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Note

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

Application to 2-amino-ω-phosphonoalkanoic acid homologues and α-amino-β-N-methylaminopropanoic acid (β-methylaminoalanine)

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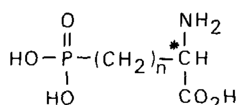
Excitatory amino acid receptors have been classified into different subtypes[1,2]. The N-methyl-D-aspartate (NMDA) receptors, which are activated by NMDA and blocked by 2-amino-ω-phosphonoalkanoic acids, are involved in synaptic excitation in the spinal cord and it has been proposed that they are activated physiologically by L-aspartate and/or L-glutamate[2]. The use of the NMDA antagonists 2-amino-ω-phosphonoalkanoic acids [Fig. 1: 2-amino-4-phosphonobutanoic acid (2APB), **1a**, **b**; 2-amino-5-phosphonovaleric acid (2APV), **2a**, **b**; and 2-amino-7-phosphonoheptanoic acid (2APH), **3a**, **b**] are of considerable value in the investigation of amino



compound	n	optical configuration
1a	2	L-
1b	2	D-
2a	3	L-
2b	3	D-
3a	5	L-
3b	5	D-

Fig. 1. Structure of the 2-amino-ω-phosphonoalkanoic acids (asterisk denotes chiral centre).

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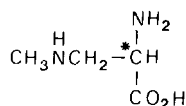

compound	optical configuration
4a	L-
4b	D-

Fig. 2. Structure of MeDAP (asterisk denotes chiral centre).

acid-mediated synaptic excitation[1,4]. The antagonist activity of these compounds resides with the D-enantiomers[4], although previous biochemical studies employed racemic DL-mixtures[1,4]. However, it is essential to establish the enantiomeric purity of these compounds. For example, although the D-(−)-enantiomer of 2APB acts as an antagonist at the NMDA receptor, its corresponding L-(+)-enantiomer is a potent synaptic depressant, possibly at another non-NMDA receptor site[2]. The L-(+)-isomer of β-amino-β-methylaminopropanoic acid (MeDAP, Fig. 2) appears to act at the NMDA receptor and it became the focus of much interest in view of the high incidence of degenerative neural diseases on the Pacific island of Guam[5]. During World War II, the inhabitants were forced to consume large quantities of seeds from the false sago palm (*Cycas circinalis*), which is a natural source of MeDAP. Primates given repeated oral doses of MeDAP exhibited many signs characteristic of upper and lower motor systems disease together with associated neurodegenerative changes[6]. The neurotoxicity of MeDAP is associated with the L-(+)-enantiomer only[7,8].

It can be seen therefore that since both MeDAP and the 2-amino-ω-phosphonoalkanoic acid series are prepared synthetically[4,9] as the racemate and then resolved, the enantiomeric purity of the resultant L- and D-enantiomers must be assessed prior to pharmacological or biochemical studies. To date, the optical purity of these compounds has been ensured by optical rotation measurements. However, this method is insensitive to contamination with small quantities of minor enantiomers arising from racemisation or incomplete resolution[7].

Recently, we have described the use of an enantioselective high-performance liquid chromatographic (HPLC) determination of the non-NMDA agonists β-N-oxalyl-L-α,β-diaminopropanoic acid, γ-N-oxalyl-L-α,γ-diaminobutanoic acid and related compounds by a pre-column derivatisation technique, utilising *o*-phthaldialdehyde (OPA) and chiral thiols to yield diastereoisomeric isoindole derivatives, which are separable by reversed-phase HPLC and detected using fluorimetry[10]. It was decided to extend this approach to the development of a new, enantioselective assay for the 2-amino-ω-phosphonoalkanoic acids and MeDAP.

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 (NAC) and N-*tert.*-butyloxycarbonyl-S-benzyl-L-cysteine from Fluka. DL-, D- and L-2APB, 2APV, 2APH and L-MeDAP were obtained from Cambridge Biochemical Reserch. N-*tert.*-Butyloxycarbonyl-L-cysteine (BocC) was prepared as described by Buck and Krummen[11]. Synthetic DL-, D- and L-MeDAP were prepared using an established method[7].

