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Note

Resolution of lombricine enantiomers by high-performance liquid chromatography utilising pre-column derivatisation with *o*-phthaldialdehyde–chiral thiols

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Lombricine (Fig.1) is present in a number of invertebrates[1–4] and is thought to act as a regulator of adenosine triphosphate levels[1,5]. Serine, the amino acid in lombricine, occurs in either the L or D configuration depending upon the phylum of the particular invertebrate[3]. Since D-amino acids are not commonly found in nature[6], a convenient and specific method of determining the configuration of the seryl moiety in crude biological extracts was sought as part of our on-going research into novel helminthic high energy phosphates[7,8].

To date, the configuration of the seryl moiety has been laboriously determined by optical rotation measurements on purified isolated lombricine and comparison to authentic D- and L-lombricine whose configuration was unambiguously determined by classical hydrolysis on isolated lombricine[4,9]. The liberated serine was purified by ion-exchange and paper chromatography and the configuration of the purified serine was then established by optical rotation measurements and the action of D-amino acid oxidase.

Recently, there has been interest in enantioselective high-performance liquid chromatographic (HPLC) determination of amino acids[10–14] and amino enantiomers[13,15] by pre-column derivatisation with *o*-phthaldialdehyde (OPA) and chiral thiols to yield diastereoisomeric isoindole derivatives which are separable on reversed-phase HPLC, and can be detected using fluorometry. It was therefore decided to evaluate the use of this new methodology in the development of a new and enantioselective assay for D- and L-lombricine.

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 and standard D- and

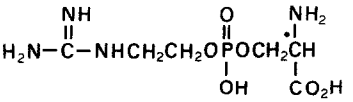

Fig. 1. Structure of lombricine (asterisk denotes chiral centre).

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L-amino acids were 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[13]. Authentic natural D- and L-lombricines were kindly supplied by Y. Robin and H. Rosenberg. Synthetic D/L, D- and L-lombricines were prepared according to the method of Euerby *et al.*[16].

Chromatographic systems

HPLC was performed on an apparatus as described by Euerby *et al.*[8]. 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). A fluorescence sensitivity of either 0.2 or 0.1 r.f.u. was employed.

Preparation of standard amino acids and derivatives

Stock solutions of the individual enantiomers were prepared in water at a concentration of 60 μmol/ml and were stable for at least one month of continual use if stored at −14°C. Standard mixtures were prepared by mixing the appropriate stock solutions, followed by dilution with water to yield a final concentration of 30–60 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 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 is shown in Table I.

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 should only be added immediately prior to derivatisation). These reagents were stored at 4°C in the dark until use. The standard amino acid solutions (20 μl) or centrifuged biological extracts (20 μl) were

TABLE I

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR THE ANALYSIS OF D- AND L-LOMBRICINE

Duration (min)	*From (%solvent A:B)*	*To (%solvent A:B)*
0–30	90:10	75:25
30–35	75:25	70:30
35–45	70:30	70:30
45–50	70:30	90:10
50–60	90:10	90:10

mixed with the derivatisation reagent (40 μl) and borate buffer (60 μl, pH 9.5, adjusted with 2 *M* sodium hydroxide), and incubated for 5 min at ambient temperature in the dark before immediate injection onto the column.

Preparation of biological extracts

The "earthworms" used were collected in the Leicestershire area and were of mixed species but were predominantly *Allolobophora caliginosa*, *Octolasium cyaneum* and *Lumbricus terrestris*[17]. Live pre-weighed earthworms (approximately 10.0 g) were homogenised with ice-cold water (10 ml) in a Polytron® homogeniser (Kinematica) for 5 min. The homogenate was adjusted to pH 3.0 with concentrated sulphuric acid, boiled for 5 min and centrifuged for 25 min at 31 000 *g* using a Beckmann J2-21 centrifuge (JA-20 head). The pellet was re-extracted with two volumes of water and centrifuged as before. The combined supernatants were brought to pH 8.0 with cold-saturated barium hydroxide solution and centrifuged, as before, to remove barium sulphate. The supernatent was then neutralised to pH 7.0 with dilute hydrochloric acid and then lyophilised (Edward EF 4 Module freeze drier operating at −60°C). The prepared extracts were dissolved in water (10 ml) and centrifuged to give the stock extract solutions which were diluted 1 in 1000 when required with water and then derivatised.

