Journal of Chromatography, 353 (1986) 201–206
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 744

AUTOMATED ON-COLUMN DERIVATIZATION OF THE ANTIBIOTICS PHOSPHINOTHRICIN AND PHOSPHINOTHRICYL-ALANYL-ALANINE WITH *o*-PHTHALALDEHYDE AND MICROBORE COLUMN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR QUANTITATIVE DETERMINATION IN BIOLOGICAL CULTURES

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SUMMARY

The concentration of the antibiotic phosphinothricyl-alanyl-alanine in biological cultures has been determined by reversed-phase microbore-column high-performance liquid chromatography. The procedure, involving precolumn derivatization with *o*-phthalaldehyde, mercaptoethanol reagent, and UV detection at 338 nm, features a rapid and convenient separation, together with a high degree of precision and accuracy, and is suitable for routine analyses.

INTRODUCTION

The antibiotic phosphinothricyl-alanyl-alanine (PTC-Ala-Ala), produced by *Streptomyces viridochromogenes*, is active against Gram-positive and Gram-negative bacteria as well as fungi[1]. The tripeptide represents the form in which the antibiotic is transported into the bacterial cell, where the free acid, phosphinothricin (PTC), is released. PTC is a potent inhibitor of glutamine synthetase and shows strong herbicidal activity[1–3]. The structures of PTC and PTC-Ala-Ala are shown in Fig. 1.

Quantitative determination of the compounds in biological samples during the fermentative production period requires selectivity, sensitivity, a short analysis time, reproducibility, and, preferably, automation.

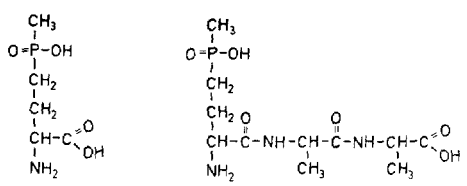

Fig. 1. Structures of PTC (left) and PTC-Ala-Ala (right).

0021-9673/86/$03.50

Precolumn derivatization of amino acids with *o*-phthalaldehyde (OPA) in the presence of mercaptoethanol, with subsequent separation of the derivatives in a reversed-phase high-performance liquid chromatographic (HPLC) system with fluorescence detection has gained wide popularity[4–10]. The same derivatization procedure has been described for the determination of the component ratio of aminoglycoside antibiotics[11]. However, certain disadvantages remain. The OPA reaction is limited to primary amino acids, and the derivatives are stable for only 10–15 min[12]. The instability can be improved when the derivatives are retained on the column without solvent flow, if the pH of the solvent and the concentration ratio OPA:amino acids are optimized[13]. Nevertheless, for routine analytical determinations of amino acids, an automated derivatization procedure is indispensable, as described for the on-column derivatization of amino acids with OPA on microbore columns[14].

EXPERIMENTAL

Chemicals

Methanol (HPLC grade) and sodium acetate (analytical grade) were obtained from Merck (Darmstadt, F.R.G.). Water was purified by means of a Milli-Q system (Millipore, Eschborn, F.R.G.). The derivatizing agent was Fluoraldehyd™, a ready-to-use mixture of *o*-phthalaldehyde, 2-mercaptoethanol and Brij 35 in a borate buffer (Pierce, Rockford, IL, U.S.A.). PTC and PTC-Ala-Ala were obtained from Hoechst (Frankfurt, F.R.G.).

Chromatographic system

A HP-1090A liquid chromatograph (Hewlett-Packard, Waldbronn, F.R.G.) was used with a DR5 solvent delivery system, a variable-volume auto-injector, an autosampler, and a thermostatically controlled column compartment.

The OPA derivatives were identified by means of a HP-1040A diode-array detection system. A detection wavelength of 338 nm was used, and the ratio wavelength:bandwidth was 338:10.

Detector signals were processed and recorded on a HP-3392A recording integrator. Signals and spectra were stored on a HP-9121 flexible disk drive and plotted with a HP-7470A plotter.

Columns

The microbore column (100 × 2.1 mm I.D.) was fitted with a guard-column (20 × 2.1 mm I.D.) and filled with 5 μm Hypersil ODS (Hewlett-Packard). The column-compartment temperature was set to 40°C.

Mobile phases

The OPA derivatives were separated by gradient elution. Solvent A was 0.1 *M* sodium acetate buffer, solvent B was methanol. The linear gradient was from 20 to 60% solvent B in 5 min, increasing in 2 min to 100% solvent B with a 2-min hold at 100% solvent B. The flow-rate was 350 μl/min.

