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DETERMINATION OF AGMATINE, ARGININE, CITRULLINE AND ORNITHINE BY REVERSED-PHASE LIQUID CHROMATOGRAPHY USING AUTOMATED PRE-COLUMN DERIVATIZATION WITH *o*-PHTHALALDEHYDE

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SUMMARY

A method is presented for the pre-column derivatization of agmatine, arginine, citrulline and ornithine with *o*-phthalaldehyde–2-mercaptoethanol, and subsequent separation of the derivatives by reversed-phase liquid chromatography. Fluorescent response is linear from 10 to 150 pmol of injected analyte and detection limits range from 28 to 100 fmol. Response factors relative to the internal standard, homocysteic acid, were 1.16 (agmatine and arginine), 1.03 (citrulline) and 0.34 (ornithine). The applicability of the method to the measurement of arginase, arginine deiminase, arginine decarboxylase and other enzyme activities in bacterial extracts was examined.

INTRODUCTION

The fluorogenic pre-column derivatization of compounds possessing primary amino groups with *o*-phthalaldehyde–thiol (OPA–thiol) reagents followed by reversed-phase liquid chromatography (RPLC) has become a popular method for the analysis of amino acids [1–6], various metabolic products [7,8] and drugs [9]. Recently, this technique has also been used for the sensitive detection and assay of specific enzymes by quantitation of reaction products [10–13]. During a project to detect arginine-catabolising enzymes in extracts of thermophilic bacteria, the need arose to simultaneously measure enzymatically formed agmatine, citrulline and ornithine in the presence of excess substrate arginine in stopped-assay mixtures. The use of RPLC and pre-column derivatization with OPA to analyse amines in physiological fluids and tissues has shown the feasibility of separating arginine, citrulline and ornithine derivatives by this method [1,3,14,15]. Although there are few studies which record the elution behaviour of OPA derivatives of agmatine, the work of Griffin et al. [16] suggested that of

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the amines investigated in this study only ornithine would elute close to agmatine. The above method therefore appeared most suitable. In developing the procedure described in this paper, we also examined the mixing of OPA reagent and sample during automated pre-column derivatization and the effect high concentrations of arginine had on the response and elution behaviour of other analytes.

EXPERIMENTAL

Apparatus

Analyses were performed using a Waters Assoc. liquid chromatograph equipped with two M510 pumps, a WISP 710B autoinjector, an M420-AC fluorescence detector ($\lambda_{ex}=338$ nm, $\lambda_{em}=425$ nm) with the span control set at maximum and a gain of 16 unless otherwise indicated, an M720 system controller and an M730 data module. A Nova-Pak C_{18}, 5-μm, 150 mm $\times$ 4.6 mm I.D. stainless-steel column (Waters Assoc., Milford, MA, U.S.A.) enclosed in an M1122 oven (Waters Assoc.) set at 30°C was used for chromatographic separations.

Buffers and eluents

All water used was from a Milli-Q water purification system (Millipore, Milford, MA, U.S.A.). Potassium borate buffer (0.2 *M*, pH 9.4 at 20°C) was prepared by dissolving 3.092 g boric acid (Aristar grade, BDH, Poole, U.K.) in water and adjusting the pH with a saturated solution of potassium hydroxide (Analar grade, BDH) in a final volume of 250 ml. The buffer was passed through a 0.2-μm filter (Gelman Sciences, Australia) and stored at 4°C. A stock solution of eluent A was prepared by dissolving 13.609 g anhydrous potassium dihydrogenphosphate (analytical-reagent grade, Ajax Chemicals, Sydney, Australia) in 950 ml of water and adding potassium acetate (AR grade, Peking Chemical Works, Peking, China) to adjust the pH to 5.93 at 20°C in a final volume of 1 l. After filtering through a 0.45-μm membrane filter (Millipore) this solution was stored at 4°C in the dark in a sterile glass container. Eluent A was freshly prepared by a ten-fold dilution of stock A with water, vacuum-degassed by filtration before use and helium-sparged during use. Eluent B was a 4:3:3 (v/v/v) mixture of acetonitrile, methanol (both HPLC grade, Waters Assoc.) and water. Each component was filtered through a 0.22-μm aqueous Durapore filter (Millipore) before mixing, and eluent B was degassed for 25 min in an ultrasonic bath before use.

