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Direct separation of amino acid enantiomers using a chiral crown ether stationary phase

Application to 2-amino-ω-phosphonoalkanoic acids

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ABSTRACT

The resolution of a series of 2-amino-ω-phosphonoalkanoic acid enantiomers using a crown ether chiral stationary phase is described. The method is applicable to other primary amino acid and shows some advantages over chiral derivatization with fluorometric detection. Optical isomers of under 0.5% may be quantified.

INTRODUCTION

The putative neurotransmitter(s) mediating fast excitatory impulses in the mammalian central nervous system are L-glutamate and (or) L-aspartate [1]. The excitatory amino acid receptors have been classified into four subtypes, two of which are the N-methyl-D-aspartate (NMDA) and the L-2-amino-4-phosphonobutanoic acid (L-AP4) receptors [2,3]. Structure-activity studies have established that the *R*-isomers of 2-amino-ω-phosphonoalkanoic acids block the NMDA receptor [4] while the *S*-isomers of some members of the series have activity as agonists or antagonists on the L-AP4 receptor [3]. The enantiospecific synthesis of neuro-active amino acids requires the quantification of enantiomeric purity, often when one enantiomer is present in a large excess of the other. This paper details a new method for the direct separation of the configurational isomers of members of the 2-amino-ω-phosphonoalkanoic acid series using a newly introduced chiral stationary phase (CSP); the method is applicable to other primary amino acids.

Methods used to assess enantiomeric purity of amino acids

Previous methods used for the separation of amino acid enantiomers have included ligand exchange chromatography [5] and achiral derivatization followed by resolution on a CSP, *e.g.* using a Pirkle-type CSP [6]. However, the determination of the optical purity of amino acids has predominantly relied on pre-column derivatization followed by separation using conventional reversed-phase chromatography.

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Derivatizing agents commonly used have included *o*-phthaldialdehyde with various chiral thiols [7–9], a chiral isothiocyanate [10] and a chiral chloroformate [11]. These methods, although often giving excellent results, require additional validation. It is important to establish not only that the chiral reagent is enantiomerically pure, but also that the derivatizing reaction does not induce any racemisation in either of the chiral centres of the diastereoisomer formed. Many of the diastereoisomer products tend to be very hydrophobic and thereby exhibit large retention volumes on reversed-phase media. Often elaborate gradient programming of the mobile phase is required to remove the products from the column. A more insidious handicap of the quantification of the enantiomeric ratios of amino acids by chiral derivatization is the realisation that the two diastereoisomers formed may exhibit dissimilar fluorescence efficiencies [7,8]. This means that only derivatives obtained from the same enantiomer may be compared. However, because of the general availability of racemates, differences in the fluorescence signal may be easily corrected for. It is interesting to note that difference between diastereoisomers in their absorptivities is much less pronounced and generally not detected on UV–VIS high-performance liquid chromatographic (HPLC) detectors.

The direct separation of enantiomers circumvents these difficulties provided neither the mobile phase nor the CSP induces racemisation or decomposition of the analyte. A new CSP (Crownpak, Daicel Chemical Industries) has become available which is able to resolve many molecules which contain a primary amino group proximate to a chiral centre. The newly introduced phase uses a chiral crown ether moiety as a chiral selector; discrimination between enantiomers relies on the formation of two diastereoisomeric inclusion complexes between the ammonium ion moiety of the amino acid and the chiral crown ether entity of the stationary phase. It is necessary to carry out chromatography in a highly acidic medium, not only to ensure the presence of the ammonium ion on the amino acid, but also to protonate the nearby α-carboxylic acid group, the electronegativity of which would otherwise inhibit the formation of the inclusion complex with the CSP [12].

EXPERIMENTAL

Chromatography

Analytical HPLC was performed using an LDC constaMetric III solvent pump and a spectroMonitor D UV–VIS detector. The wavelength of detection was 200 nm, the amino acids studied show no appreciable absorptivity above *ca.* 208 nm. The column was a 150 × 4 mm I.D. Crownpak CR(+) (5 μm) column (Daicel Chemical Industries, Tokyo, Japan). Injections were made using a Rheodyne 7125 injector (20 μl loop). Chromatograms were recorded on a Perkin-Elmer R-100 chart recorder and the peak areas were calculated using an Axxiom Chromatography Model 727 data station (Axxiom Chromatography, Calabasas, CA, U.S.A.). Mobile phase elution was performed isocratically using filtered (0.45 μm), degassed perchloric acid, diluted to obtain the required pH. No organic modifier was included in the mobile phase. The flow-rate was 0.4 ml/min. Temperature control of the column was effected by the use of a water jacket.

