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SEPARATION OF AMINO ACIDS AND ANTIBIOTICS BY NARROW-BORE AND NORMAL-BORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PRE-COLUMN DERIVATIZATION

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SUMMARY

The selectivity, efficiency and lifetime of normal- and narrow-bore columns for high-performance liquid chromatography were investigated for the separation and quantification of amino acids and the amino acid-like antibiotics phosphinothricin and phosphinothricylalanylalanine in biological samples. These compounds were determined by an automated pre-column derivatization with *o*-phthalaldehyde–2-mercaptoethanol reagent and UV detection at 338 nm.

INTRODUCTION

Pre-column derivatization of amino acids with a mixture of *o*-phthalaldehyde and 2-mercaptoethanol or 3-mercaptopropionic acid and separation by reversed-phase high-performance liquid chromatography (RP-HPLC) has gained increasing importance[1–7], because of the shorter analysis time and increased sensitivity compared with the classical amino acid analyzers. In a previous paper, an automated pre-column derivatization technique with the above-mentioned reagents and separation on reversed-phase microbore columns was described for the quantification of the antibiotics phosphinothricin (PTC) and phosphinothricylalanylalanine (PTC-Ala-Ala) during their fermentative production[8]. The structures of these amino acid-like antibiotics are shown in Fig. 1.

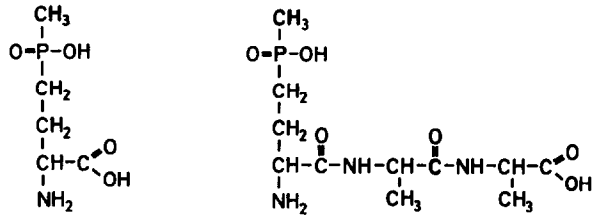

Fig. 1. Structures of PTC (left) and PTC-Ala-Ala (right).

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This method featured a rapid and convenient separation, together with a high degree of precision and accuracy, and seemed to be suitable for routine analyses. However, routine separation requires data on the selectivity, efficiency and lifetime of columns packed with different materials as well as those from different manufacturers. Narrow-bore and normal-bore HPLC columns should be compared during 1000 injections of biological samples onto each column. We were interested in the advantages and disadvantages of these columns for routine analyses.

EXPERIMENTAL

Chemicals

Acetonitrile and tetrahydrofuran (HPLC grade), sodium dihydrogenphosphate and disodium hydrogenphosphate dihydrate (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 Fluoraldehyde™, a ready-to-use mixture of *o*-phthalaldehyde (OPA), 2-mercaptoethanol and Brij 35 in a borate buffer; the reagent and amino acid standard H were obtained from Pierce Chemicals (Rockford, IL, U.S.A.). PTC and PTC-Ala-Ala were a gift from Hoechst (Frankfurt, F.R.G.).

Chromatographic system

An HP-1090A liquid chromatograph (Hewlett-Packard, Waldbronn, F.R.G.) was used with a DR5 solvent-delivery system, a variable-volume autoinjector, an autosampler and a thermostatically controlled column compartment. The OPA derivatives were identified by means of an HP-1040A diode-array detection system. A detection wavelength of 338 nm and a bandwidth of 10 nm were used, with 550 nm and a bandwidth of 100 nm as reference. Detector signals were processed and recorded on an HP-3392A recording integrator, stored on an HP-9121 flexible-disk drive and plotted with an HP-7470A plotter.

Guard columns

The normal-bore cartridges (20 mm × 4.6 mm I.D.) and narrow-bore cartridges (10 mm × 3.0 mm I.D.), filled with Shandon Hypersil ODS (5 μm) or Spherisorb ODS II (5 μm), were obtained from Bischoff (Leonberg, F.R.G.).

Normal-bore columns

The Hyperchrome HPLC columns (125 mm × 4.6 mm I.D.), filled with Shandon Hypersil ODS (5 μm) and Spherisorb ODS II (5 μm), were also obtained from Bischoff.

Narrow-bore columns

The following narrow-bore columns were used: CGC glass cartridge (150 mm × 3.0 mm I.D.), filled with LiChrosorb RP-18 (5 μm) from Merck; microbore HPLC column (150 mm × 2.0 mm I.D.), filled with Ultrasphere ODS (5 μm) from Beckman Instruments (Munich, F.R.G.); microbore HPLC column (100 mm × 2.1 mm I.D.), filled with Shandon Hypersil ODS (5 μm) from Hewlett-Packard; microbore HPLC column (100 mm × 2.0 mm I.D.), filled with Shandon Hypersil ODS (5 μm) from

Knauer (Berlin, F.R.G.); microbore HPLC column (100 mm × 1.8 mm I.D.), filled with Shandon Hypersil ODS (5 μm) from Bischoff; microbore HPLC column (100 mm × 2.1 mm I.D.), filled with Spherisorb ODS II (5 μm) from Bischoff.

Mobile phases

Solvent A was 12.5 m*M* sodium phosphate buffer (pH 7.2) with addition of 0.5% tetrahydrofuran; solvent B was acetonitrile.

The separation of amino acids was performed with linear gradient elution from 5 to 30% solvent B in 20 min, increasing in 1 min to 80% solvent B with a 1-min hold. The flow-rate was 2 ml/min in the case of 4.6 mm I.D. columns, 0.8 ml/min for 3.0 mm I.D. columns and 0.3 ml/min for 2.1, 2.0 and 1.8 mm I.D. columns, respectively.

The separation of PTC and PTC-Ala-Ala in biological samples was performed with linear gradient elution from 5 to 35% solvent B in 9 min in the case of 4.6 mm I.D. columns or narrow-bore columns with a length of 150 mm, and in 7 min using narrow-bore columns with a length of 100 mm, respectively, increasing in 1 min to 80% solvent B with a 1-min hold; the flow-rates were as mentioned above.

