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Note

High-performance liquid chromatographic determination of lombricine and N-phosphoryl lombricine in the earthworm by pre-column fluorescence derivatisation with *o*-phthaldialdehyde–ethanethiol

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Lombricine (2-guanidinoethyl-2-amino-2-carboxyethyl hydrogen phosphate) and its N-phosphorylated form (Fig. 1) have been reported to be present in a number of oligochaetes[1–6] including many species of earthworm. The physiological role of these compounds has been postulated[1,3,7] to be similar to that of creatine and arginine and their corresponding N-phosphorylated forms in vertebrates and invertebrates respectively, in that they act as intracellular regulators of adenosine triphosphate levels.

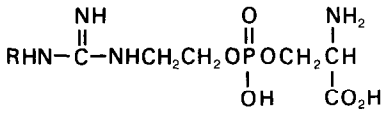

Fig. 1. Structures of lombricine (R = H) and N-phosphoryl lombricine (R = H_2O_3P).

To compliment our research into the occurrence of high energy phosphates in helminths[8] a specific assay was required for lombricine and its N-phosphorylated form in biological samples. To date, levels of these compounds have been measured by laborious isolation procedures[2,4,5] and by non-specific colourimetric estimations of guanidines and inorganic phosphate levels before and after hydrolysis of the N-phosphoryl guanidines[1,3,6].

Recently, there has been considerable interest in the analysis of both O- and N-phosphoryl amino acids in biological samples by high-performance liquid chromatography (HPLC)[9–11], involving the utilisation of pre-column derivatisation with an *o*-phthaldialdehyde (OPA)–thiol reaction followed by reversed-phase HPLC and fluorometric detection. It was decided to evaluate the use of this HPLC methodology in the development of a new and specific assay for lombricine and its N-phosphorylated form.

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EXPERIMENTAL

Reagents/chemicals

All chemicals and solvents were of analytical or HPLC grade. Ultrapure water was obtained by means of a Milli-Q system (Millipore). Ethanethiol was purchased from Aldrich; OPA, N-phosphoryl arginine and standard amino acids from Sigma and a standard protein hydrolysate mixture from LKB Biochrom. Authentic lombricine was kindly supplied by Professors Robin and Rosenberg. Synthetic lombricine was prepared according to the method of Euerby *et al.*[12].

Chromatographic system

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 210 single channel recorder at a sensitivity of 10 mV, a chart speed of 5 mm/min and a fluorescence sensitivity of 0.05 range units. Integration of peak area and measurement of peak height was performed using a Spectra-Physics Minigrator. A Spherisorb ODS II, 5 μm (12.5 cm $\times$ 4.6 mm I.D.) column was purchased from Hichrom (Reading, U.K.) and fitted with a guard column (5 cm $\times$ 2 mm I.D.) packed with CO : PELL ODS sorbent (particle size 40 μm; Hichrom).

Mobile phases

Solvents A and B were prepared freshly every other day, filtered through a 0.22-μm membrane filter and degassed by purging with helium. Solvent A consisted of 0.3 *M* sodium dihydrogen phosphate buffer (pH 7.2, adjusted with 2 *M* sodium hydroxide)–tetrahydrofuran–water (100:25:1875, v/v). Solvent B consisted of 0.3 *M* sodium dihydrogen phosphate buffer (pH 7.2, adjusted with 2 *M* sodium hydroxide)–acetonitrile–water (45:1100:855, v/v).

The flow-rate was 2 ml/min and the column pressure was approximately 1400 p.s.i. when only solvent A was being pumped. The gradient elution programme employed is shown in Table I.

TABLE I

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR THE HPLC ANALYSIS OF LOMBRICINE AND N-PHOSPHORYL LOMBRICINNE

Duration (min)	*From (ratio solvent A/B)*	*To (ratio solvent A/B)*
0–4	100:0	100:0
4–25	100:0	70:30
25–28	70:30	20:80
28–31	20:80	20:80
31–35	20:80	100:0
35–40	100:0	100:0

Pre-column derivatisation procedure

The derivisation reagent was freshly prepared on a daily basis by dissolving 3 mg of OPA in 0.5 ml of methanol, 5 ml of borate buffer (pH 9.5, adjusted with 2 *M* sodium hydroxide) and 7.5 μl ethanethiol. This reagent was stored at 4°C in the dark until use. The amino acid standard solutions (50 μl) or the centrifuged biological extracts (50 μl) were mixed with 50 μl of the derivatising solution and incubated for 5 min at ambient temperature in the dark before immediate injection onto the column.