Reagents and solutions

Amino acids and agmatine sulphate were supplied by Sigma (St. Louis, MO, U.S.A.) except for L-arginine (chromatographically homogeneous, BDH) and L-ornithine hydrochloride (BDH and Sigma). Urea (AR grade) was obtained from Ajax Chemicals and ammonium chloride (GR grade) from Merck (Darmstadt, F.R.G.). Stock solutions (20 m*M*) of single amino acids and amines were prepared in 50 m*M* nitric acid (Aristar grade, BDH). Sample solutions were prepared for derivatization by mixing with an equal volume of 0.2 m*M* homocysteic acid in 4% (w/v) perchloric acid (AR grade, BDH), diluting ten-fold with filtered water and placing 100 μl in a Waters Assoc. small-volume-insert sample

vial. The samples were centrifuged (10 000 *g*, 5 min) before placing them onto the WISP autoinjector sample carousel.

Research-grade 2-mercaptoethanol (ME) was obtained from Serva (Heidelberg, F.R.G.). OPA (Sigma) was vacuum-sublimed before use. The OPA–ME derivatizing reagent was prepared by dissolving 50 mg OPA in 1 ml of filtered methanol, then adding 53 μl of ME and 9 ml of 0.2 *M* potassium borate buffer (pH 9.4) and was stored at 4 °C for not more than two days before use.

Chromatographic procedure

The WISP autoinjector was programmed to charge the sample needle with 7 μl of the OPA–ME derivatizing reagent, then 5 μl of sample, followed by immediate injection. Concomitantly with injection, the flow-rate was raised from 0 to 0.2 ml/min for 0.2 min, then reduced to 0.1 ml/min for 1 min. After injection (1.2 min) the flow-rate was increased linearly to 1 ml/min over 2.3 min at 20% eluent B in the mobile phase. This was followed by a 1.5-min linear gradient to 27% B and a 10.5-min isocratic step, then an 11.5-min linear gradient to 100% B. After 4 min at 100% B, reequilibration was initiated by a 9-min linear reverse gradient to 20% B and completed when 18 ml of this eluent had been passed through the column (see Fig. 1). The flow-rate was then reduced to 0 over 3 min and held for 1 min in preparation for the next injection. Total time between two injections was 60 min.

RESULTS AND DISCUSSION

The procedure described here separated the four derivatized amines (Fig. 1A). Elution times (see Table I) were highly reproducible, although a high concentration of arginine in the sample decreased the reproducibility. The isocratic step in the elution gradient was required to separate citrulline and arginine when high concentrations of arginine were present in the sample (Fig. 1B), and this led to broadening of these peaks. The presence of 5 nmol arginine in the sample also resulted in an additional peak which eluted before agmatine. The internal standard, homocysteic acid, eluted well away from the analyte peaks.

While an internal standard compensates for variation in sample volume and mixing of sample and derivatizing reagent between injections, the most important effect in our study was to compensate for a drop in OPA–ME reagent strength. Typically, a 9% decrease in sensitivity was observed over the first 4 h after placing fresh OPA–ME reagent in the sample compartment. Subsequently, sensitivity decreased by about 1% per hour. The initial loss of reagent effectiveness is probably due to the oxidation of thiols in the reagent [17]. This decreases the ME/OPA ratio below 2, which is the optimum for reaction rate [18], and thus for fluorescent response [19]. This oxidation was accelerated in the WISP sample compartment, which has a normal operating temperature of 30 °C. At this temperature significant evaporation from sample vials was observed over a 24-h period.

When peak areas were normalised with respect to homocysteic acid, the coefficients of variation ($n=5$) at the 50-pmol level were 7% for ornithine, 2% for agmatine, 0.9% for citrulline and 0.5% for arginine. Poor reproducibility for or-

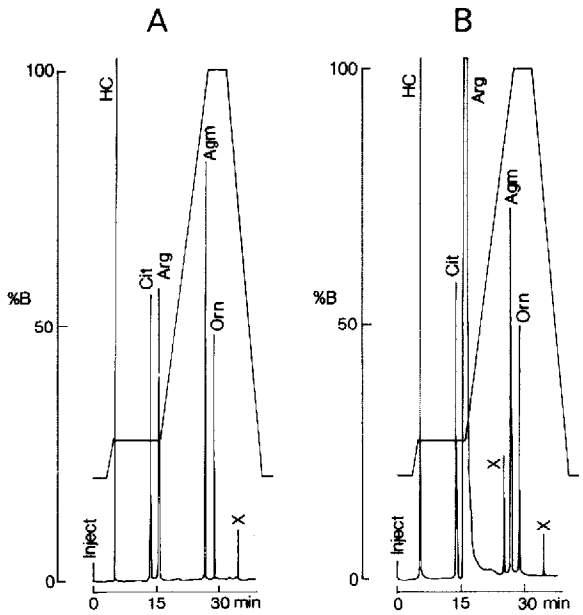

Fig. 1. Elution profiles of 50 pmol of OPA-ME-derivatized homocysteic acid (HC), citrulline (Cit), agmatine (Agm) and ornithine (Orn) in the presence of (A) 50 pmol arginine (Arg) and (B) 5 nmol arginine. X = Unknown peak. Gradient profile superimposed.

nithine has been observed previously [1]. An unidentified peak eluting at 33.8 min occurred in all analyses.