Derivatization procedure

The amino acid standard solution or the centrifuged biological samples and the OPA reagent were automatically and sequentially drawn up by the injection system from two different vials; in the case of normal-bore HPLC, first, 4 μl of OPA reagent, secondly, 2 μl of sample and thirdly, 4 μl of OPA reagent. In the case of narrow-bore HPLC, the volumes were 2 μl, 1 μl and 2 μl respectively. After a reaction time of 1 min, the mixture was transferred to the HPLC column within 1 min at a flow-rate of 10 μl/min. Then the flow-rate was automatically increased to the values described above.

RESULTS

The column efficiency was tested using a conventional system with aromatic

TABLE I

EFFICIENCY OF NORMAL- AND NARROW-BORE REVERSED-PHASE HPLC COLUMNS

N = number of plates.

Packing material	*Column dimensions (mm × mm)*	*N per column*	*N per metre*
Normal-bore columns			
Shandon Hypersil ODS	125 × 4.6	8000	64000
Spherisorb ODS II	125 × 4.6	8700	69600
Narrow-bore columns			
LiChrosorb RP-18	150 × 3.0	7280	48500
Ultrasphere ODS	150 × 2.0	11300	75300
Shandon Hypersil ODS	100 × 2.1	6700	67000
Shandon Hypersil ODS	100 × 2.0	6000	60000
Shandon Hypersil ODS	100 × 1.8	5500	55000
Spherisorb ODS II	100 × 2.1	6650	66500

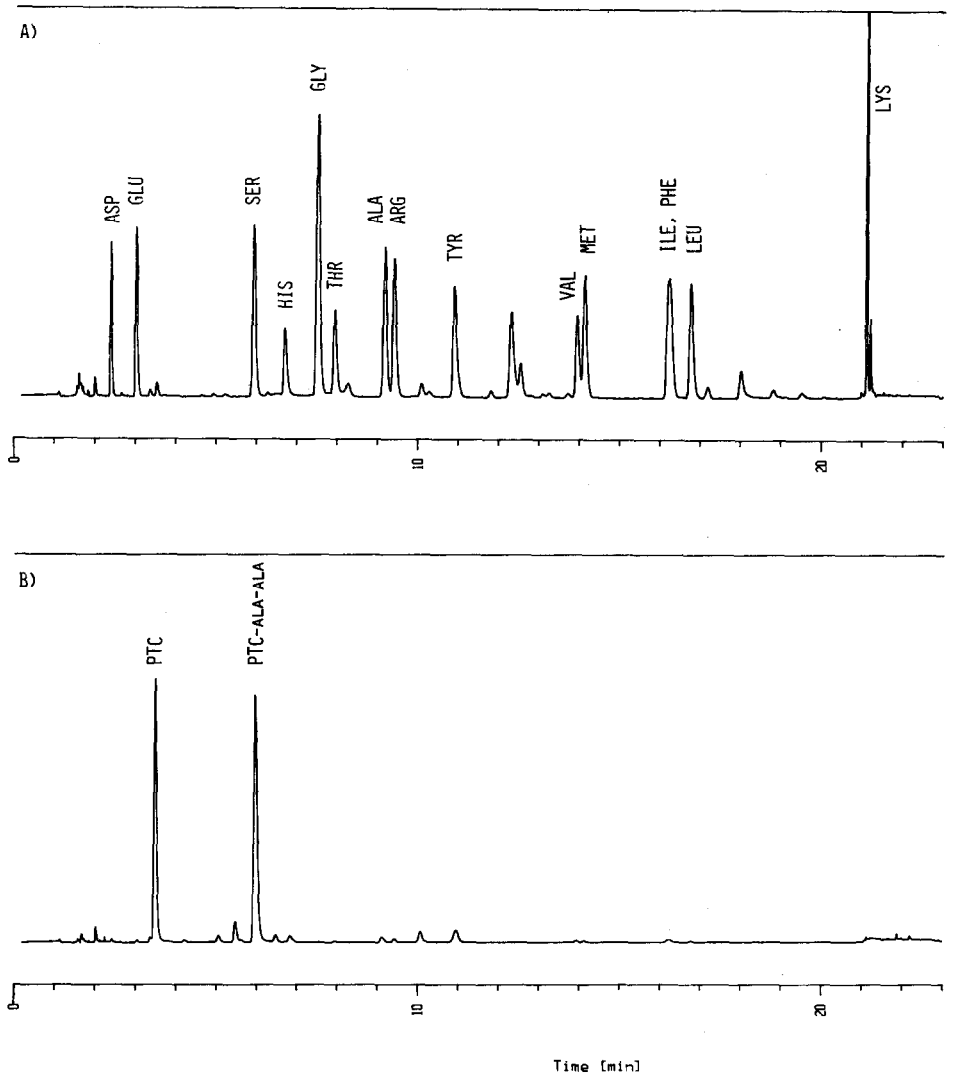

Fig. 2. Normal-bore HPLC on Shandon Hypersil ODS (125 mm × 4.6 mm). Sample volume: 2 μl. (a) Amino acid standard (2.5 μmol/ml), attenuation 75 mAU. (b) PTC (2.5 μmol/ml) and PTC-Ala-Ala (5.0 μmol/ml), attenuation 100 mAU.

standards and, depending on the packing material as well as on the column manufacturers, by comparing identical stationary phases, as shown in Table I.

The selectivity of the different reversed-phase materials for the separation of OPA derivatives of amino acids, PTC and PTC-Ala-Ala was investigated using linear gradient elution. If baseline separation of all amino acids is desired, the gradient profile must be modified, as detailed in other publications[3,7,9]. Cysteine, proline and hydroxyproline cannot be detected by OPA derivatization.