Reagents and materials

Water used in the mobile phase was purified by passage through a Milli-Q unit (Millipore, Bedford, MA, U.S.A.). The perchloric acid used was of analytical grade (BDH), Poole, U.K.). Concentrated perchloric acid (70%, 16.3 g) was diluted in water (total volume 1 l) to give a pH 1 solution, which could be diluted further to give a pH 2 solution. Perchloric acid is the acidifier of choice because of its transparency in the far UV region of the spectrum.

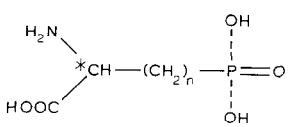
The 2-amino-ω-phosphonoalkanoic acid series (I–VII, Table I) was obtained from Tocris Neuramin (Buckhurst Hill, Essex, U.K.). With the exception of AP6 and AP8, the racemate together with at least one of the enantiomers was available. All amino acids were dissolved in the mobile phase to a concentration of 0.2–1.0 mM and passed through a 0.45-μm micropore filter prior to injection.

RESULTS

The enantiomeric separation of AP3–AP8, AP5-ene and Glu on the crown ether CSP is depicted in Table II and Fig. 1. With the exceptions of AP3 and AP5-ene, adequate resolution could be achieved using a pH 2 perchloric acid mobile phase. However to effect a baseline separation of the enantiomers of AP3 and AP5-ene it was necessary to change the mobile phase to a pH 1 solution of perchloric acid. In the manufacturers instruction manual the column is described as being stable from pH 1 to 9, however it also points out that "the lower pH results in good resolution but in a shorter column life". Our experience is that the column still performs satisfactorily after *ca.* 500 injections, of which maybe 50 were carried out at pH 1. The column was cooled to 3–4°C in all separations.

TABLE I

STRUCTURE OF PHOSPHONO AMINO ACIDS

Compound	Structure[a]	Name	Designation
			
I	$n = 1$	2-Amino-3-phosphonopropanoic acid	AP3
II	$n = 2$	2-Amino-4-phosphonobutanoic acid	AP4
III	$n = 3$	2-Amino-5-phosphonopentanoic acid	AP5
IV	$n = 4$	2-Amino-6-phosphonohexanoic acid	AP6
V	$n = 5$	2-Amino-7-phosphonoheptanoic acid	AP7
VI	$n = 6$	2-Amino-8-phosphonooctanoic acid	AP8
VII	$n = 3$	2-Amino-5-phosphonopent-4-enoic acid	AP5-ene

[a] The asterisk denotes the chiral centre.

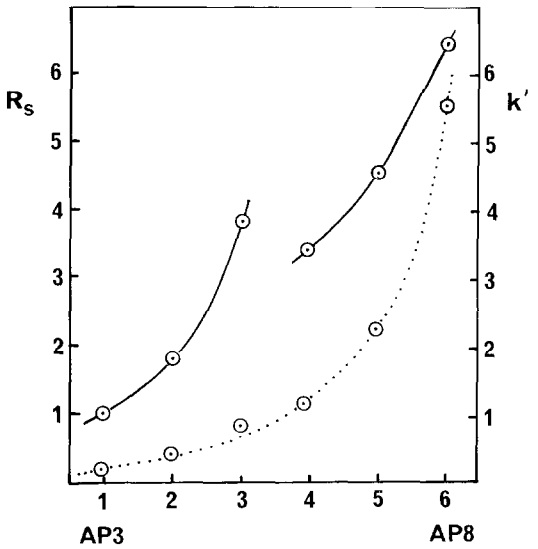


Fig. 1. Relationship between R_s (———), k'_s (· · · · · ·) and alkyl chain length (n) of 2-amino-ω-phosphonoalkanoic acids. Column, Crownpak CR(+); eluent, pH 2 perchloric acid; flow-rate, 0.4 ml/min; temperature, 4°C; detection, 200 nm.