Preparation of standard amino acids and derivatives

Stock solutions of the individual components were prepared in water at a concentration of 60 μmol/ml and were stable for approximately 1 month of continual use if stored at −20°C, with the exception of the phosphoryl amino acids and derivatives which were prepared freshly each day. Standard mixtures used for calibrations during the quantitative analysis were prepared by mixing the appropriate stock solutions, followed by dilution with water to yield a final concentration of 2–8 nmol/ml for each individual component. Typical concentrations of individual components injected onto the column were between 20–80 pmol.

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*[13]. Live pre-weighed worms were snap-frozen in liquid nitrogen and ground to a fine powder under liquid nitrogen using a liquid nitrogen-cooled pestle and mortar. The powder was then transferred to 5 volumes of ice-cold 0.75 *M* perchloric acid contained in a 50 ml polycarbonate centrifuge tube. The suspension was homogenised on ice using a Polytron® (Kinnematica). The homogenate was then neutralised to litmus paper using 10 *M* potassium hydroxide and 1 *M* potassium carbonate and stored on ice for 1 h to allow precipitation of protein and potassium perchlorate. The homogenate was centrifuged for 20 min at 20400 *g* using a JA 20 head (Beckmann J2-21 centrifuge). The supernatant was reserved on ice and the pellet re-extracted with an equal volume of water. This was then centrifuged as above and the supernatents combined. The combined supernatents were then lyophilised (Edward EF 4 Module freeze dryer operating at −60°C) and stored at −20°C until analysed. The prepared extracts were dissolved in water (20 ml/g of wet weight of earthworm) and centrifuged to give the stock extract solutions which were then diluted as required in water (1 in 40), to bring them within the range of the calibration curve and then derivatised.

Hydrolysis of N-phosphoryl lombricine

The stock extract solution was diluted with water (1 in 20), 50 μl was added to 25 μl of 1 *M* hydrochloric acid and mixed, the solution was heated at 100°C for 5 min, cooled, 25 μl of 1 *M* sodium hydroxide added, followed by further mixing before precolumn derivatisation.

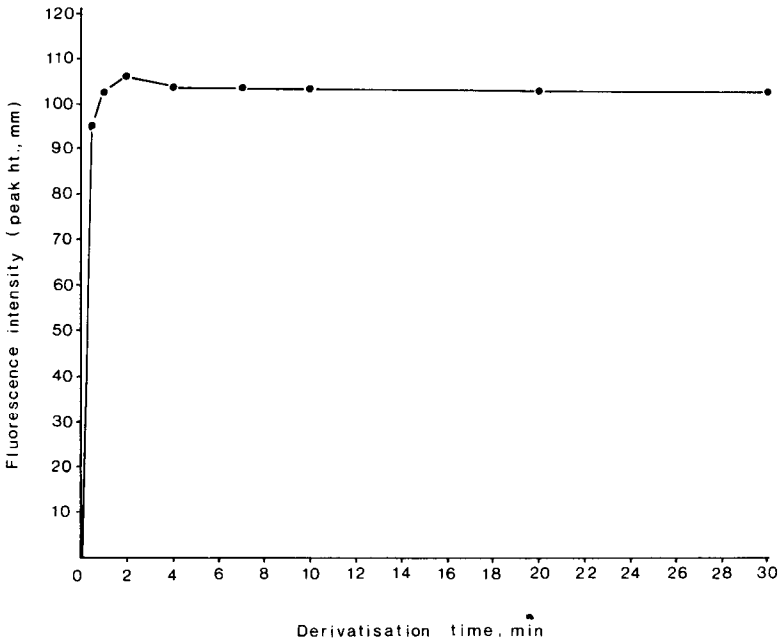

Fig. 2. Fluorescence response of the OPA–ethanethiol derivatisation as a function of reaction time for lombricine. Chromatographic conditions as in Experimental section.