In Figs. 2 and 3 the separations of the amino acid standard, PTC and PTC-

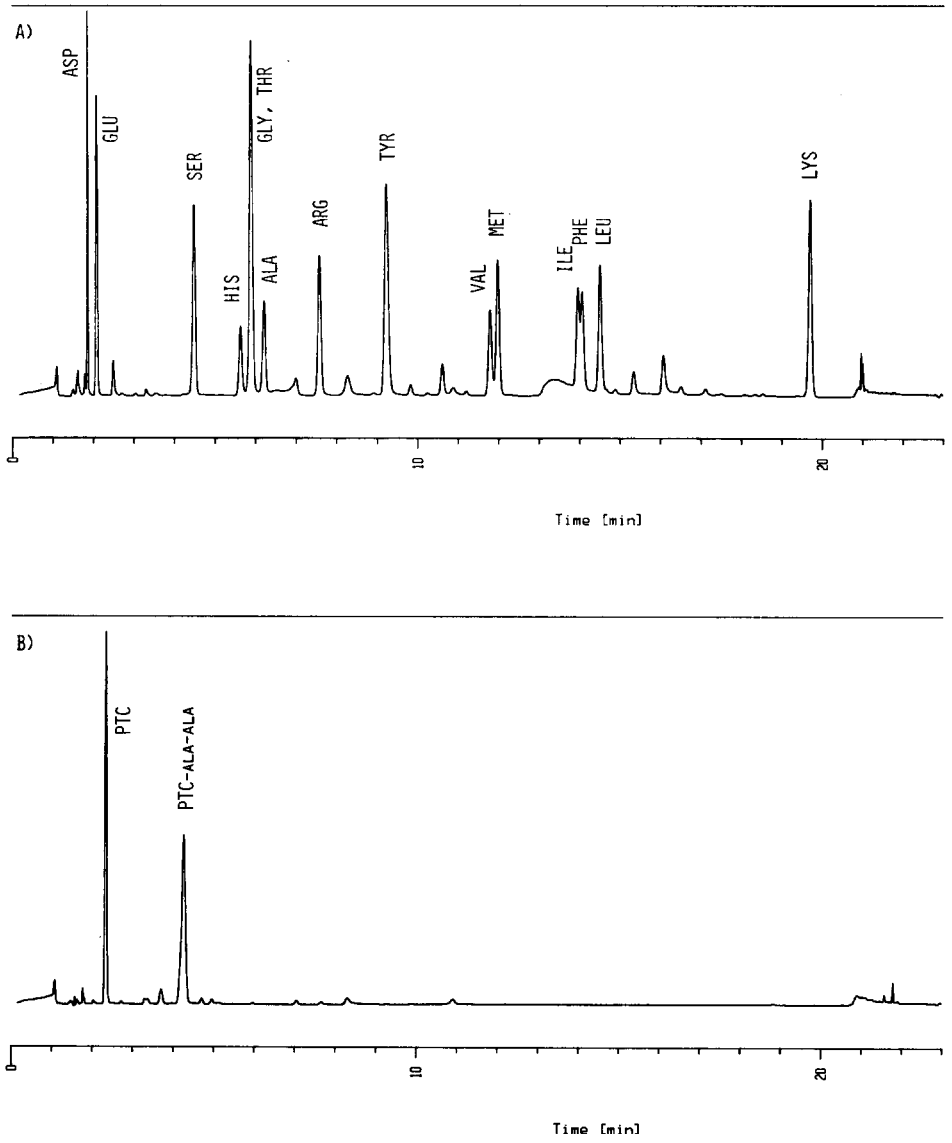

Fig. 3. Normal-bore HPLC on Spherisorb ODS II (125 mm × 4.6 mm). Details as in Fig. 2.

Ala-Ala on normal-bore columns, packed with Shandon Hypersil ODS and Spherisorb ODS II, are shown, respectively.

The separations of the same samples on narrow-bore columns, but in lower concentrations and smaller injection volumes, are shown in Figs. 4–8, with LiChrosorb RP-18, Ultrasphere ODS and Shandon Hypersil ODS from different column manufacturers, and Spherisorb ODS II as stationary phases. The differences in peak sharpness, and symmetry are due to differences in the packing material, packing procedure or column hardware. Shandon Hypersil ODS and Spherisorb ODS II seem

