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Note

Effect of differing thiols on the reversed-phase high-performance liquid chromatographic behaviour of *o*-phthaldialdehyde-thiol–amino acids

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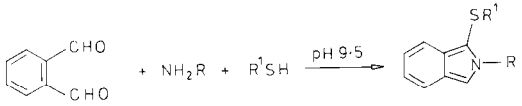
Over the last decade increasing use has been made of the *o*-phthaldialdehyde (OPA)-thiol pre-column derivatisation of biologically important primary amino compounds[1–5], with special relevance to amino acids[1,2,4,6–10]. The resultant fluorescent derivatives are separated by reversed-phase high-performance liquid chromatography (HPLC) followed by fluorimetric detection and are believed to be N-alkyl-2-alkylthio substituted isoindole derivatives (Fig. 1).

To date, the thiols most commonly employed are mercaptoethanol[5–9], ethanethiol[2,10,11] and 3-mercaptopropionic acid[3,4,12] and their selection appears to be



'OPA'

Amino acid	R=	Thiol	R^1 =
Lombricine	$CH(CO_2H)CH_2O\overset{\overset{O}{\|}}{\underset{\underset{OH}{\|}}{P}}OCH_2CH_2NH\overset{\overset{NH}{\|}}{C}NH_2$	Methanethiol	CH_3
		Ethanethiol	CH_2CH_3
Aspartic acid	$CH(CO_2H)CH_2CO_2H$		
		1-Propanethiol	$CH_2CH_2CH_3$
Serine	$CH(CO_2H)CH_2OH$		
		2-Mercaptoethanol	CH_2CH_2OH
Arginine	$CH(CO_2H)CH_2CH_2CH_2NH\overset{\overset{NH}{\|}}{C}NH_2$		
		3-Mercaptopropionic acid	$CH_2CH_2CO_2H$
		2-Mercaptoethane sulphonic acid	$CH_2CH_2\overset{\overset{O}{\|}}{\underset{\underset{OH}{\|}}{S}}OH$

Fig. 1. Proposed structures of the amino acid derivatives formed with OPA-thiols.

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determined solely by the stability of the resultant derivatives, rather than by other physicochemical properties which they may impart. Mercaptoethanol has been claimed to be less stable than either ethanethiol or 3-mercaptopropionic acid[12–14] but still remains the most widely used thiol. Recently, however, N-*tert.*-butyloxycarbonyl-L-cysteine, N-acetyl-L-cysteine and N-acetyl-D-penicillamine have been employed with OPA as the chiral derivatisation reagent for the chromatographic resolution of amino acids[15,16], amino compounds[16,17] and lombricine (a novel multifunctional amino acid derivative found in certain invertebrates)[18].

The retention times of the OPA-thiol–amino adducts can be altered by changing the organic modifier and by the addition of anions to the eluent[1,19]. It would be expected that the use of differing thiols would produce differing isoindoles with differing chromatographic properties; however, there are no reports in the literature which investigate this idea, with the exception of a paper describing the use of OPA and 2-aminoethanol or taurine to detect thiols in urine and marine sediment porewaters[19].

This article reports the findings of a study which compares the effects of a series of thiol homologues and two ionisable thiol derivatives with the commonly used mercaptoethanol on the retention times of aspartic acid, serine, arginine and lombricine (Fig. 1).

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, standard amino acids, mercaptoethanol, mercaptoethanesulphonic acid sodium salt were purchased from Sigma; methanethiol sodium salt, ethanethiol, propanethiol and 3-mercaptopropionic acid from Aldrich. Lombricine was prepared according to the method of Euerby *et al.*[20].

Chromatographic systems

HPLC apparatus and experimental conditions were as described for the assay of lombricine[21].

