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Note

Resolution of neuroexcitatory non-protein amino acid enantiomers by high-performance liquid chromatography utilising pre-column derivatisation with *o*-phthaldialdehyde chiral thiols

Application to ω-N-oxalyl diamino acids

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The ω-N-oxalyl derivatives of L-α,β-diaminopropanoic acid (L-β-ODAP, **1a**) and L-α,γ-diaminobutanoic acid (L-γ-ODAB, **2a**) are both natural products which were first isolated from the seeds of *Lathyrus* species[1–3], L-β-ODAP is believed to be the major causative agent of neurolathyrism, a crippling neurological disease[4–6]. The compound is a powerful convulsant[1] and is neuroexcitatory to central nervous system neurones[7–9], acting at the quisqualate and kainate receptors[8–10] [*i.e.* as distinct from N-methyl-D-aspartate (NMDA) receptors].

Pharmacological and biochemical interest in the longer chain L-ω-N-oxalyl derivatives of diamino acids, together with the possibility that the corresponding D isomers might act as important antagonists[11], prompted us to synthesise the D- and L-ω-N-oxalyl derivatives of diaminobutanoic acid (γ-ODAB, **2a,b**), ornithine (δ-OORN, **3a,b**) and lysine (ε-OLYS, **4a,b**) (see Fig. 1). The synthetic, pharmacological and biochemical properties of these compounds will be described elsewhere[12,13]. A knowledge of their optical purity was essential before a study of the pharmacological and biochemical activity could be undertaken, since L-β-ODAP activates quisqualate and kainate receptors to differing degrees and in a concentration-dependent manner[10], whereas D-β-ODAP is a weak antagonist at the NMDA receptor[14].

To date, optical purity has been ensured by the use of "optically pure" starting materials, optical rotation and rotary dispersion measurements. However, these methods are insensitive to contamination with small quantities of the minor enantiomer, either as a result of being induced by racemisation in the synthetic procedure, or being present in the "optically pure" starting materials.

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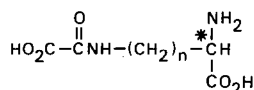


compound	n	optical configuration
1a	1	L
1b	1	D
2a	2	L
2b	2	D
3a	3	L
3b	3	D
4a	4	L
4b	4	D

Fig. 1. Structure of the ω-N-oxalyl diamino acids (asterisk denotes chiral centre).

Recently, there has been interest in enantioselective high-performance liquid chromatographic (HPLC) determination of amino acids[15–19] and related compounds[18,20–23] by pre-column derivatisation with *o*-phthaldialdehyde (OPA) and chiral thiols to yield diastereoisomeric isoindole derivatives which are separable by reversed-phase HPLC, and detected using fluorometry. It was therefore decided to evaluate the use of this methodology in the development of a new enantioselective assay of ω-N-oxalyl diamino acids.

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents were of analytical or HPLC grade. Ultra-pure water was obtained by means of a Milli-Q system (Millipore). OPA was purchased from Sigma; N-acetyl-L-cysteine, N-acetyl-D-penicillamine and N-*tert*.-butyloxycarbonyl-S-benzyl-L-cysteine from Fluka. N-*tert*.-butyloxycarbonyl-L-cysteine was prepared as described by Buck and Krummen[18]. Synthetic ω-N-oxalyl DL, D- and L-diamino acids were prepared according to the method of Nunn *et al.*[13].

Authentic samples of L-β-ODAP and L-γ-ODAB were obtained from *L. latifolius* seeds (500 g) which were extracted with 50% (v/v) aqueous ethanol (6 × 500 ml), the resultant extracts were combined and evaporated at 40°C under reduced pressure and the resulting residue taken up in water (2000 ml). This was loaded onto a column (4 × 25 ml) of Zeo-Karb 225 (H^+) and eluted with 0.062 *M* acetic acid. Fractions were analysed for the presence of L-β-ODAP and L-γ-ODAB and were further purified as described previously[24].

Chromatographic systems

HPLC was performed using a Gilson gradient system (Anachem, Luton, U.K.) which consisted of two Model 301 single piston pumps (5-ml heads), a Rheodyne 7125 loop injector (20 μl), a Model 801 pressure module and a Model 121 fluorescence detector fitted with OPA filters (excitation at 344 nm and emission at 443 nm). The gradient was controlled by an Apple IIe computer using Gilson gradient manager software.