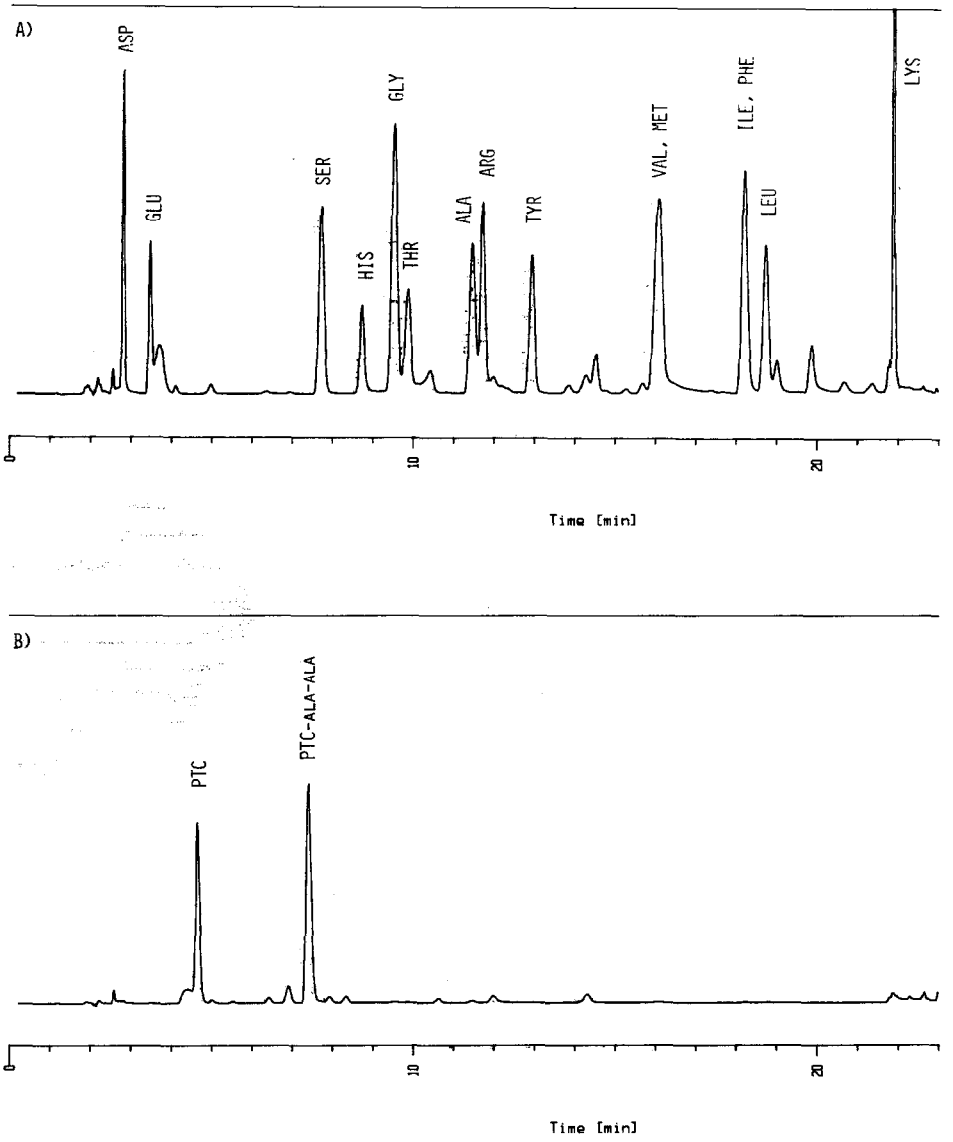

Fig. 4. Narrow-bore HPLC on LiChrosorb RP-18 (150 mm × 3.0 mm). Sample volume; 1 μl. (a) Amino acid standard (2.5 μmol/ml), attenuation 100 mAU. (b) PTC (2.5 μmol/ml) and PTC-Ala-Ala (5.0 μmol/ml), attenuation 200 mAU.

to be the most suitable stationary phases for the separation of OPA derivatives of amino acids, PTC and PTC -Ala-Ala.

The detection sensitivity as measured by the peak area was enhanced by a factor of 8 in the case of PTC and PTC-Ala-Ala and by a factor of 8–12 in case of amino acids in the standard solution, comparing 1.8, 2.0 and 2.1 mm I.D. columns with 4.6 mm I.D. columns, and by a factor of 2 in the case of PTC, PTC-Ala-Ala