Pre-column derivatisaton procedure

The derivatisation reagents were prepared daily by dissolving 18.9 mg of OPA in 3.5 ml of methanol and 35 ml of borate buffer (pH 9.5 adjusted with 2 *M* sodium hydroxide). To 5.5 ml of this solution was added 200 nmol of the appropriate thiol, and the mixture stored at 4°C in the dark until use. The standard amino acid solutions (50 μl) were mixed with the derivatisation reagent (50 μl) and incubated for 5 min at ambient temperature in the dark before immediate injection onto the column.

RESULTS AND DISCUSSION

The reversed-phase gradient HPLC assay described previously[21] was used to investigate the effects of differing thiols on the retention times of aspartic acid, lombricine, serine and arginine. All the thiols investigated reacted with the amino acids

and OPA in alkaline conditions (pH 9.5) to give highly fluorescent derivatives which reached their maximum fluorescence within 5 min; all the derivatives formed were ammenable to reversed-phase HPLC on a 125 × 4.6 mm I.D. Spherisorb ODS II

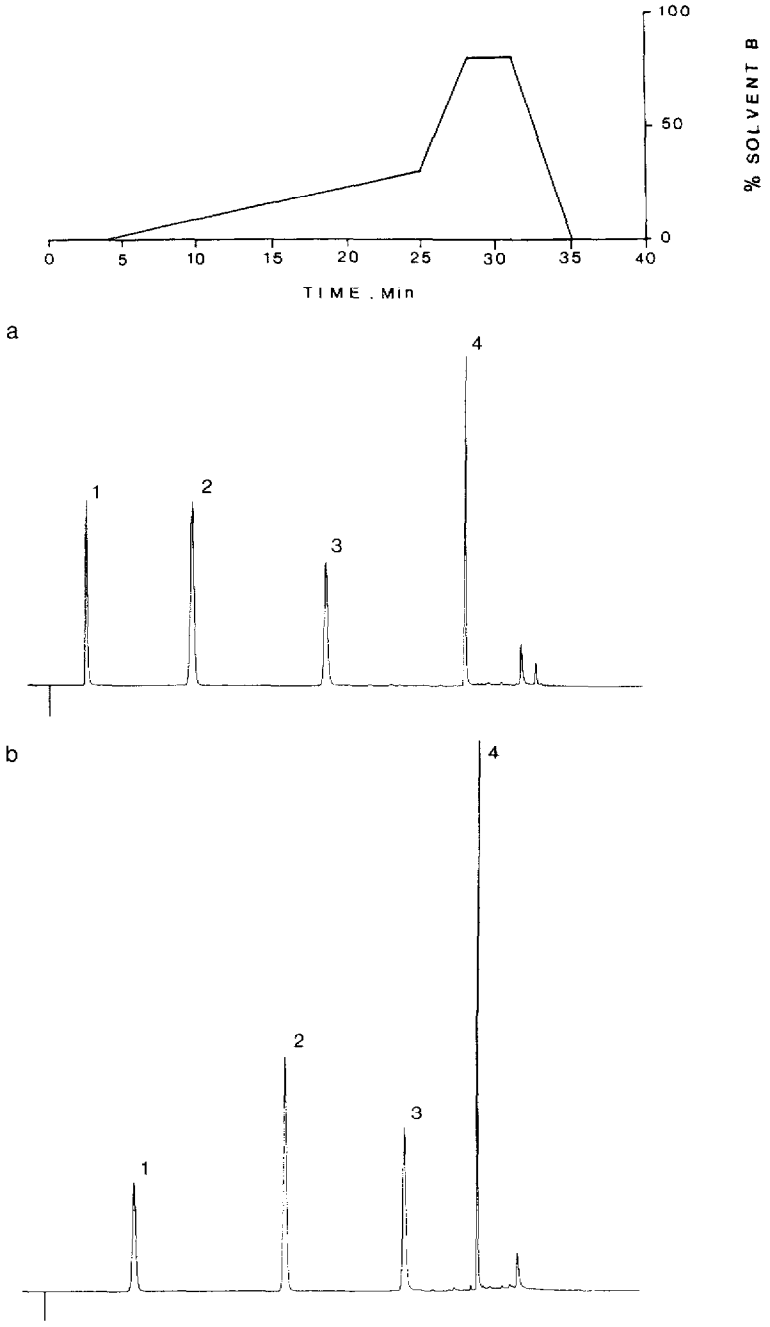

Fig. 1.