TABLE I

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR THE ANALYSIS OF ω-N-OXALYL DIAMINO ACIDS

Duration (min)	*From (% solvent A:B)*	*To (% solvent A:B)*
0–30	90:10	75:25
30–35	75:25	65:35
35–50	65:35	65:35
50–55	65:35	40:60
55–60	40:60	40:60
60–65	40:60	90:10
65–75	90:10	90:10

Chromatograms were recorded on an LKB 2210 single-channel recorder at a sensitivity of 10 mV, a chart speed of 5 mm/min and a fluorescence sensitivity of 0.2 or 0.5 range units. A Spherisorb ODS II EXCEL, 5-μm (25 cm × 4.6 mm I.D.) column (Hichrom, Reading, U.K.), was used with a guard column (5 cm × 2 mm I.D.) packed with CO:PELL ODS sorbent (particle size 40 μm; Hichrom).

Preparation of standard ω-N-oxalyl diamino acid (1a–4b) solutions

Stock solutions of the individual enantiomers were prepared freshly each day in water at a concentration of 50 μmol/ml. Standard mixtures were prepared by mixing the appropriate stock solutions, followed by dilution with water to yield a final concentration of 100–300 nmol/ml for each individual component.

Mobile phases

Solvents A and B were prepared freshly every other day, filtered through a 0.22-μm membrane filter and degassed by continuous purging with helium. Solvents A and B consisted of 50 m*M* sodium acetate (pH 7.2, adjusted with dilute acetic acid) and methanol, respectively. The flow-rate was 1 ml/min and the column pressure was approximately 1600 p.s.i. at the beginning of the gradient. The gradient elution programme employed for the separation of enantiomers of γ-ODAB, δ-OORN and ε-OLYS is shown in Table I and for β-ODAP in Table II.

TABLE II

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR THE ANALYSIS OF THE D AND L ENANTIOMERS OF β-ODAP

Duration (min)	*From (% solvent A:B)*	*To (% solvent A:B)*
0–40	100:0	91:9
40–45	91:9	40:60
45–50	40:60	40:60
50–55	40:60	100:0
55–60	100:0	100:0

Pre-column derivatisation procedure

The derivatisation reagents were freshly prepared every other day by dissolving 10 mg of OPA and the chiral thiol in 1 ml of methanol (in order to preserve the optical purity of the chiral thiols, the alkaline borate buffer was added immediately prior to derivatisation). These reagents were stored at 4°C in the dark until use. The standard and isolated ω-N-oxalyl diamino acid solutions (20 μl) were mixed with the derivatisation reagent (40 μl) and borate buffer (60 μl, pH 8.2, adjusted with 2 *M* sodium hydroxide), and incubated for 5 min at ambient temperature in the dark before immediate injection onto the column.

RESULTS AND DISCUSSION

All the ω-N-oxalyl diamino acids reacted with OPA and the chiral thiols N-acetyl-L-cysteine (NAC), N-acetyl-D-penicillamine (NAP) and N-*tert*.-butyloxycarbonyl-L-cysteine (BocC) in alkaline conditions to yield highly fluorescent derivatives. The reactions occurred rapidly and quantitatively at ambient temperature in the dark, reaching their maximum fluorescence within 1–2 min, and were stable for at least 10 min. The fluorescence intensity of the derivatives was observed to be similar to that obtained with other commonly occurring amino acids (the NAP adducts had a lower fluorescent intensity than the corresponding NAC and BocC adducts). Efficient separation of the L and D enantiomers of γ-ODAB, δ-OORN and ε-OLYS was achieved by the use of the OPA–BocC reagent, with a 50 mmol sodium acetate (pH 7.2)–methanol gradient (which enhances the separation of early eluting "acidic" amino acids) and a Spherisorb ODS II EXCEL column (Fig. 2A and Table I). All