Derivatization procedure

The centrifuged biological samples and the OPA reagent were automatically

and sequentially drawn up by the injection system from two different vials; first, 4 μl of OPA reagent; second, 0.5 μl of sample; third, 4 μl of OPA reagent. The mixture was transferred to the column at a flow-rate of 10 μl/min. After a reaction time of 1 min, the flow-rate was automatically increased to 350 μl/min and the derivatives were separated by gradient elution within 8 min. Because of the instability of the OPA derivatives, it was necessary to prepare them freshly prior to the analysis in an automated derivatization procedure.

RESULTS

The elution profiles and UV spectra of the OPA derivatives of PTC and PTC-Ala-Ala at as level of 2.5 nmol are shown in Fig. 2. Both substances are well separated at 3.7 min (PTC) and 5.2 min (PTC-Ala-Ala) during the very short gradient elution programme of 8 min.

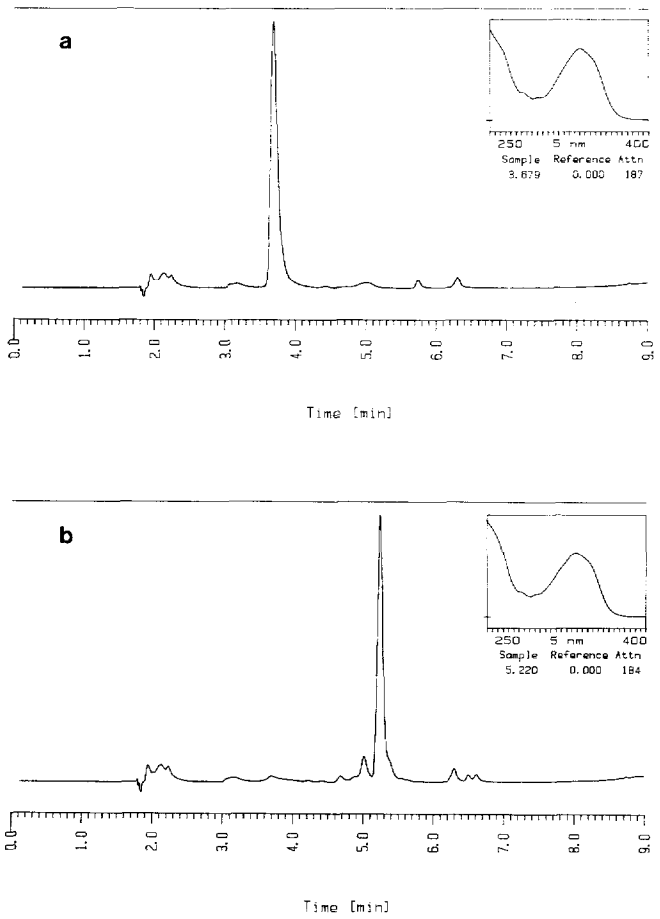

Fig. 2. Separation of PTC and PTC-Ala-Ala standards. (a) 1.25 nmol PTC, attn. 138 mAU; (b) 2.50 nmol PTC-Ala-Ala, attn. 117 mAU.

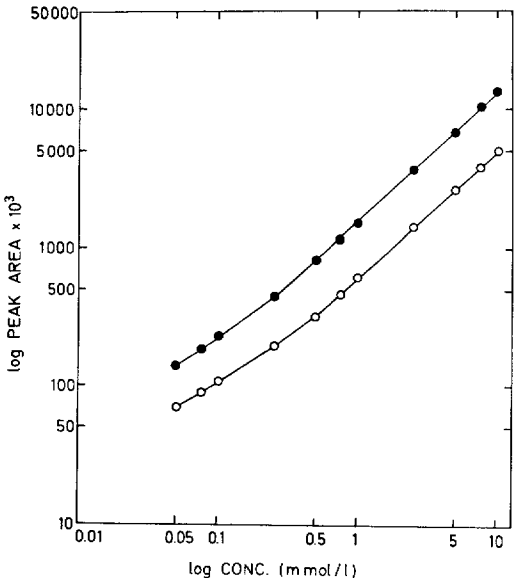

Fig. 3. Linear dynamic range of PTC and PTC-Ala-Ala determination. ●—●, PTC; ○—○, PTC-Ala-Ala.