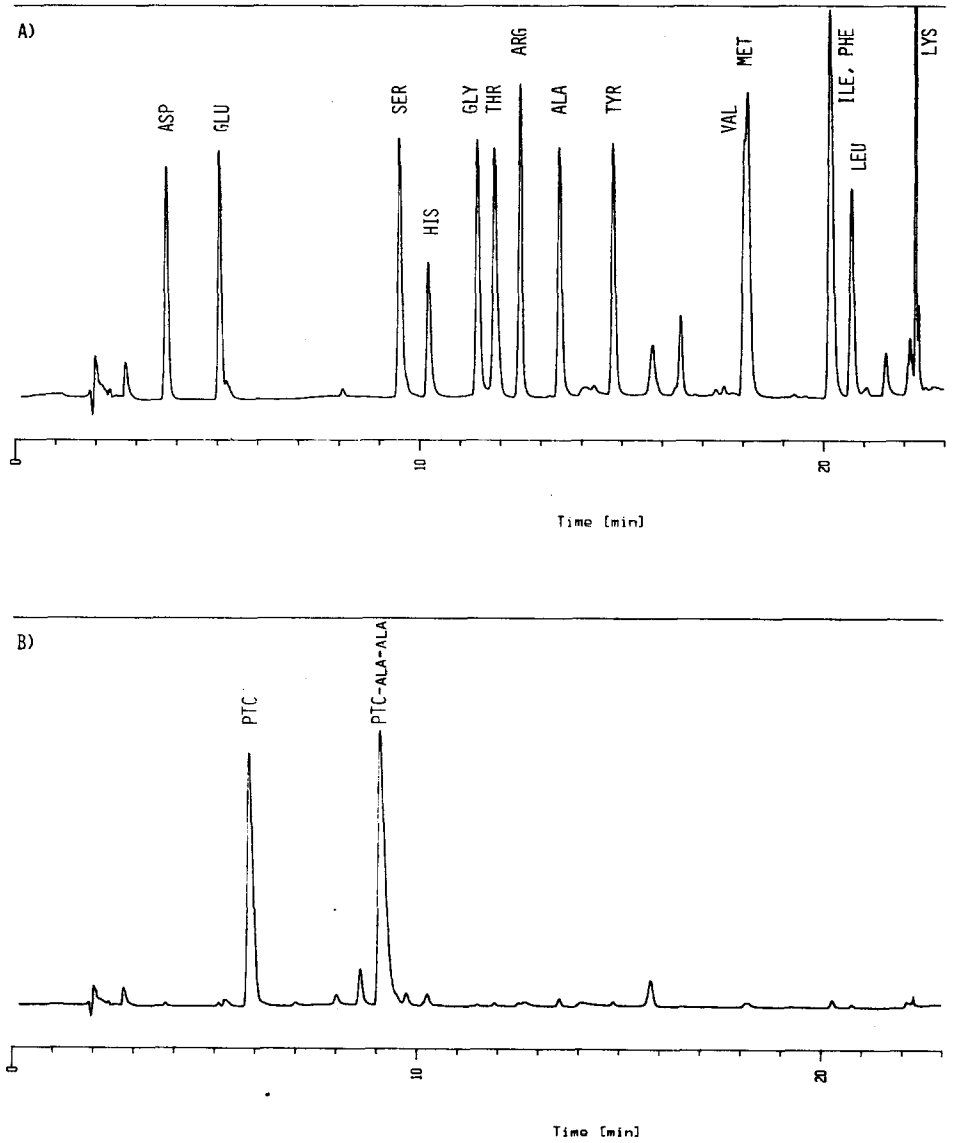

Fig. 5. Narrow-bore HPLC on Ultrasphere ODS (150 mm × 2.0 mm). Sample volume; 1 μl. (a) Amino acid standard (0.75 μmol/ml), attenuation 100 mAU. (b) PTC (1.25 μmol/ml) and PTC-Ala-Ala (2.5 μmol/ml), attenuation 200 mAU.

and amino acids using 3.0 mm I.D. columns, when the same sample volume was injected. This enhancement using narrow-bore columns is in variance with theoretical considerations. Normal- and narrow-bore columns filled with the same packing material generated about the same plate number, and so had the same efficiency. The internal volume, calculated from the ratio of the cross-sectional area, differed by factors of 4.8, 5.3 and 6.5 when comparing 4.6 mm I.D. columns with 2.1, 2.0 and

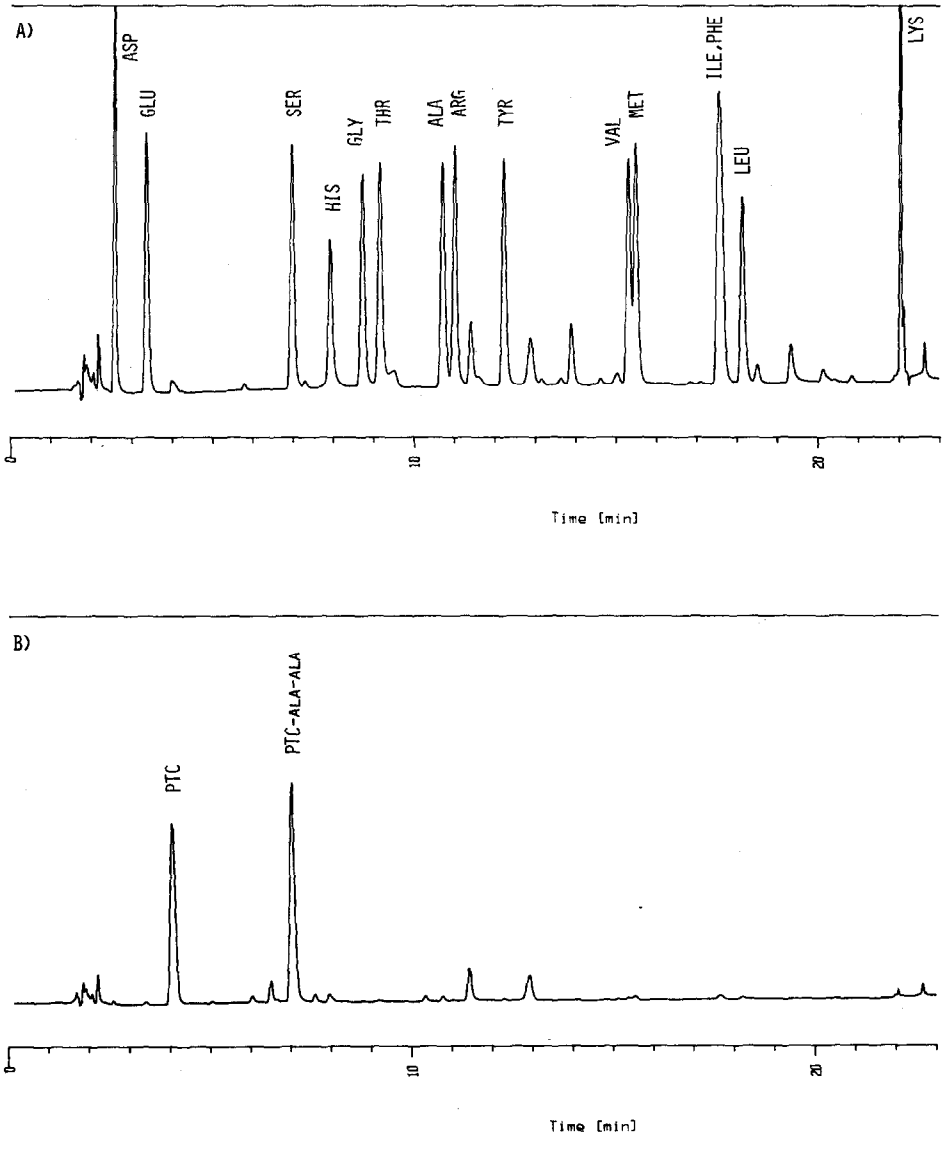

Fig. 6. Narrow-bore HPLC on Shandon Hypersil ODS (100 mm × 2.1 mm). Details as in Fig. 5.