RESULTS AND DISCUSSION

We have recently shown that lombricine can be detected in biological extracts using OPA–ethanethiol derivatisation[8]. The seryl moiety of lombricine should, in principle, form diastereoisomers with OPA and the optically pure chiral thiols, N-acetyl-L-cysteine (NAC), N-acetyl-D-penicillamine (NAP) and N-*tert.*-butyloxycarbonyl-L-cysteine (BocC), using the same methodology as described for the amino acids and amino alcohols[10–15]. The resultant diastereoisomers should then be separable on standard reversed-phase HPLC stationary phases, alleviating the need for expensive chiral stationary phases. D- and L-lombricine were, in fact, shown to form highly fluorescent derivatives with all three thiols at pH 9.5 (the NAP adduct had a lower fluorescent intensity than NAC and BocC). The reactions occurred rapidly and quantitatively at ambient temperatures in the dark reaching their maximum fluorescence within 1–2 min and were stable for at least 10 min. Despite the use of the many mobile phase combinations described in the literature[10–15], the separation of the D- and L-lombricine diastereoisomers of NAC and NAP could not be achieved. However, the D- and L-isomers of lombricine could be easily separated, giving near baseline separation (Fig.2A, Table II), by the use of a 50 m*M* sodium acetate–methanol gradient and OPA–BocC derivatisation. The BocC chiral thiol has previously been observed to give better resolution of enantiomers than NAC[12]. A possible explanation for this is that the N-*tert.*-butyloxycarbonyl group of BocC is considerably more bulky than the acetyl groups of NAC and NAP and so forms diastereoisomers with more restricted conformations.

The limit of detection of L-lombricine in a mixture of D- and L-lombricine is better than 0.5% as can be seen in Fig. 2B and a blank run, using water in place of sample for the derivatisation, showed no interfering peaks in this region of interest. It was established that racemisation did not occur since only one enantiomer of

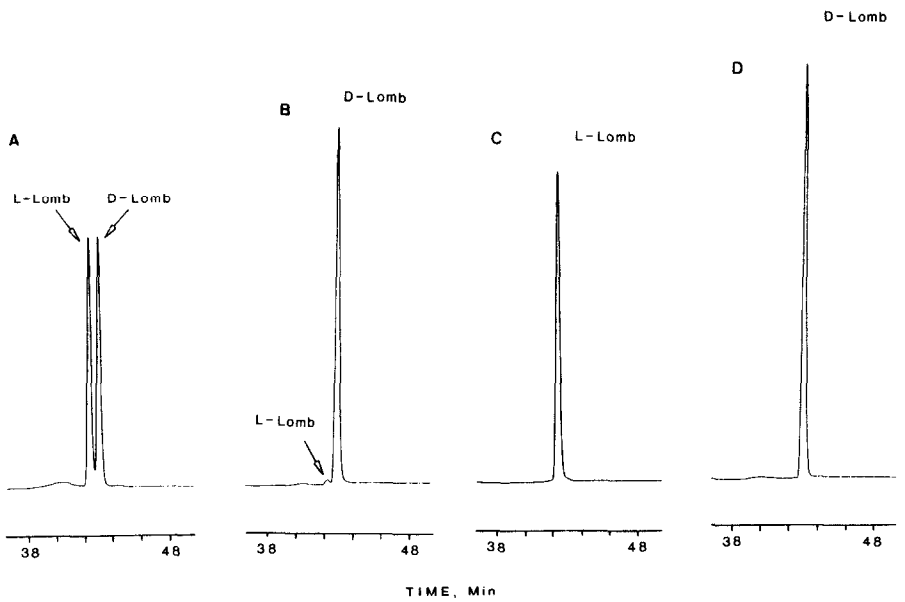

Fig. 2. HPLC of OPA–BocC derivatives of D- and L-lombricine on a Spherisorb ODS II EXCEL reversed-phase column. Chromatographic conditions as in the Experimental section. Non-standard abbreviations used: D-Lomb and L-Lomb = D- and L-lombricine respectively. (a) Synthetic racemic D/L-lombricine; (b) synthetic L- and D-lombricine (0.5:99.5 ratio); (c) L-lombricine from *Urechis caupo*; (d) D-lombricine from *Lumbricus terrestris*.

lombricine was detected on subjecting the single enantiomers to extraction and pre-column derivatisation. This confirms the findings of Pack and Krummen[12,13]. The initial gradient phase (10 to 25% methanol in 50 m*M* sodium acetate) was vital to avoid co-elution of the diastereoisomers. As observed with other OPA–BocC amino acids[13], the L enantiomer of lombricine eluted before its corresponding D enantiomer. 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

TABLE II

SEPARATION OF DIASTEREOISOMERIC DERIVATIVES FORMED FROM LOMBRICINE, ASPARTIC ACID AND GLUTAMIC ACID

t_0 = 3.20 min; k', α and R_s are the capacity ratio, separation factor and resolution, respectively, for a pair of enantiomers; chromatographic conditions are as in the Experimental section.