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.

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 and 0.5 range units for MeDAP and 2-amino-ω-phosphonoalkanoic acids respectively. A Spherisorb ODS II "EXCEL", 5 μm (25 cm × 4.6 mm I.D.) column was purchased from Hichrom (Reading, U.K.) and fitted with a guard column (5 cm × 2 mm I.D.) packed with CO:PELL ODS sorbent (particle size 40 μm; Hichrom).

Preparation of standard amino acids derivatives

Stock solutions of the individual enantiomers were prepared, freshly each day, in water at a concentration of 2.5–5.0 μmol/ml. Standard mixtures were prepared by diluting the appropriate stock solutions with water to yield a final concentration of 60–250 nmol/ml and 150–450 nmol/ml for each individual enantiomer of the 2-amino-ω-phosphonoalkanoic acids and MeDAP respectively.

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 7.1 and 6.8, adjusted with dilute acetic acid for the 2-amino-ω-phosphoalkanoic acids and MeDAP, respectively) and

TABLE I

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR THE ANALYSIS OF 2-AMINO-ω-PHOSPHONOALKANOIC ACIDS AND MeDAP

Duration (min)	*Solvent A:B*	
	From	*To*
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

TABLE II

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR THE ANALYSIS OF 2APB

Duration (min)	*Solvent A:B*	
	From	*To*
0–40	90:10	75:25
40–45	75:25	40:60
45–50	40:60	40:60
50–55	40:60	90:10
55–65	90:10	90:10

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 the enantiomers of 2APV, 2APH and MeDAP is shown in Table I and for 2APB in Table II.

Pre-column derivatisation procedure

The derivatisation reagents were freshly prepared every other day by dissolving 10 mg of OPA and 10 mg of the chiral thiol in 1 ml of HPLC-grade 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 2-amino-ω-phosphonoalkanoic acid and MeDAP 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

The acidic 2-amino-ω-phosphonoalkanoic acids gave intensely fluorescent diastereoisomeric isoindole derivatives when reacted with OPA and the chiral thiol, BocC in alkaline conditions. At ambient temperature in the dark they reached their maximum fluorescence within 2–3 min and were stable for up to at least 10 min. The resultant diastereoisomeric isoindole derivatives were separable by reversed-phase HPLC using a 50 m*M* sodium acetate (pH 7.1)–methanol gradient (optimised for separation of acidic compounds) and a Spherisorb ODS II column as described previously for the ω-N-oxalyl-diamino acid series[10]. It has previously been shown that this type of pre-column diastereosiomer formation induces no detectable degree of racemisation[11,12].

Using these conditions the commercially available racemic DL-2APV and DL-2APH (Table I) gave baseline separation for the D- and L-enantiomers, whereas those of the short chain homologue, 2APB, were not fully resolved (Tables I and III, and Fig. 3A). However, on employing a gradient with a lower rate of change of buffer-methanol, naseline separation was achieved (Tables II and III, and Fig. 3D). The reproducibility of the retention times and peak heights for the enantiomers of 2APB, 2APV and 2APH was better than 1% coefficient of variation (C.V.). The