TABLE II

INFLUENCE OF ALKYL CHAIN LENGTH ON THE ENANTIOSEPARATION OF 2-AMINO-ω-PHOSPHONOALKANOIC ACIDS

Column, Crownpak CR(+); mobile phase, perchloric acid; temperature, 3–4°C; k'_R, k'_S, capacity factors of the least and most retained isomers, respectively. R and S are the configurations at the amino acid chiral centre; α, separation factor; R_s, the resolution factor, calculated from: $R_s = \frac{t'_2 - t'_1}{\frac{1}{2}(w_2 + w_1)}$, where t'_i are the corrected retention times of the enantiomers and w_i are the widths of the peaks at their bases.

	pH 2				pH 1			
	k'_R	k'_S	α	R_S	k'_R	k'_S	α	R_S
AP5-ene	0.14	0.32	2.3	0.8	0.45	1.48	3.3	3.90
AP3	0.07	0.17	2.4	1.0	0.10	0.41	4.1	2.30
AP4	0.07	0.41	5.9	1.79				
AP5	0.14	0.86	6.1	3.82				
AP6[a]	0.24	1.14	4.8	3.42				
AP7	0.70	2.31	3.3	4.61				
AP8[a]	2.10	5.55	2.6	6.45				
Glu	0.28	1.76	6.3	2.93				

[a] Only racemate was available.

DISCUSSION

Retentions and resolutions

The value of the capacity factor of the second peak eluted (k'_s) and the resolution factor (R_s) for the series is presented in Fig. 1. The resolution factor is a better indicator of the quality of the enantioseparation than the separation factor α because of the very low retentions exhibited by the 2-amino phosphonoalkanoic acids on the crown ether CSP. The general improvement in resolution (Table II and Fig. 1) observed in ascending the series from AP3 to AP8 may principally arise as a consequence of the increasing size of the alkyl side chain of the amino acid. It has been pointed out that amino acids with the largest side groups generally exhibit the highest degree of chiral recognition on chiral crown ether phases [13]. The discontinuity in the general progression of the R_s values between AP5 and AP6 is most likely to be due to a conformational effect.

Resolution between enantiomer peaks is defined by the formula:

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{(\alpha - 1)}{\alpha} \cdot \frac{k'}{(1 + k')}$$

From this equation it may be seen that at low retentions, the resolution will be controlled by the capacity factor k'. The phosphono function is considerably more hydrophilic than a carboxylic acid group and so it is to be expected that glutamic acid isomers have greater retention on the Crownpak column and consequently exhibit better enantioseparation. In the resolution of *R* and *S* glutamic acid on the CR(+) column at pH 2 the capacity and the separation factors ($k'_R = 0.28$, $k'_S = 1.76$, $\alpha = 6.3$) are larger than observed in the phosphono analogue AP4 ($k'_R = 0.07$, $k'_S = 0.41$, $\alpha = 5.9$). The improvement in resolution is in spite of the fact that the phosphono group is larger than the carboxylic group (see above).

The presence of a double bond in AP5-ene ($\alpha = 2.3$ at pH 2) has the effect of diminishing the quality of the separation compared to the saturated analogue AP5 ($\alpha = 6.1$).

Order of elution

The designation of k'_R and k'_S as the most and the least retained enantiomers respectively was verified by chromatographing the racemate and one or both enantiomers. However for AP6 and AP8 only the racemate was available. The elution of the *R* isomer prior to the *S* isomer is the expected order of elution for almost all amino acids on the CR(+) column [12].

Sensitivity

Fig. 2 illustrates the enantioseparation of AP7. An enantiomeric impurity of 3% was found in this particular preparation of R-AP7. Using the crown ether phase we were able to quantify minor enantiomers of less than 1%; impurities down to 0.2% may also be possible. However the lack of a suitable chromophore in many amino compounds limits the sensitivity of direct resolution methods. It has been suggested the use of post-column derivatization will allow for the determination of enantiomeric impurities in amino acids down to about 0.01% (100 ppm) [14].

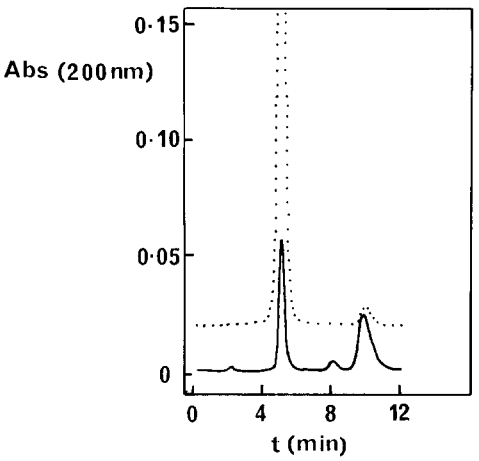


Fig. 2. Chromatograms of the separation of AP7 enantiomers on a Crownpak CR(+) column (150 × 4 mm I.D.). Eluent, pH 2 perchloric acid; flow-rate, 0.4 ml/min; temperature, 4°C; detection, 200 nm; (———), rac-AP7, (· · · · · ·), "*R*"-AP7.

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