Detection was carried out by means of diode-array technology at 338 nm, which is the UV maximum of the OPA derivatives. Quantitative determination of the compounds requires linearity in the range of analytical interest. With UV detection, this range is achieved in a concentration range between 0.3 and 10 mmol/l, which corresponds to between 150 pmol and 5 nmol PTC or PTC-Ala-Ala per sam-

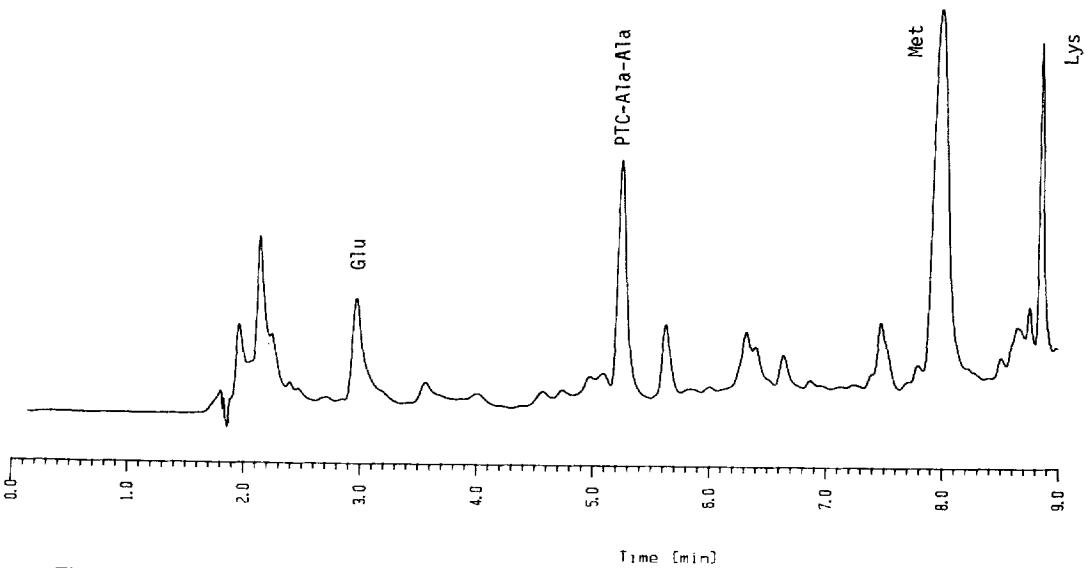

Fig. 4. Typical chromatogram of a biological sample. Determination of PTC-Ala-Ala in the culture filtrate of *S. viridochromogenes*.

TABLE I

PRECISION OF RETENTION TIMES AND PEAK AREAS

	*Relative standard deviation (%)**	
	Retention time	*Peak areas*
PTC, standard (2.5 mmol/l)	0.24	2.62
PTC-Ala-Ala, standard (2.5 mmol/l)	0.15	1.50
PTC-Ala-Ala, biological sample	0.20	1.75

* Based on twenty replicate analyses.

ple, as shown in Fig. 3. By defining the detection limit as having a signal-to-noise ratio of 5, the detectable limit for PTC would be 10 pmol and for PTC-Ala-Ala 25 pmol. However, for quantitation during fermentative production processes, this sensitivity was more than adequate, because antibiotic concentrations should be increased to a level of more than 1 mmol/l.

Fig. 4 shows the elution profile of an OPA-derivatized biological sample of culture filtrate from *S. viridochromogenes* in the course of PTC-Ala-Ala fermentation. In this case, the PTC-Ala-Ala concentration was 1.6 mmol/l.

The precision of the method was tested by assaying the antibiotic standards and biological samples twenty times, as shown in Table I. The reproducibility of both the retention time and the peak area proved the system to be suitable for routine analysis.

CONCLUSIONS

A rapid and convenient separation technique for the quantitative determination of the antibiotics PTC and PTC-Ala-Ala has been developed, based on automated precolumn derivatization with OPA. Whereas the detection limit of 1 pmol (or in the femtomol range), which can be achieved by fluorescence detection of amino acid OPA derivatives, was not achieved by UV detection at 338 nm, the detection limits of PTC at 10 pmol and of PTC-Ala-Ala at 25 pmol with a linear response in the concentration range between 0.3 and 10 mmol/l was adequate for the quantitative determination of the compounds during the fermentative production process.

ACKNOWLEDGEMENTS

We thank Hoechst for providing samples of PTC and PTC-Ala-Ala. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 76).

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