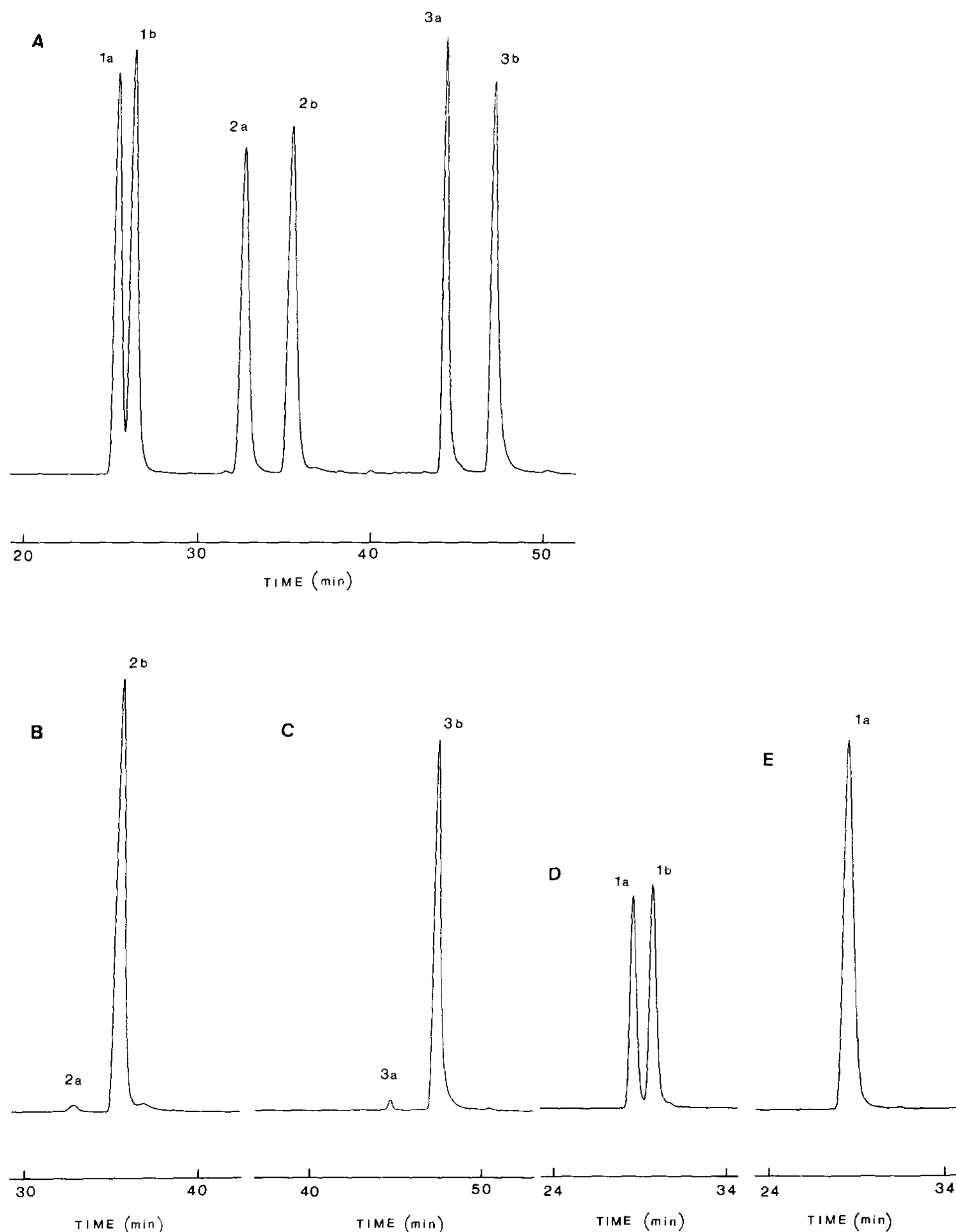

Fig. 3. HPLC of OPA-BocC derivatives of 2-amino-ω-phosphonoalkanoic acids on a Spherisorb ODS II "EXCEL" reversed-phase column. Chromatographic conditions as in the Experimental section. (A) Standard mixture of 2-amino-ω-phosphonoalkanoic acids using conditions as in Table I. Each peak corresponds to 500 pmol. (B and C) Commercial samples of D-2APV and D-2APH. (D) Standard mixture of DL-2APB using conditions as in Table II. Each peak corresponds to 300 pmol. (E) Commercial sample of L-2APB. Peaks: **1a**, **b** = L-, D-2APB; **2a**, **b** = L-, D-2APV; **3a**, **b** = L-, D-2APH.