Elution times and response factors for agmatine, citrulline and ornithine relative to homocysteic acid determined at the 50-pmol level in the presence of 50 pmol and 5 nmol of arginine were not significantly different (Tables I and II).

Linearity of response in the 10–150 pmol range (corresponding to a concentration range of 0.04–0.6 m*M* in samples prior to addition of the internal standard solution) was excellent for all four analytes, the critical coefficient (r^2) being greater than 0.997 in every case. This was also the case for citrulline, agmatine and ornithine over the same concentration range in the presence of 5 nmol of arginine. At these high arginine concentrations the molar ratio of OPA to total derivatizable amino groups drops to approximately 40. According to Lindroth and Mopper [20], linearity of response can only be expected if OPA is present in at

TABLE I

ELUTION TIMES

Amount of arginine in injected sample	Elution time (mean ± S.D., $n = 5$) (min)				
	Homocysteic acid	Citrulline	Arginine	Agmatine	Ornithine
50 pmol	5.08 ± 0.02	13.36 ± 0.03	15.22 ± 0.04	25.98 ± 0.03	28.07 ± 0.00
5 nmol	5.12 ± 0.06	13.42 ± 0.10	15.18 ± 0.20	26.04 ± 0.06	28.09 ± 0.09

TABLE II

RESPONSE FACTORS RELATIVE TO HOMOCYSTEIC ACID

Amount of arginine in injected sample	Response factor (mean ± S.D., $n=5$)			
	Citrulline	Arginine	Agmatine	Ornithine
50 pmol	1.03 ± 0.01	1.16 ± 0.007	1.16 ± 0.02	0.34 ± 0.02
5 nmol	1.06 ± 0.01	–	1.20 ± 0.04	0.35 ± 0.01

least a 100- to 200-fold excess over analytes. However, our results are consistent with those of Cooper et al. [19], who have shown that OPA-to-amino acid ratios as low as 18 produce a linear response under correct reaction conditions.

The low fluorescent response of ornithine has been observed previously [3,15] and it is suggested that quenching due to the presence of two fluorescent isoindole structures in the derivative is responsible [21]. To test the purity of arginine, citrulline, agmatine and ornithine, 5 nmol of each were injected. The former three each gave a single major peak, but the elution profile of ornithine consisted of two major and at least seven minor peaks compared with the single peak seen for injections of picomole amounts of ornithine. The largest of the additional peaks eluted at 19.5 min with an area equivalent to the ornithine peak at 28 min. These multiple peaks were observed for both Sigma and BDH ornithine and may be due to incomplete derivatization of sample ornithine when present at high levels, with the rapidly formed, more highly fluorescent [21] and less hydrophobic mono-derivative of the δ-NH_2 group of ornithine accounting for the largest additional peak and eluting at 19.5 min.

Detection limits and sample–reagent mixing during derivatization

The method described was highly sensitive, with all analyte peaks readily detected at the 100-fmol level (Fig. 2). Apart from the analyte peaks, a number of contaminant peaks and baseline shifts became apparent at this sensitivity. The largest interfering peak, which may in part be due to the OPA–ammonia deriva-

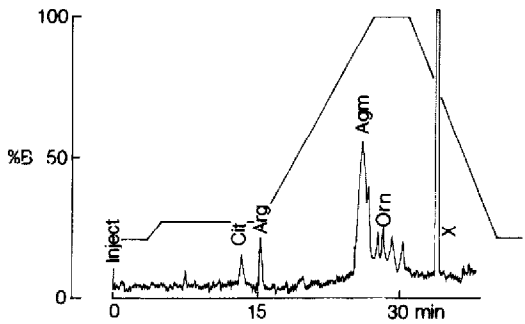

Fig. 2. Elution profile of 100 fmol of OPA–ME-derivatized citrulline (Cit), arginine (Arg), agmatine (Agm) and ornithine (Orn). Detector setting: gain = 128. X = Unknown peak. Gradient profile superimposed.

tive, coeluted with agmatine and caused a relatively high detection limit of about 100 fmol for agmatine. The detection limits for arginine, citrulline and ornithine calculated by the method of Knoll [22] with a peak width multiple of 10 were 28, 38 and 60 fmol, respectively. These are comparable with other automated pre-column derivatization analyses using OPA [1,2,23,24].