RESULTS AND DISCUSSION

The derivatisation reagent OPA–ethanethiol was found to form a highly fluorescent derivative with synthetic and isolated lombricine at pH 9.5. Ethanethiol was used in place of the more commonly employed 2-mercaptoethanol since there have been reports of the OPA–ethanethiol amino acid adduct being more stable than that of the corresponding 2-mercaptoethanol adduct[14,15]. The derivatisation occurred rapidly and quantitatively at ambient temperature in the dark reaching its maximum fluorescence within 1 to 2 min and was stable for at least 30 min (Fig. 2). The fluorescence intensity of the lombricine derivative was observed to be similar to that obtained with other commonly occurring amino acids.

Unlike the O-phosphoryl amino acids (*i.e.* O-phosphoryl serine), the N-phosphoryl amino acids (*i.e.* N-phosphoryl arginine) are extremely acid-labile and base-stable. In order to minimise the hydrolysis of the labile N-phosphoryl guanidine bonds, mobile phases of pH 7.2 were employed in the assay as reported previously[9] for O- and N-phosphoryl amino acids. Adaptations were made to the method of the previous workers; a silica gel based reversed-phase column was used and a gradient elution which enhanced the separation of the early eluting amino acids as seen in Fig. 3.

The run-time between injections was 40 min including wash and re-equilibrium phases. Authentic lombricine was shown to elute (t_R = 17.20 min) clear of a standard amino acid protein hydrolysate under the conditions employed. In addition, the possible precursors of lombricine: serine ethanolamine phosphate (t_R = 29.00 min), O-

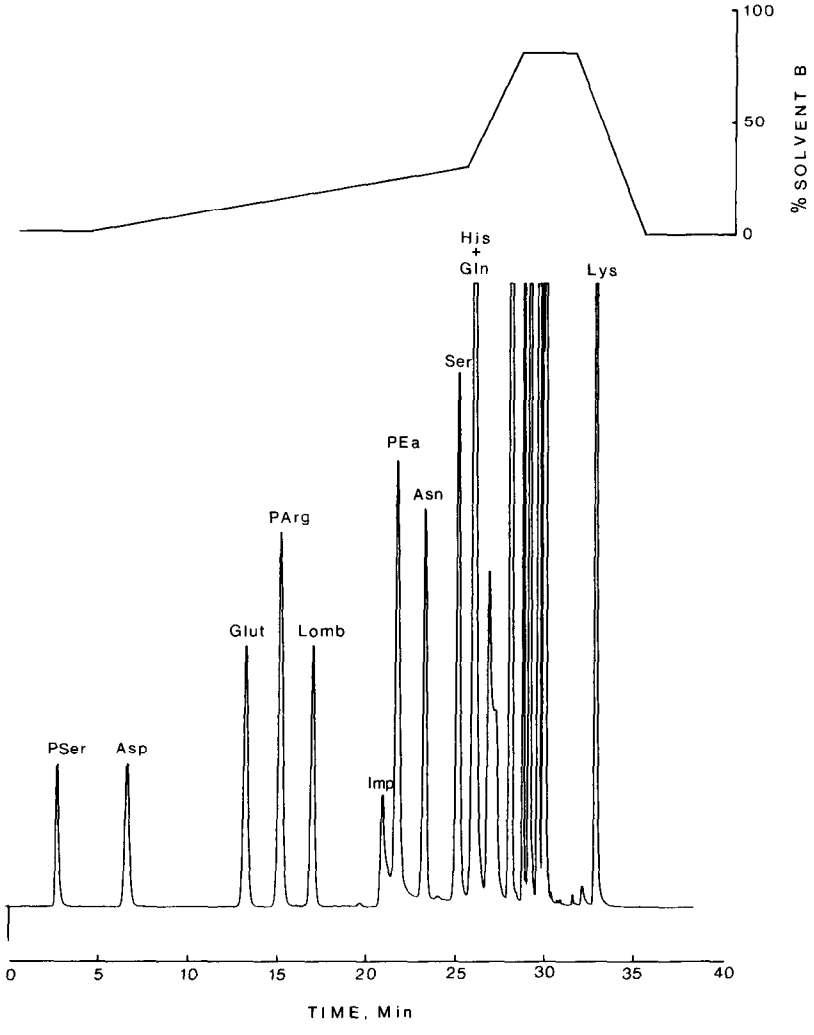

Fig. 3. HPLC analysis of standard OPA–ethanethiol amino acids and derivatives on a Spherisorb ODS II reversed-phase column. Chromatographic conditions as in Experimental section. Non-standard abbreviations used, peaks: PSer = O-phosphoryl serine; PArg = N-phosphoryl arginine; Lomb = Lombricine; PLomb = N-phosphoryl lombricine; PEa = O-phosphoryl ethanolamine; Imp = impurity arising from standard amino acid protein hydrolysate.