response (at 338 nm) to both derivatives was the same. In contrast, the BocC derivatives gave peaks of equal area for both enantiomers. This phenomenon of unequal fluorescence response was also observed by Buck and Krummen[11] for the amino compounds phenylalaninol and tryptophane but the difference in intensities was much lower than observed for the NAC derivatives of D- and L-MeDAP. The separation of the enantiomers achieved with the chiral thiol NAC was excellent (Table III and Fig. 4A) giving baseline separation and a limit of detection of the minor isomer of better than 0.1%. The elution order of the OPA–BocC derivatives of MeDAP was L- before the D-enantiomer whereas the order for the OPA–NAC derivatives was D- before the L-enantiomer. The order of elution with OPA–NAC derivatives has been previously related to the hydrophilicity of the amino acid. Hydrophilic amino acids result in the L-eluting before the D-enantiomer whereas the hydrophobic amino acids elute with the D- before the L-enantiomer[11].

The assay of MeDAP was highly pH dependent, using a mobile phase of pH values 6–7.1 a single peak was seen for each enantiomer. However, at pH values above

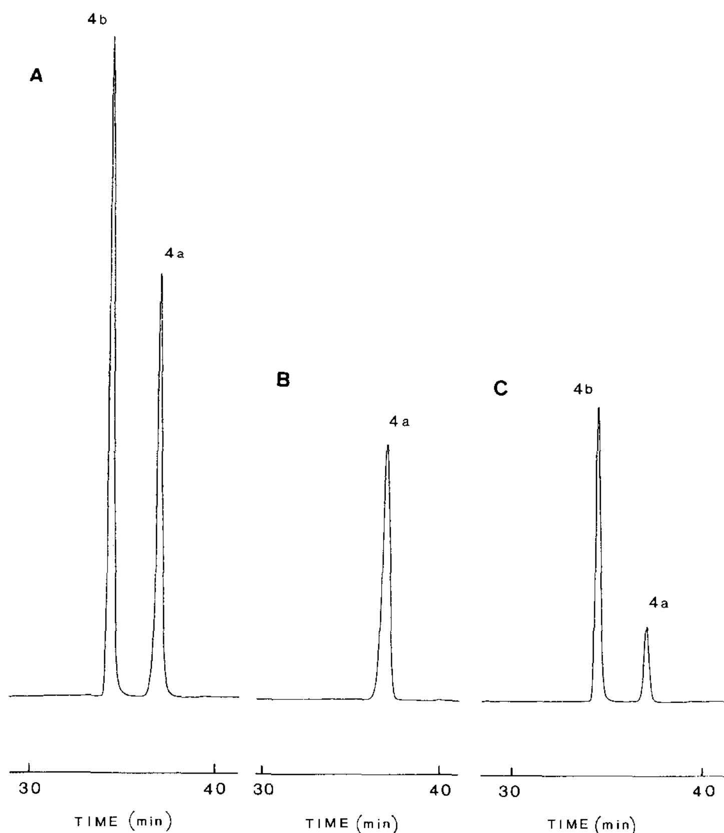

Fig. 4. HPLC of OPA–NAC derivatives of MeDAP (**4a**, **b**) on a Spherisorb ODS II "EXCEL" reversed-phase column. Chromatographic conditions as in Experimental section. (A) Standard mixture of DL-MeDAP (**4a** and **b**). Each peak corresponds to 1.5 nmol. (B) Commercially available L-MeDAP (**4a**). (C) Synthetically prepared D-MeDAP (**4b**).

TABLE III

SEPARATION OF DIASTEREOISOMERIC DERIVATIVES FORMED FROM 2-AMINO-ω-PHOSPHONOALKANOIC ACIDS–BocC AND MeDAP–NAC

t_0 = 3.2 min; k^* and R_s are the capacity and resolution factors, respectively, for a pair of enantiomers; chromatographic conditions are as in the Experimental section and Table I.

Compound	K_L^*	K_D^*	R_s
APB	6.97	7.28	1.04
APB[a]	7.88	8.13	1.64
APV	9.25	10.13	3.33
APH	12.94	13.88	3.77
MeDAP	10.25	9.48	4.33

[a] Chromatographic conditions are as in the Experimental section and Table II.

calibration graph for the enantiomers of 2APB, 2APV and 2APH showed good linearity between peak height and concentration within the range of 0.2–1.1 nmol per injection (r^2 > 0.997). The limit of detection of the minor isomer was better than 0.1% and injection of a blankillustrated that there were no interfering peaks in the region of interest.