The use of automated on-line pre-column derivatization with OPA decreases the extent of decay of OPA derivatives and allows highly reproducible reaction timing. However, to achieve the high sensitivity and reproducibility the method is capable of, good mixing between the OPA–thiol reagent and sample in automated procedures is required. A number of workers have used purpose-built devices or pumping systems to ensure complete mixing [2,7,23,24], while others have employed the WISP autoinjector used in this study and a mixing chamber containing glass beads between the WISP and column [1,4,5]. One group [3] describes a successful pre-column derivatization procedure using the WISP without a mixing chamber, in which 10-μl volumes of OPA reagent and sample were "mixed in the sample needle for 2 min" before injection. Such mixing could not occur by diffusion, as the tubing forming the WISP sample needle has an internal cross-sectional area of approximately $2 \cdot 10^{-3}$ cm^2. A 10-μl sample occupies a length of 5 cm in this tubing.

In the absence of any additional pre-column mixing aids, we found that programming the flow-rate for a slow passage (0.1 ml/min) through the Nova-Pak column frit and onto the column resulted in adequate and reproducible mixing of the OPA reagent and sample. The success of this approach to mixing relies on the OPA and sample solutions being spread by the column frit to form narrow zones and the speed of the derivatizing reaction. Before the sample and OPA solutions pass onto the column packing they are spread by the column frit to zones of approximately 0.5 mm thickness. Mixing of the zones by diffusion is now possible, and this is assisted by the mechanical mixing that occurs by passage through the 2 μm mesh of the filter insert (Waters Assoc.) and the perforated packing retainer plate (Waters Assoc.). Mixing and reaction may continue as the solutions enter the column packing. The reaction time in this study was less than 1 min, but this is sufficient for complete OPA derivatization when factors such as the OPA-to-thiol ratio, reaction pH and OPA-to-amine ratio are optimised [19]. In addition, the reaction was conducted at 30°C in our experiments. A 1-min reaction time may even be excessive for ornithine, as the OPA derivative of this amino acid is very unstable in the reaction mixture [14,19,20]. This instability emphasises the importance of the precisely timed derivatization reactions afforded by automating the procedure.

We are currently applying the method described here to the simultaneous measurement of arginase, arginine deiminase and arginine decarboxylase activities in bacterial extracts (Fig. 3). These enzymes catabolise arginine to ornithine, citrulline and agmatine, respectively. As the former two enzymes also produce urea and ammonia, respectively, we examined the behaviour of these products in our RPLC system. Urea was not detected at the 5-nmol level. The OPA derivatives of ammonia and agmatine coeluted, but the response factor of the ammonia derivative (0.02 relative to homocysteic acid) was too low to interfere with the

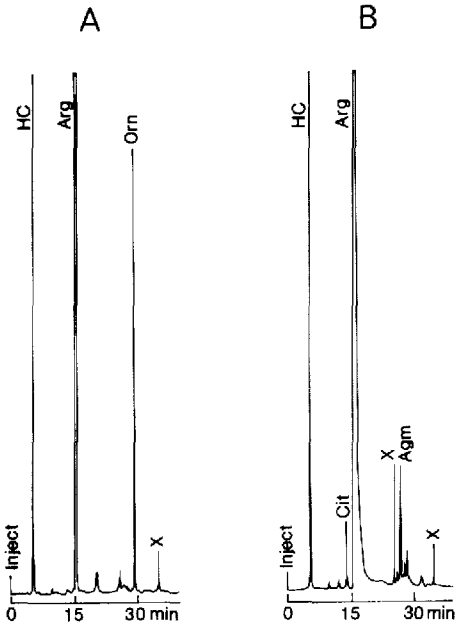

Fig. 3. Elution profiles of deproteinized stopped-assay supernatants resulting from the incubation of cell-free bacterial extracts with buffered 20 m*M* L-arginine. (A) *Bacillus licheniformis* DSM13* extract incubated for 10 min at pH 7.0 at 37°C. Assay solution was diluted 200-fold and a 5-μl sample was derivatized. (B) Tok12S1 (an extremely thermophilic, anaerobic, sulphur-dependent archaebacterium) extract incubated for 10 min at pH 6.2 at 80°C. Assay solution was diluted 20-fold and a 5-μl sample was derivatized. Peaks: HC = homocysteic acid; Arg = arginine; Orn = ornithine; Cit = citrulline; Agm = agmatine; X = unknown peak.

quantitation of agmatine. However, high levels of arginase activity have precluded the detection of the agmatine derivative because it coelutes with one of the minor additional peaks resulting from large amounts of sample ornithine. The method has been successfully used to detect two other enzymes of arginine catabolism, namely ornithine aminotransferase by measurement of glutamate (elution time 5.55 min, response factor 0.9) formed from ornithine and 2-oxoglutarate, and catabolic ornithine carbamyltransferase by measurement of ornithine formed from citrulline in a phosphate buffer.

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