phosphoryl serine (t_R = 2.72 min) and O-phosphoryl ethanolamine (t_R 21.80 min) were shown not to interfere with the lombricine assay (Fig. 3). The reproducibility of the retention times, peak heights and areas for lombricine (Lomb), N-phosphoryl lombricine (PLomb) and N-phosphoryl arginine (PArg) is shown in Table II.

The calibration graph for lombricine showed good linearity between both peak height and area with concentrations in the range of 20–80 pmol per injection (r^2 = 0.999 for both peak height and area). The detection limit was in the lower femtomole range (signal-to-noise ratio of 3:1).

TABLE II

REPRODUCIBILITY OF RETENTION TIME, PEAK AREA AND HEIGHT

Compound	*Coefficient of variation (%, n=6)*		
	Retention time	*Peak area*	*Peak height*
Lombricine	0.53	0.51	1.06
N-Phosphoryl lombricine	0.61	0.68	1.45
N-Phosphoryl arginine	0.86	0.70	1.32

The extraction procedure involved, maintaining the integrity of the N-phosphoryl guanidine bonds by freeze-clamping and grinding the worms in liquid nitrogen, followed by ice-cold perchloric acid extraction and potassium hydroxide neutralisation (the whole procedure taking less than 4 min). The recovery of lombricine from the extraction procedure was in excess of 99%. A typical chromatogram is shown in Fig. 4. The homogeneity of the assigned peaks (Asp, t_R = 7.10 min; Glut, t_R = 13.80 min; PArg, t_R = 15.80 min; Lomb, t_R = 17.20 min; Asn, t_R = 23.60 min; Ser, t_R = 25.56 min; His + Gln, t_R = 26.20 min and Lys, t_R = 32.10 min) was confirmed by comparison with the retention times of authentic standards and chromatography of spiked samples. N-Phosphoryl arginine was additionally confirmed by collection of the peak fraction which was subjected to acid hydrolysis and re-analysed giving a peak corresponding to arginine (Arg, t_R = 29.44 min). It is of interest to note that in our assay O-phosphoryl serine (PSer) and aspartic acid (Asp) were completely resolved (Fig. 3) whereas in the hands of Carlomagno *et al.*[9] they were shown to co-elute.

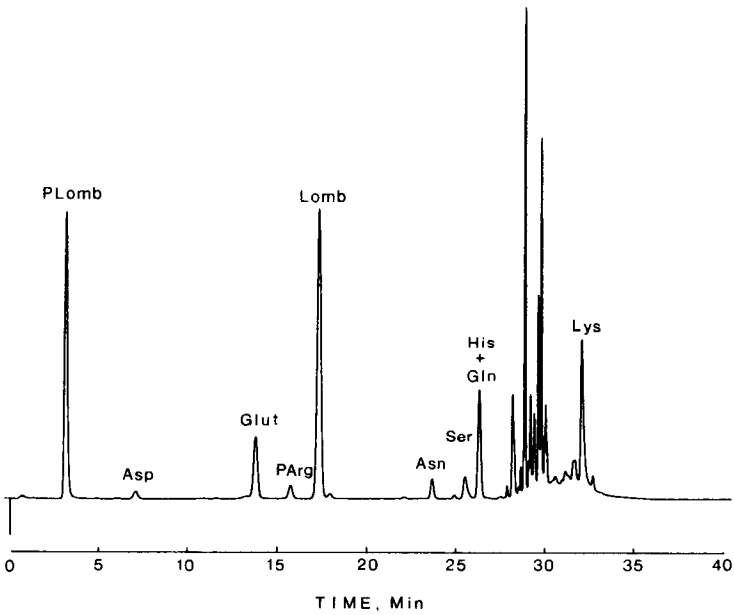

Fig. 4. HPLC analysis of OPA–ethanethiol amino acids and derivatives, in a freeze-clamped perchloric acid extract of earthworm. Conditions and abbreviations as in Fig. 3.