5-μm column (Fig. 2a–f). A series of four gradient runs were performed with the four amino acids for all the thiols. The derivatisation was performed prior to each injection and the reaction time was 5 min. Using peak heights, the average coefficient of variation was 1.5% and the average coefficient of variation for the retention time was 1.1%.

The partition coefficient values (log P) for mercaptoethanol and the thiol homologues methanethiol, ethanethiol and propanethiol were calculated from their hydrophobic fragmentation coefficients as described by Rekker[22] and were plotted against the retention times of the amino acids (Fig. 3 and Table I). It can be noted that there is a good relationship between the log P values of the thiols and retention

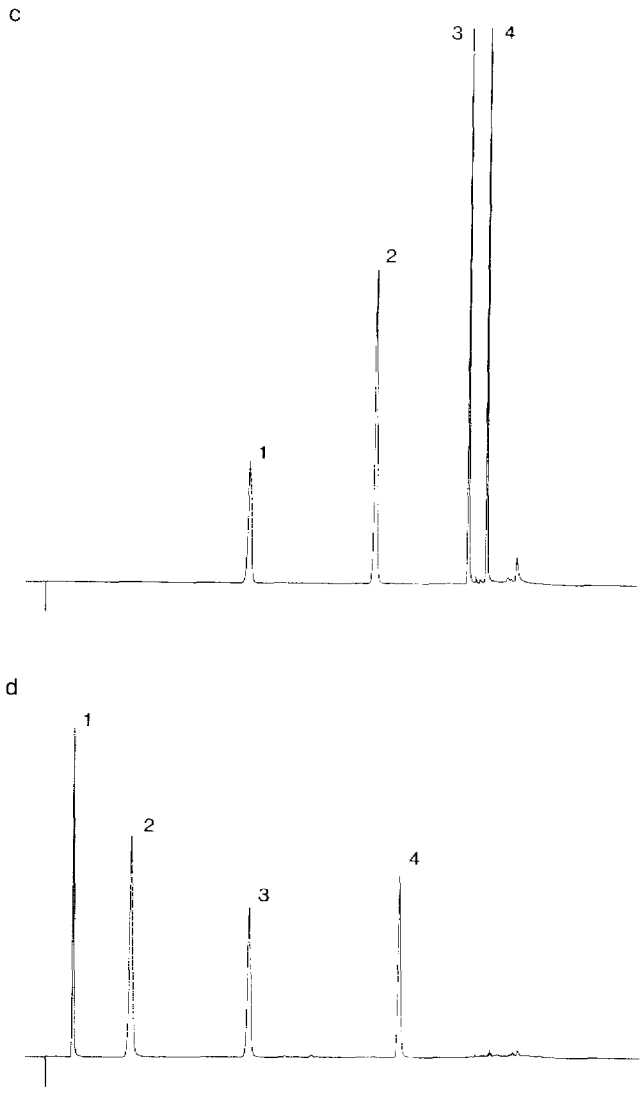

Fig. 1.