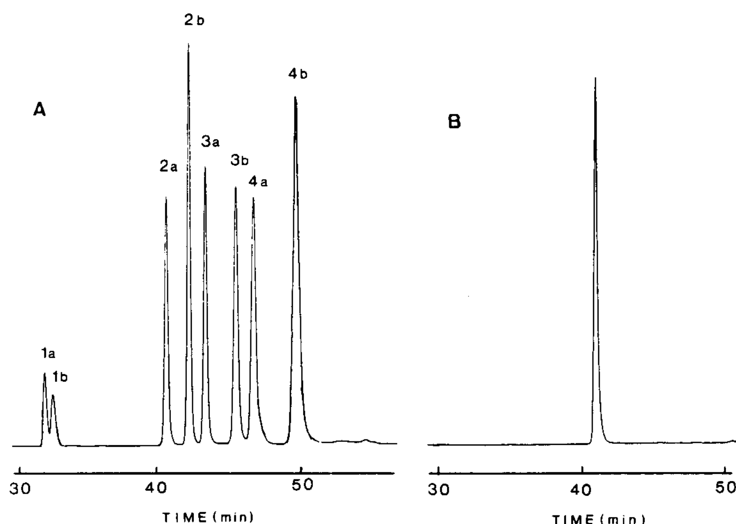


Fig. 2. HPLC of OPA–BocC derivatives of ω-N-oxalyl diamino acids on a Spherisorb ODS II EXCEL reversed-phase column. Chromatographic conditions as in the Experimental section. (a) Standard mixture of ω-N-oxalyl diamino acids. Peaks **1a,b** = L-, D-β-ODAP; **2a,b** = L-, D-γ-ODAB; **3a,b** = L-, D-δ-OORN; **4a,b** = L-, D-ε-OLYS. Each peak coresponds to 1 nmol expect for peaks **1b**,**2b** and **4b** which corresponds to 0.75, 1.5 and 1.4 nmol respectively. (B) "Isolated natural" L-γ-ODAB (**2a**) from *Lathyrus latifolius*.

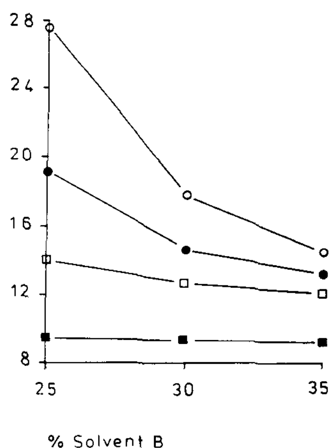
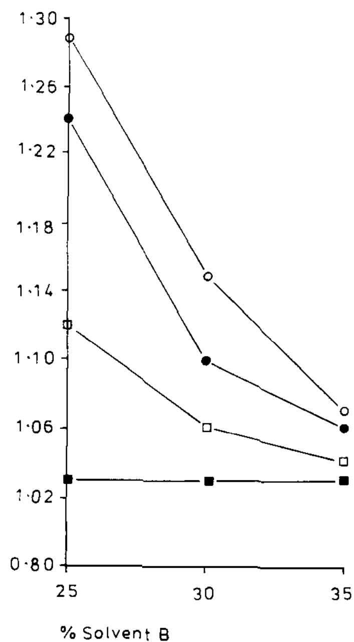


Fig. 3. The effect of methanol percentage at the plateau (35–50 min) on the capacity factors for the D enantiomers of the ω-N-oxalyl diamino acids: β-ODAP (■), γ-ODAB (□), δ-OORN (●) and ε-OLYS (○).



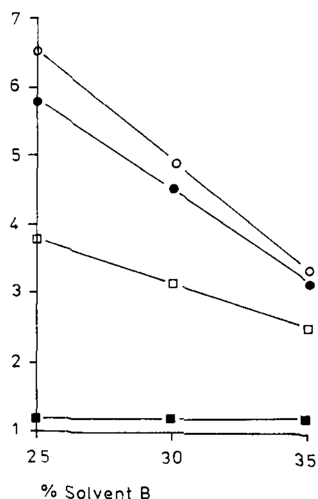


Fig. 4. The effect of methanol percentage at the plateau (35–50 min) on the selectivity factor of the enantiomers of the ω-N-oxalyl diamino acids: β-ODAP (■), γ-ODAB (□), δ-OORN (●) and ε-OLYS (○).

Fig. 5. The effect of methanol percentage at the plateau (35–50 min) on the resolution of the enantiomers of the ω-N-oxalyl diamino acids. Key as in Fig. 4.

enantiomers were separated in a run-time of 75 min which included wash and re-equilibrium phases. The coefficients of variation for the retention times and peak heights for all the ω-N-oxalyl compounds were below 0.7 and 1.2%, respectively. The calibration graph for the ω-N-oxalyl diamino acids showed good linearity between peak height and concentration in the range of 0.2 to 1.0 nmol per injection (r^2 < 0.995). As expected, the capacity and separation factor for the enantiomers of γ-ODAB, δ-OORN and ε-OLYS increased on lowering the methanol content of the mobile phase at the plateau (35–50 min), and also the resolution was observed to increase linearly (Figs. 3–5 and Table III). The capacity, separation and resolution for the enantiomers of β-ODAP remained constant (Figs. 3–5 and Table III). In an attempt to achieve better separation, other chiral thiols were investigated. OPA–NAC resulted in co-elution of the D and L enantiomer and also failed to completely resolve the enantiomers of γ-ODAB. In contrast, the OPA–NAP reagent resulted in baseline separation (separation factor and resolution of 1.37 and 1.43, respectively, for β-ODAP) using a modified sodium acetate–methanol gradient (Fig. 6A and Table II). OPA–NAP also effectively separated the enantiomers of γ-ODAB. As observed with other OPA–BocC amino acids[18,21], the L enantiomers (*S* configuration at the tertiary carbon atom bearing the primary amino group) of compounds **2–4** eluted before their corresponding D enantiomers. This is probably due to stronger hydrogen-bonds in the D diastereoisomers, resulting in a more hydrophobic molecule which would be expected to interact more strongly with the reversed-phase column and have a longer retention time than its corresponding L diastereoisomer. It has been suggested previously[18] that OPA–NAP derivatives of hydrophobic amino acids result in the D enantiomer eluting before the L enantiomer, the opposite is observed for the hydrophilic amino acids as can be seen for β-ODAP.