1.8 mm I.D. columns, respectively. A reduction of the flow-rate in proportion to the column diameter reduces the volume in which an eluting peak is dissolved and so the peak heights and areas should be increased by the same factors. However, we found enhancements of 8–12. These results were confirmed by using columns from different batches of the packing materials.

The separation of a biological sample with short gradient elution for the quantification of PTC and PTC-Ala-Ala during their fermentative production is shown

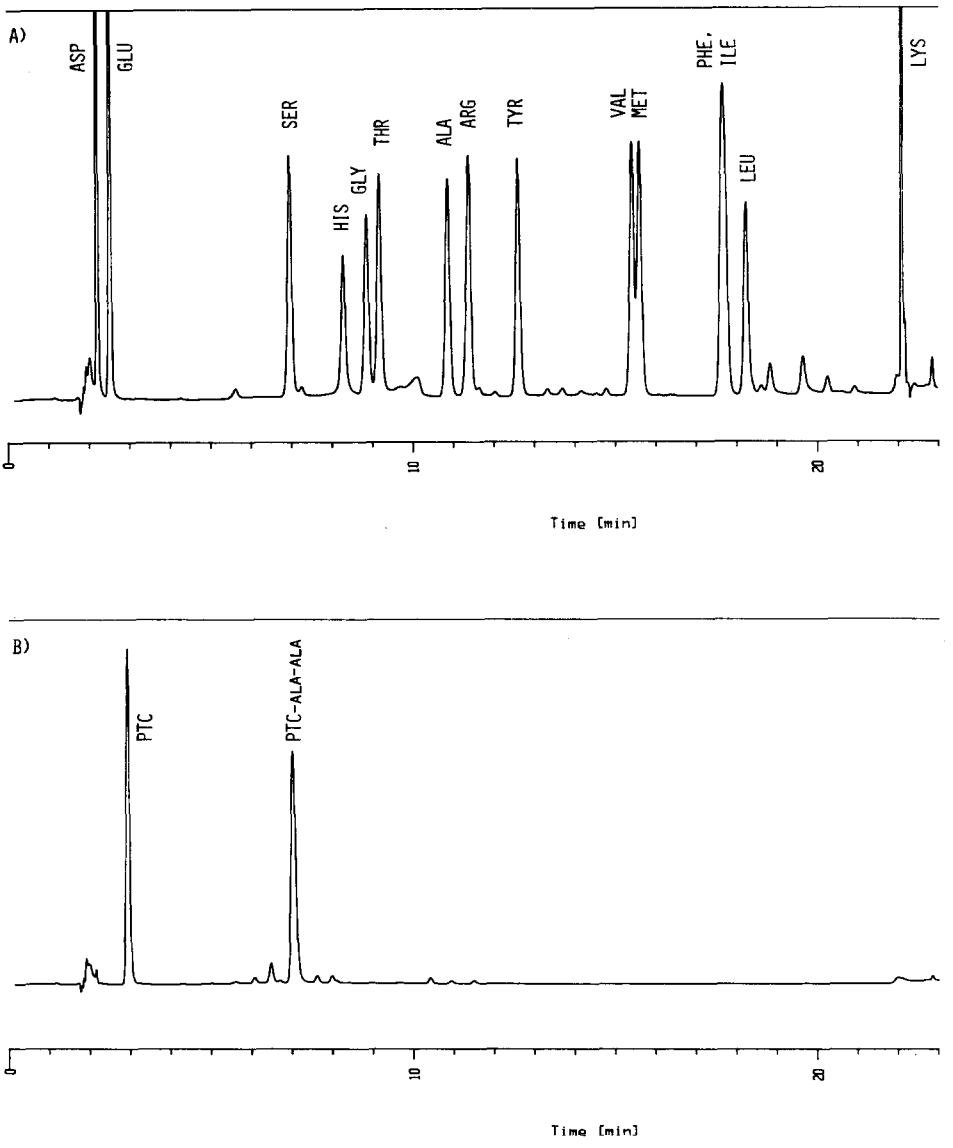

Fig. 7. Narrow-bore HPLC on Shandon Hypersil ODS (100 mm × 2.0 mm). Details as in Fig. 5.

in Fig. 9. An identical sample was injected onto a normal-bore and a narrow-bore column, packed with the same stationary phase.

The separation of biological samples in a routine analytical program demands investigation of the lifetime of normal- and narrow-bore columns. The columns were fitted with a guard column to protect the main column and to enhance lifetime. Up to 1000 samples of centrifuged fermentation broth were injected onto each column, and the efficiency was determined periodically. After 80 injections of biological samples, the columns and guard columns were cleaned during 20 min with methanol and acetonitrile; they were stored in acetonitrile.

