu and NA-βAsp-Glu, as their trianionic salts, with HCl. Upper curve, NA-αAsp-Glu; lower curve, NA-βAsp-Glu.

the resolution between the peaks for the α- and β-isomers (Fig. 4). The maxima of the two curves are separated by about 0.25 pH unit, but the correlation between the resolution and the net charge difference for the two solutes is good. Hence the α- and β-isomers of NA-Asp-Glu must be regarded as very simple acids being resolved by ion-exchange chromatography without uncontrolled effects of the two peptidic linkages that exist in each molecule.

The methods proposed by various workers[6–11] for the determination of the pK_a

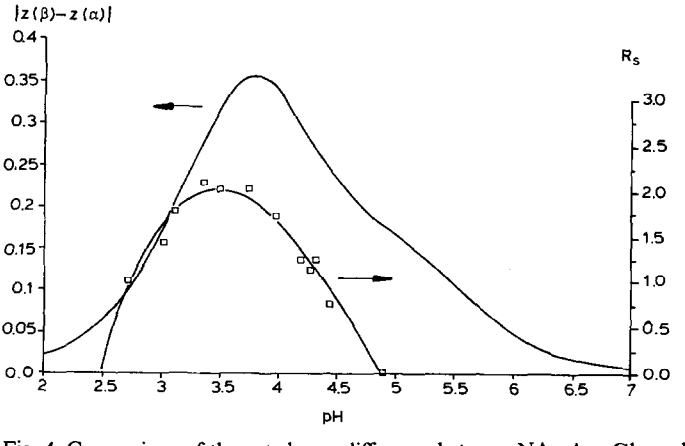


Fig. 4. Comparison of the net charge difference between NA-αAsp-Glu and NA-βAsp-Glu with the resolution of the chromatographic peaks. The net charge difference curve is calculated from the pK_a data. Chromatographic conditions as in Table I.

values of acids and bases from their k' *versus* pH dependences would certainly not work for the NA-Asp-Glu isomers, because the three pK_a values for each compound are too close. In such a situation, the simulated titration curve method is more efficient, the agreement of the calculated values with the chromatographic properties being a good verification.

CONCLUSION

The separation of the α- and β-isomers of N-acetylaspartylglutamic acid by conventional ion-exchange chromatography is very simple and gives good results without needing a very efficient column (ours had only 700 plates). The determination of close pK_a values with the software developed in our laboratory seems to be reliable and versatile and the use of the net charge difference calculated from these results is a rapid means of optimizing the resolution in ion-exchange chromatography.

REFERENCES

1 THEA (Thérapeutique et Application) S.A., *Fr. Pat.*, 2 546 407 (1983).
2 R. Schwarzenbach, *J. Chromatogr.*, 251 (1982) 339.
3 K. Lenda, *J. Liq. Chromatogr.*, 4 (1981) 863.
4 M. Miyake, H. Morino, M. Mutsuhiko and Y. Kakimoto, *Clin. Chim. Acta*, 120 (1982) 119.
5 E. J. Olson and L. H. Wade, *Arch. Biochem. Biopohys.*, 119 (1967) 22.
6 O. Samuelson, *Ion Exchange Separations in Analytical Chemistry*, Wiley, New York, 1963.
7 Z. J. Zmrhal, J. G. Heathcote and R. J. Washington, in Z. Deyl, K. Macek and J. Janak (Editors), *Liquid Column Chromatography*, Elsevier, Amsterdam, 1975, Ch. 32.
8 B. L. Karger, J. N. Le Page and N. Tanaka, in Cs. Horváth (Editor), *HPLC, Advances and Perspectives*, Vol. I, Academic Press, New York, 1980, p. 113.
9 C. Horvath, W. Melander and I. Molnar, *Anal. Chem.*, 49 (1977) 142.
10 S. H. Unger and T. F. Feuerman, *J. Chromatogr.*, 176 (1979) 426.
11 K. Miyake, K. Okumura and H. Terada, *Chem. Pharm. Bull.*, 33 (1985) 76.
12 R. Rosset, D. Bauer and J. Desbarres, *Chimie Analytique et Microinformatique*, Masson, Paris, 1979.
13 A. P. Arnold, S. A. Daignault and D. L. Rabenstein, *Anal. Chem.*, 57 (1985) 1112.
14 R. Rosset, D. Bauer and J. Desbarres, *TOT Software*, ESPCI, Paris, 1986.