Sample	k'		α	R_s
	L	*D*		
Lombricine	12.16	12.38	1.02	1.17
Aspartic acid	7.97	8.47	1.06	1.74
Glutamic acid	10.21	10.88	1.07	1.77

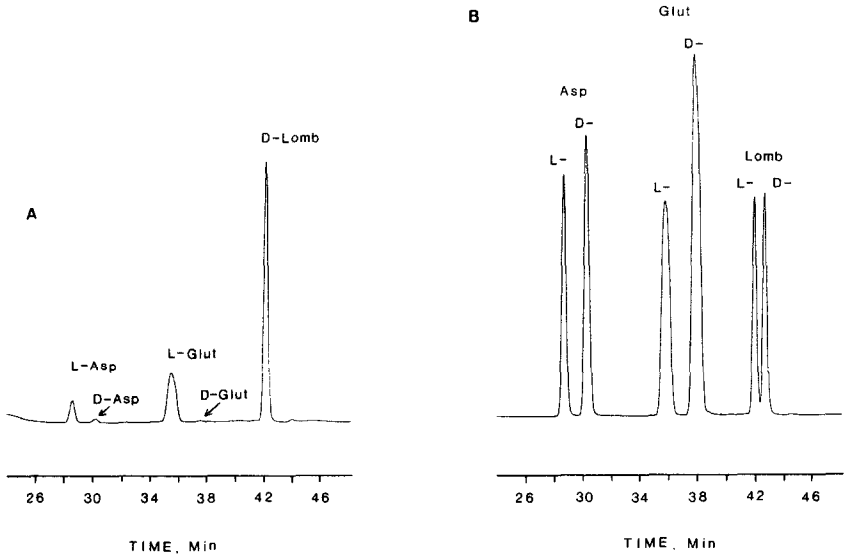

Fig. 3. HPLC of OPA–BocC derivatives in (A) sulphuric acid extract of earthworms and (B) L- and D-lombricine and aspartic acid (120 pmol of each enantiomer injected) and L- and D-glutamic acid (100 and 150 pmol of each enantiomer respectively injected). Conditions and abbreviations as in Fig. 2.

the reversed-phase column and have a longer retention time than its corresponding L diastereoisomer.

The presence of D-lombricine has been previously established in the phylum *Annelida*, whereas L-lombricine is only found in the phylum *Echiuoidea*. Samples of isolated L-lombricine from *Urechis caupo* and D-lombricine from *Lumbricus terrestris* were shown to be enantiomerically pure and had retention times of 42.10 and 42.80 min respectively (Fig. 2C and D) which, on comparison with the retention time of unambiguously synthesised D- and L-lombricine[16], verifies the previously assigned isolated lombricine configurations.

A sulphuric acid extraction of a mixed batch of earthworms [*Allolobophora caliginosa*, *Octolasum cyaneum* and *Lumbricus terrestris* of the *Annelida* phylum, as described by Hoffmann[2] (with the exception that the crude extract was lyophilised instead of being subjected to isolation of lombricine)], was subjected to the assay in order to determine the configuration of lombricine in the crude extract. A typical chromatogram is shown in Fig. 3A. The homogeneity of the assigned peaks (L-Asp, t_R = 28.70 min; D-Asp, 30.30 min; L-Glut, 35.88 min; D-Glut, 38.00 min and D-Lomb, 42.80 min) was confirmed by comparison with the retention times of authentic D and L standards and chromatography of spiked samples. It is of interest to note that in addition to possessing the D enantiomer of lombricine the earthworms studied also possessed a significantly higher level of the D enantiomer of aspartic acid than would be found in mammalian tissues. A standard chromatogram of the enantiomers of aspartic and glutamic acids and lombricine (Fig. 3B) illustrated that elution of their OPA–BocC diastereoisomers occurred in the same order as previously noted for their OPA–ethanethiol adducts[8].

The HPLC assay described represents a specific method for detecting enantiomers of lombricine in crude biological extracts and assessing the enantiomeric purity of synthetic and isolated L- and D-lombricine. The assay also permits the separation and detection of the enantiomers of aspartic acid and glutamic acid in earthworm extract.

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