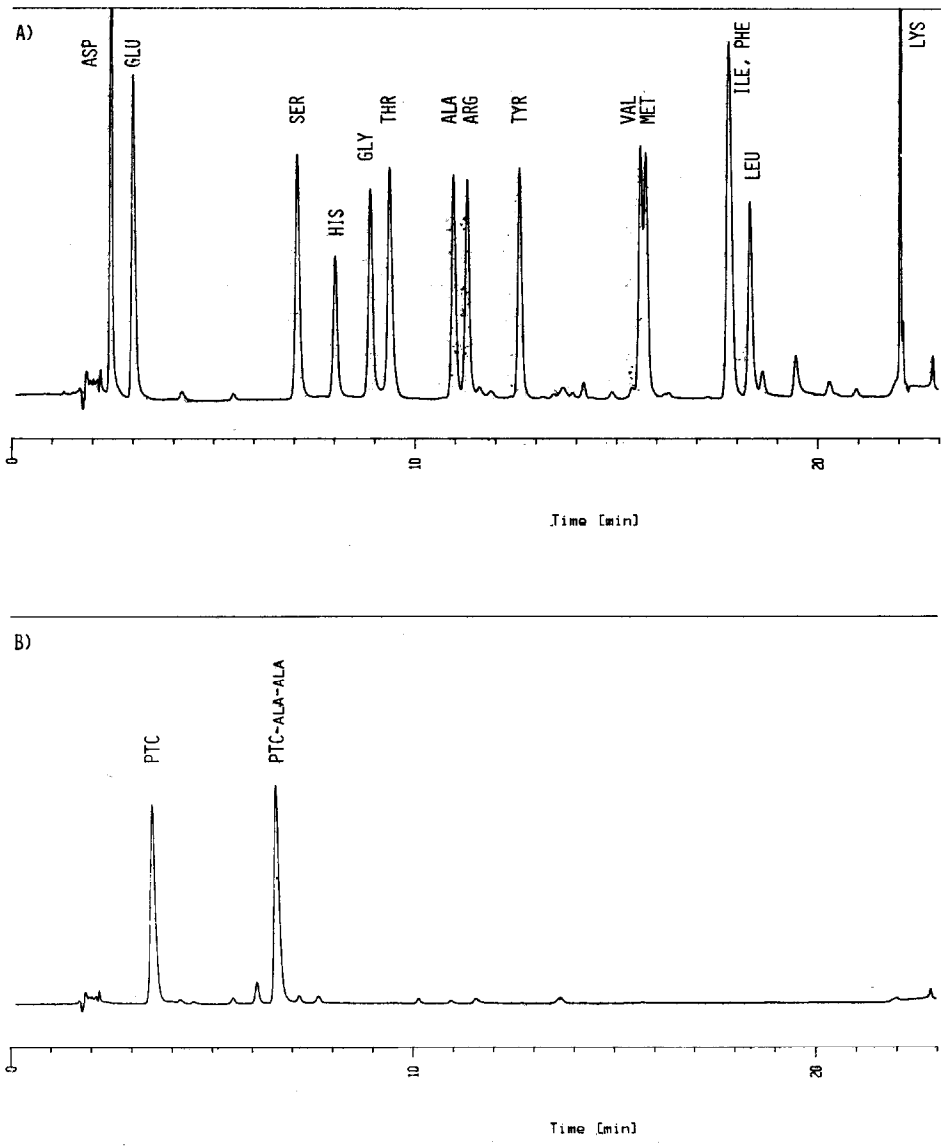

Fig. 8. Narrow-bore HPLC on Spherisorb ODS II (100 mm × 2.1 mm). Details as in Fig. 5.

The results are shown in Fig. 10, and they may be summarized as follows.

(1) Normal-bore HPLC columns had a longer lifetime than narrow-bore columns, comparing the same packing material. The same effect was found in the case of guard columns. After 1000 injections of biological samples, no decrease in the efficiency of a normal-bore column, packed with Shandon Hypersil ODS, was observed; narrow-bore columns packed with the same material could be used for no more than 400 injections. Problems of unstable packing of narrow-bore columns may