TABLE III

SEPARATION OF DIASTEREOISOMERIC DERIVATIVES FORMED FROM ω-N-OXALYL DIAMINO ACIDS AND OPA–BocC

t_0 = 3.2 min; k', α and R_s are the capacity, separation and resolution factors, respectively, for a pair of enantiomers; chromatographic conditions are as in the Experimental section.

Compound	*Methanol in buffer at plateau (%)*	k'_L	k'_D	α	R_s
β-ODAP	35	9.00	9.25	1.03	1.19
	30	9.00	9.25	1.03	1.19
	25	9.06	9.31	1.03	1.19
γ-ODAB	35	11.66	12.13	1.04	2.50
	30	11.88	12.63	1.06	3.64
	25	12.56	14.03	1.12	3.76
δ-OORN	35	12.50	13.19	1.06	3.14
	30	13.25	14.63	1.10	4.78
	25	15.47	19.19	1.24	5.73
ε-OLYS	35	13.56	14.50	1.07	3.33
	30	15.50	17.81	1.15	4.93
	25	21.38	27.50	1.29	6.53

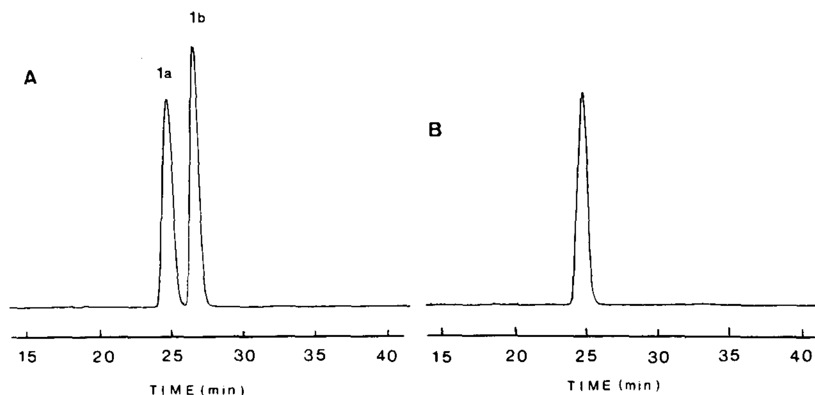


Fig. 6. HPLC of OPA–NAP derivatives of ω-N-oxalyl diamino propanoic acid (**1a,b**) on a Spherisorb ODS II EXCEL reversed-phase column. Chromatographic conditions as in the Experimental section. (A) Standard mixture of D- and L-β-ODAP (**1a,b**). (B) "Isolated natural" L-β-ODAP (**1a**) from *Lathyrus latifolius*.

Samples of natural L-β-ODAP and L-γ-ODAB were isolated from an aqueous ethanolic extract of *L. latifolius* seeds. Purification and separation was achieved by the use of a Zeo-Karb 225 (H^+) resin eluting with 0.062 *M* acetic acid. This process separated the L-β-ODAP from L-γ-ODAB, the samples were further purified as described previously[24]. The isolated L-β-ODAP and L-γ-ODAB were shown to be enantiomerically pure (Figs. 6B and 2B) and the homogeneity of the assigned peaks (L-β-ODAP, t_R = 24.5 min and L-γ-ODAB, t_R = 41.1 min) was confirmed by comparison with the retention times of synthetic standards and chromatography of "spiked samples" and verifies the previously assigned L configuration in isolated materials.

The optical purity of all the synthetic ω-N-oxalyl diamino acids was shown to be in excess of 99.8%. The quantification of the minor enantiomers was achieved by comparison of peak heights in conjunction with standard additions. The limit of detection of the minor enantiomers was better than 0.1%.

The HPLC assay described represents a specific method for detecting enantiomers of ω-N-oxalyl diamino acids from natural sources and for assessing the optical purity of synthetically prepared analogues.

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