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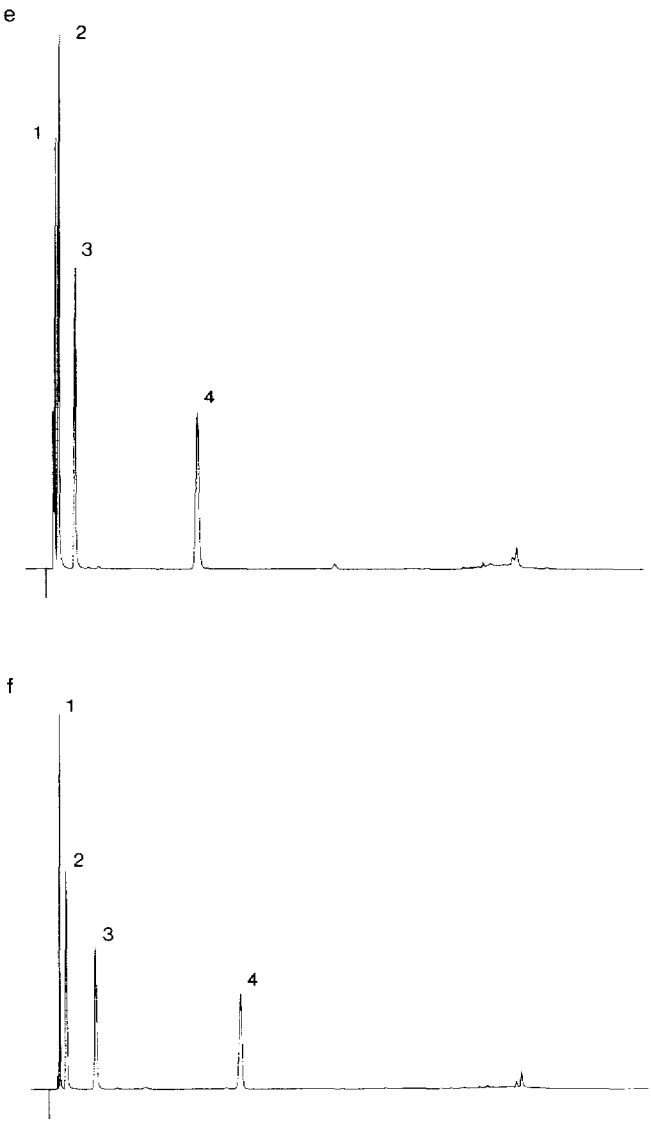

Fig. 2. HPLC of OPA–amino acid derivatives with differing thiols [(a) Methanethiol; (b) ethanethiol; (c) 1-propanethiol; (d) 2-mercaptoethanol; (e) 3-mercaptopropionic acid; (f) 2-mercaptoethanesulphonic acid] on a Spherisorb ODS II reversed-phase column (particle size 5 μm, 125 × 4.6 mm I.D.). Mobile phases: (A) 0.3 *M* sodium dihydrogenphosphate buffer (pH 7.2)–tetrahydrofuran–water (100:25:1875, v/v/v), (B) 0.3 *M* sodium dihydrogenphosphate buffer (pH 7.2)–acetonitrile–water (45:1100:855, v/v/v). Gradient: 0–4 min, 0% B; 4–25 min, 0–30% B; 25–28 min, 30–80% B; 28–31 min, 80% B; 31–35 min, 80–0% B; 35–40 min, 0% B. Flow-rate, 2 ml/min. Peaks: 1 = aspartic acid; 2 = lombricine; 3 = serine; 4 = arginine. Each peak corresponds to 50 pmol except for the serine and lombricine peaks which correspond to 30 and 75 pmol respectively. Fluorescence sensitivity employed was 0.2 R.F.U. Chromatographic conditions as in the Experimental section.