As observed with other OPA–BocC amino acids, the L-enantiomers eluted before their corresponding D-enantiomer[10–12]. This result is probably due to the fact that the diastereoisomers from the D-enantiomers are capable of forming stronger intramolecular hydrogen bonds and therefore, being more lypophilic, are retained longer on the reversed-phase column than those formed from the L-enantiomers.

Commercial samples of the D- and L-enantiomers of 2APB, 2APV and 2APH were shown to be more than 98% optically pure and fell within the specification of the manufacturer (*i.e.*, see Fig. 3B, C and E). The use of this approach is ideally suited to the quality control assessment of the enantiomeric purity of these compounds as, in addition to the above results, we have shown that the pre-column derivatisation can be conveniently automated using a Gilson autosampler and injector (the BocC–OPA reagent is stable for at least 18 h at ambient temperature in amber vessels). The OPA–BocC reagent is added to the amino compound first, followed by the borate buffer just prior to derivatisation to avoid any possibility of racemisation of the chiral thiol.

In contrast to the acidic 2-amino-ω-phosphonoalkanoic acids and ω-N-oxalyl-diamino acids[10], MeDAP is considerably more basic[7]. The use of the lypophilic chiral thiol BocC with synthetic DL-MeDAP resulted in separation of the diastereoisomers. However, compared to the 2-amino-ω-phosphonoalkanoic acid series, they eluted late in the chromatogram (t_R = 60.00 and 61.5 min for the L- and D-enantiomers, respectively) due to their increased lypophilic nature. In addition, on performing a blank, interfering peaks were shown to co-elute with the peaks of interest. On substituting the chiral thiol NAC for BocC, the diastereoisomers produced were less fluorescent and were eluted earlier than their corresponding BocC derivatives and free from interferring peaks. It was of interest to note that the NAC derivative of the D-enantiomer of MeDAP had a fluorescence intensity 1.50 times greater than the corresponding L-enantiomer for a racemic DL-mixture (optical rotation and circular dichroism measurements confirmed a 50:50 racemic mixture) whereas the UV

7.1, two peaks are observed for each enantiomer, the second occuring very late in the "run" suggesting the additional production of a more lypophilic derivative (further investigations of these additional peaks are presently underway). Using a mobile phase of pH 6.8 the early-eluting peaks are reproducible (coefficients of variation of below 1% where obtained for the t_R and peak height) and the peak heights for each enantiomer responded linearly with concentration within the range 0.5–1.5 nmol (injected onto the column).

Synthetic and commercially available L-MeDAP contained no detectable levels of the D-enantiomer (Fig. 4B). However, the synthetic D-MeDAP produced by our laboratory contained 1.24% of the L-enantiomer. Whereas, D-MeDAP from another laboratory was shown to contain up to 20% of the L-enantiomer (Fig. 4C). During the synthesis, separation of the enantiomers from the racemic DL-α-N-acetyl-MeDAP is achieved by incubation with acylase-I which yields free L-MeDAP. This compound is separated from the impure α-N-acetyl-D-MeDAP by ion-exchange chromatography. The impure α-N-acetyl-D-MeDAP is resubjected to more acylase-I to remove further quantities of L-MeDAP[7]. The N-acetyl-D-MeDAP is then hydrolysed with acid to yield free D-MeDAP. The occurrence of considerable amounts of the L-enantiomer in the D-enantiomer suggests either racemisation in the hydrolysis stage or, more probably, an incomplete acylase-I reaction.

The HPLC assay described represents a specific and reproducible method ideally suited for quality control purposes for assessing the enantiomeric purity of the synthetically prepared NMDA agonist MeDAP, and antagonists, 2-amino-ω-phosphonoalkanoic acids.

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