Owing to the instability of N-phosphoryl lombricine and the lack of pure authentic standard, the peak at t_R = 3.20 min was confirmed to be N-phosphoryl lombricine by subjecting the extract to a solution of 1 *M* hydrochloric acid (2:1, v/v) at 100°C for 5 min, neutralisation and re-analysis. The original peak at t_R = 3.20 min disappeared and a corresponding increase in the lombricine peak was observed (see Figs. 4 and 5).

Under these conditions lombricine was shown to be stable, and therefore the levels of N-phosphoryl lombricine could be determined from the difference between the lombricine levels before and after hydrolysis. By this method the percentage of N-phosphoryl lombricine in the total lombricine content was found to be 52.99% ± 2.58% (n = 4) with a total lombricine concentration after hydrolysis of 1.1693 ± 0.0227 mg/g wet weight of worm (n = 4). This corresponded to levels of 0.550 ± 0.0170 and 0.8032 ± 0.0240 mg/g wet weight of lombricine and N-phosphoryl lombricine in the earthworms *Allobophora caliginosa, Octolasium cyaneum* and *Lumbricus terrestris*. The previously reported levels of these compounds in *A. caliginosa* and *O. cyaneum* (0.65 and 0.17–0.29 mg/g wet weight of total lombricine[4] after hydrolysis and N-phosphoryl lombricine[2] respectively) and *L. terrestris* (0.6 mg/g wet weight of total lombricine[5] after hydrolysis) are lower than those quoted in this paper and reflect the inherent losses associated with isolation in the earlier reports.

The assay also enabled the level of N-phosphoryl arginine to be determined and this was found to be 10 times lower (0.056 ± 0.003 mg/g wet weight of worm), based on a molar ratio, than N-phosphoryl lombricine in the earthworms examined. This illustrates that it is N-phosphoryl lombricine and not N-phosphoryl arginine which is the most abundant high energy phosphate regulator in the species of earthworm examined.

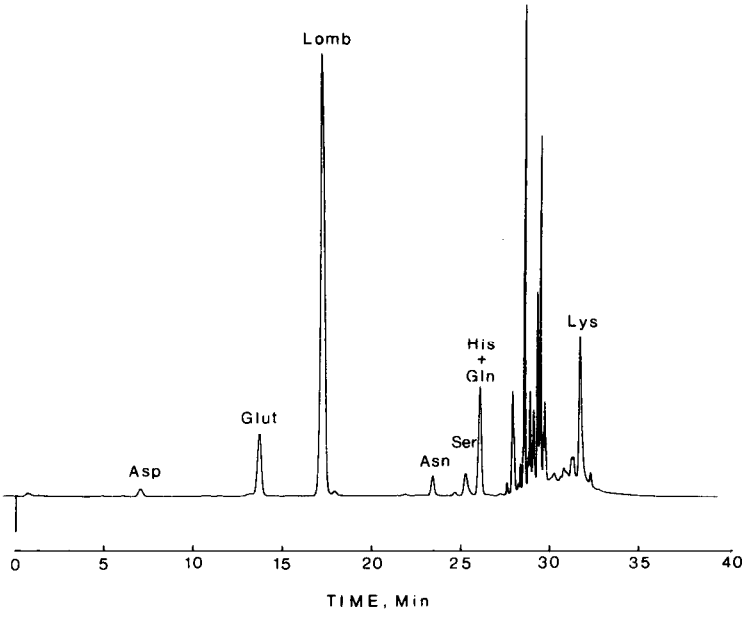

Fig. 5. HPLC analysis of OPA–ethanethiol amino acids and derivatives, in an acid hydrolysed sample of freeze-clamped perchloric acid extract of earthworm. Conditions and abbreviations as in Fig. 3.

The HPLC assay described represents a specific and reproducible method for determining lombricine and its acid-labile N-phosphoryl derivative in biological extracts. In addition, the assay proved to be an ideal method for the separation and detection of acidic amino acids and their O- and N-phosphorylated derivatives.

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