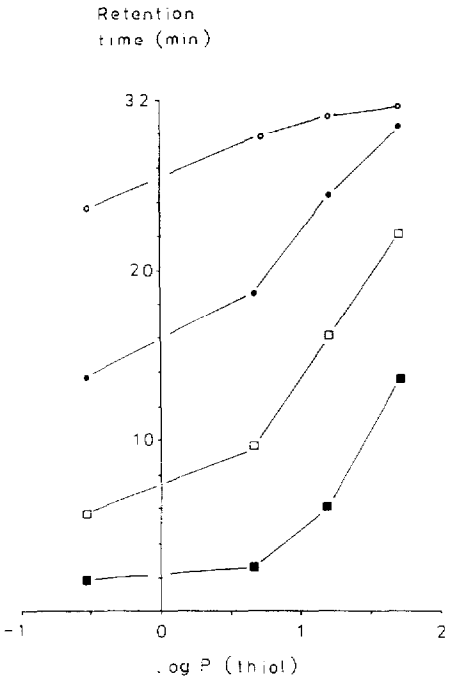

Fig. 3. The relationship of log P of the thiol (mercaptoethanol = −0.55; methanethiol = 0.66; ethanethiol = 1.18; propanethiol = 1.71) on the retention times of the resultant OPA-thiol–amino acid derivatives. Key to symbols: ○ = arginine; ● = serine; □ = lombricine; ■ = aspartic acid.

times, *i.e.* the more lypophilic the thiol the longer the retention time of the resultant derivative. The retention times for the derivatives of the thiol homologues and lombricine, which eluted on the linear gradient (4–25 min) part of the programme, were shown to be linearly related to the log P values of the thiols (regression analysis coefficient, $r^2 = 0.9998$). Whereas those of aspartic acid, serine and arginine showed no linearity owing to the fact that they eluted at differing gradients on the programme

TABLE I

EFFECT OF DIFFERING THIOLS ON THE RETENTION TIMES (min) OF AMINO ACIDS

Chromatographic conditions are as in the Experimental section.

Thiol	*Retention time (min)*			
	Aspartic acid	*Lombricine*	*Serine*	*Arginine*
Methanethiol	2.46	9.63	18.57	27.94
Ethanethiol	6.05	16.21	24.29	28.97
1-Propanethiol	13.60	22.00	28.32	29.58
2-Mercaptoethanol	1.80	5.64	13.60	23.70
3-Mercaptopropionic acid	0.52	0.76	1.90	10.12
2-Mercaptoethanesulphonic acid	0.60	1.04	3.24	12.80

TABLE II

EFFECT OF DIFFERING THIOLS ON THE SEPARATION FACTORS (α) OF AMINO ACIDS

Chromatographic conditions are as in the Experimental section.

Thiol	α		
	Aspartic acid/lombricine	*Lombricine/serine*	*Serine/arginine*
Methanethiol	3.91	1.93	1.50
Ethanethiol	2.68	1.50	1.19
1-Propanethiol	1.63	1.29	1.04
2-Mercaptoethanol	3.13	2.41	1.74
3-Mercaptopropionic acid	1.46	2.50	5.33
2-Mercaptoethanesulphonic acid	1.73	3.13	3.90

(*i.e.* the derivatives were experiencing differing rates of change of mobile composition). As expected, the ionisable thiol derivatives [3-mercaptopropionic acid (>99.5% ionised at pH 7.2) and mercaptoethanesulphonic acid] eluted faster than the lypophilic ones (*i.e.* methanethiol, ethanethiol and propanethiol); the polar thiol–mercaptoethanol derivative eluted between the ionic and lypophilic thiol derivatives.

There has recently been a report by Jinno and Tanigawa[23] describing the prediction of reversed-phase HPLC retention times of OPA-ethanethiol–amino acid derivatives. In the light of my findings it would be interesting to combine the thiol and amino acid hydrophobic parameters in Jinno and Tanigawa's[23] equations to, predict the retention times of a range of isoindoles. From Table II, it can be observed that changing the thiol employed in the pre-column derivatisation markedly effects the degree of separation achieved as a direct result of the shape of the gradient employed.

It appears, then, that by selecting a thiol of the appropriate log *P* value for use in the pre-column derivatisation reaction, it is possible to affect the retention time of the resultant derivative and also, depending on the gradient programme employed, the separation factors.

To conclude, the use of differing thiols in the OPA derivatisation of amino compounds provides a quick, convenient and complementary approach to optimising the assay of primary amino compounds.

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