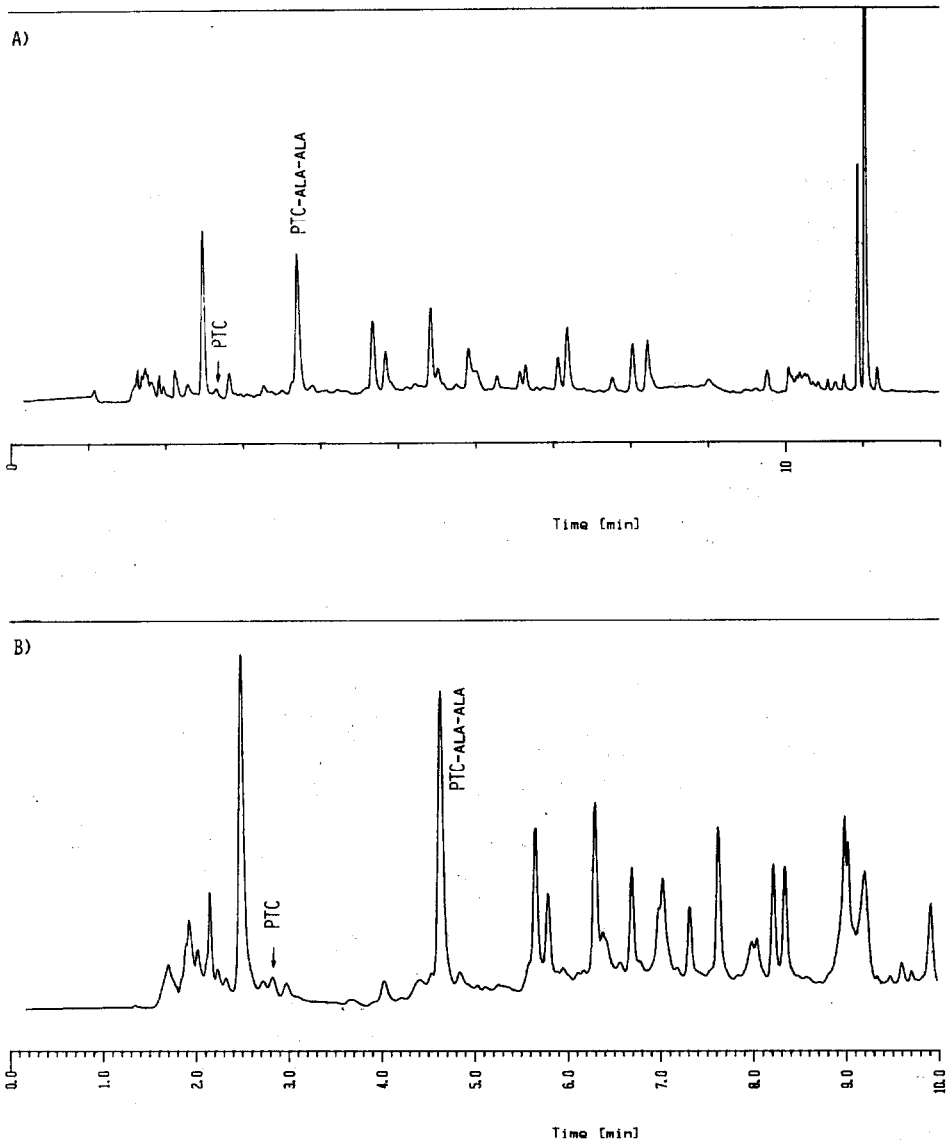

Fig. 9. Separation of a centrifuged fermentation broth on Shandon Hypersil ODS; attenuation 50 mAU. (a) Normal-bore HPLC column (125 mm × 4.6 mm), sample volume 2 μl. (b) Narrow-bore HPLC column (100 mm × 2.1 mm), sample volume 1 μl.

be caused by wall effects and the adverse ratio between the stainless-steel surface and packing material compared with normal-bore columns.

(2) Not all stationary phases were suitable for the routine separation of OPA derivatives of amino acids, PTC and PTC-Ala-Ala in biological samples. This is evident for normal- as well as narrow-bore columns, as in the case of Spherisorb ODS II. After 400 injections, the normal-bore Spherisorb ODS II column showed a

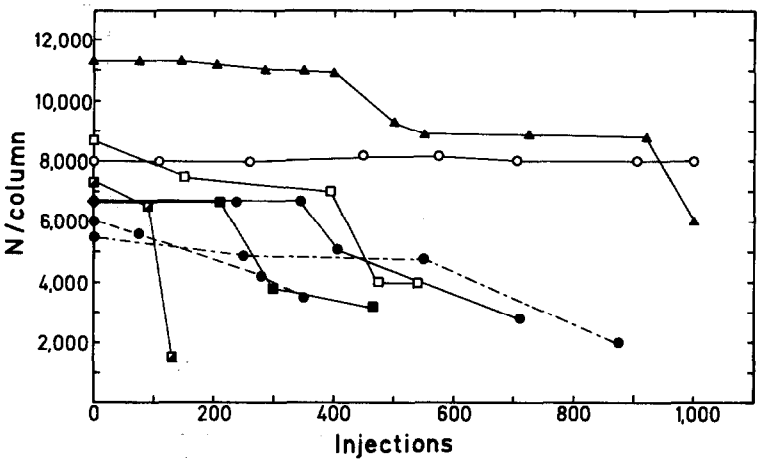

Fig. 10. Column lifetimes of normal- and narrow-bore reversed-phase HPLC columns in routine separations of biological samples. ○—○, Shandon Hypersil ODS (125 mm × 4.6 mm); □—□, Spherisorb ODS II (125 mm × 4.6 mm); ◪—◪, LiChrosorb RP-18 (150 mm × 3.0 mm); ▲—▲, Ultrasphere ODS (150 mm × 2.0 mm); ●—●, Shandon Hypersil ODS (100 mm × 2.1 mm); ●—●, Shandon Hypersil ODS (100 mm × 2.0 mm); ●- · -●, Shandon Hypersil ODS (100 mm × 1.8 mm); ■—■, Spherisorb ODS II (100 mm × 2.1 mm).

drastic reduction in efficiency, and only 210 injections could be made onto the narrow-bore column with the same packing material. The efficiency of the LiChrosorb RP-18 column became unacceptable after 100 injections. The best stationary phase in the narrow-bore columns was Ultrasphere ODS, as far as lifetime is concerned, but this material failed to give symmetrical peaks of PTC and PTC-Ala-Ala. Normal-bore guard columns must be replaced after about 400 injections and narrow-bore guard columns after 60 injections in the case of Spherisorb ODS II and after 300 injections in the case of Shandon ODS.

(3) Narrow-bore HPLC was plagued by another problem in routine analyses: the systems were highly sensitive to the smallest amounts of undissolved particles in the samples or solvents, which gave rise to a large increase in back-pressure. This problem could be avoided by adding small amounts of tetrahydrofuran or sodium azide to the samples and solvents. The back-pressure of normal-bore and narrow-bore columns ranged between 80 and 140 bar, depending on the packing material, and represented no limitation to routine analyses.

CONCLUSIONS

The combination of automated pre-column derivatization of amino acids and amino acid-like antibiotics with *o*-phthalaldehyde/2-mercaptoethanol reagent, separation of the derivatives on reversed-phase columns and UV detection at 338 nm represents an alternative to ion-exchange chromatography. The resulting separation is more rapid and is sensitive, accurate and precise.

The efficiency and lifetime of reversed-phase columns depends on the packing material as well as on column manufacture, particularly in the case of narrow-bore

columns. However, in order to establish this it would be necessary to test several columns from the same and from different batches, of the same type.

The advantages of narrow-bore HPLC are an enhancement of the detection sensitivity and the reduction of solvent consumption. Disadvantages are the decreased lifetime of the main columns and guard columns, in addition to increased back-pressure, caused by small amounts of undissolved particles in the samples or microbial contamination of the eluents, and the greater expense of the equipment, which cannot be compensated by the lower solvent consumption.

Narrow-bore HPLC is preferred when high detection sensitivity is required. Bacterial growth in the samples and solvents can be prevented by the addition of small amounts of tetrahydrofuran or sodium azide. If high detection sensitivity is not required, normal-bore HPLC seems to be a less problematic separation method for routine analyses.

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