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DETERMINATION OF 5-AMINOLAEVULINIC ACID AND PORPHOBILINOGEN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A strong anion-exchange column coupled to a strong cation-exchange column with acetate buffer as eluent or reversed-phase ion-pair chromatography on octadecylsilica with methanol–water containing 1-heptanesulphonic acid as the mobile phase is described for the simultaneous separation of 5-aminolaevulinic acid and porphobilinogen. The separation is applied to the development of a fast and simple method for determining the activity of the enzyme aminolaevulinic acid dehydrase in human erythrocytes. 5-Aminolaevulinic acid is used as the enzyme substrate and the enzyme activity is expressed as micromoles of porphobilinogen formed per ml of erythrocytes in 1 h at 38°. 5-Aminolaevulinic acid and porphobilinogen can also be separated from the urine of porphyric patients but the UV detector has insufficient sensitivity for the determination of 5-aminolaevulinic acid.

INTRODUCTION

5-Aminolaevulinic acid (ALA) and porphobilinogen (PBG) are precursors in the biosynthesis of porphyrins and haem. PBG is formed by the condensation of two molecules of ALA, catalysed by the enzyme aminolaevulinic acid dehydrase[1–3] (ALA-D). This enzyme is sensitive to heavy metals such as cadmium, copper, lead, mercury and silver which inhibit the enzyme activity[4–10]. Determination of the activity of ALA-D is important for the study of various haematological disorders, particularly the porphyrias, and for rapid differentiation between heavy metal poisoning and iron-deficiency anaemia.

There has been hitherto no method for the simultaneous determination of ALA and PBG. The standard method[11] involves separation of ALA and PBG by adsorbing PBG onto Dowex 2. The ALA is not retained and is then adsorbed onto Dowex 50, eluted with sodium acetate and condensed with ethyl acetoacetate to form a pyrrole which reacts with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) to give a colour that is measured spectrophotometrically. The PBG is determined separately by eluting it from Dowex 2 with diluted acetic acid and reacting with Ehrlich's reagent followed by spectrophotometric measurement.

This paper describes two fast and simple methods for the separation of ALA

and PBG. The first employs coupled column ion-exchange chromatography in which a bonded phase strong anion-exchange column ($-NR_3^+$) is coupled to a bonded phase strong cation-exchange column ($-SO_3^-$). ALA is not retained by the anion-exchange column but is retained by the cation-exchange column while PBG is retained by the anion-exchange column but not by the cation-exchange column. The extra column dead volume does not lead to excessive band broadening. The mobile phase used is acetate buffer pH 4.6, at a flow-rate of 1.0 ml/min. The retention of the compounds is pH-dependent and can be preciseiy controlled. The order of elution of ALA and PBG can be reversed by using acetate buffer pH 3.6.

The second method involves reverse-phase ion-pair chromatography on a C_{18} bonded phase (ODS) column. The mobile phase consists of 0.005 *M* 1-heptanesulphonic acid (pH 3.5)–methanol (95:5) at a flow-rate of 1.0 ml/min.

The separation of ALA and PBG has been applied to the determination of the enzyme, ALA-D, using ALA as the enzyme substrate. With minor modifications, the enzyme preparation and incubation procedures are as described by Tomokuni[6]. The amount of PBG formed is separated and measured by the high-performance liquid chromatography (HPLC) system and the enzyme activity calculated.

Porphyric urine can also be analysed but the detection of ALA by UV absorption lacks sensitivity.

EXPERIMENTAL

Materials and reagents

ALA and PBG were obtained from Sigma (London, Great Britain). Methanol, glacial acetic acid, sodium acetate trihydrate, $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12H_2O$ and trichloroacetic acid were of AnalaR grade from BDH (Poole, Great Britain). PIC-B7 ion-pair reagent (1-heptanesulphonic acid) was obtained from Waters Assoc. (Milford, Mass., U.S.A.) and was prepared by diluting one bottle of the reagent to 1 l with water.

Acetate buffers pH 3.6 and 4.6 were made by mixing 92.5 and 52.0 ml of acetic acid (0.2 *M*) with 7.5 and 48.0 ml of sodium acetate trihydrate solution (0.2 *M*) respectively. Phosphate buffer, 0.2 *M*, pH 6.8 was prepared by mixing $Na_2HPO_4 \times 12H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ solutions in suitable proportions.

Preparations of enzyme solution and incubation procedure

This was essentially as described by Tomokuni[6]. ALA substrate (0.5 ml, 20 mmol/l) and phosphate buffer (0.5 ml) were pipeted into each of two test-tubes. For the blank, 1 ml of trichloroacetic acid (100 g/l) was added to one of the tubes. The tubes were placed in a water-bath maintained at 38° for 5 min. The hemolysate containing the enzyme (1.5 ml) was then added to each tube and mixed thoroughly. After 1 h incubation, trichloroacetic acid (1 ml) was added to stop the reaction. The mixture was centrifuged at 3000 rpm for 5 min and 20 µl of the clear supernatant solution were injected into the liquid chromatograph.

High-performance liquid chromatography

A Pye Unicam (Cambridge, Great Britain) LC3-XP system with a variable

wavelength UV detector set at 240 nm was used. Injection was via a Rheodyne Model 7120 injection valve fitted with a 20-μl loop.

For coupled column ion-exchange chromatography a Partisil-10 SAX (strong anion-exchange) column (Whatman, Maidstone, Great Britain) was coupled to a Partisil-10 SCX (strong cation-exchange) column (both 25 cm × 4.6 mm I.D.). The eluents were either acetate buffer pH 3.6, or acetate buffer pH 4.6.

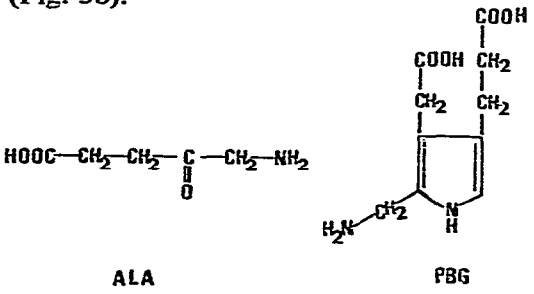
A Hypersil-APS (aminopropylsilane) column (10 cm × 5 mm I.D.; Shandon, Runcorn, Great Britain) was used for weak anion-exchange chromatography with acetate buffer pH 4.6 as the solvent system.

Reversed-phase ion-pair chromatography was performed on a 10 cm × 5 mm I.D. Hypersil-ODS (octadecylsilane) and a Partisil-10 ODS (25 cm × 4.6 mm I.D.) column. The solvents used were PIC-B7 solution–methanol (97:3) and PIC-B7 solution–methanol (95:5) respectively. Hypersil-APS and -ODS were used for the analysis of PBG in the determination of ALA-D activity. Urinary ALA and PBG were separated on Partisil-10 ODS. Urine (1 ml) was diluted to 4 ml with ethanol, centrifuged and 20 μl of the supernatant were injected.

The above separations were all run at a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

The separation of ALA and PBG (Fig. 1) on Hypersil-APS used as a weak anion-exchange column is shown (Fig. 2a). Consistent with previous observations[11] on the behaviour of ALA and PBG on ion-exchange resins, ALA is not retained. The same result is obtained when Partisil-10 SAX is used. ALA, however, can be separated satisfactorily on Partisil-10 SCX which does not retain PBG. It becomes obvious that if an anion-exchange column, for example, Partisil-10 SAX is coupled to Partisil-10 SCX, ALA and PBG can be separated simultaneously (Fig. 3a, b). In ion-exchange chromatography, selectivity is affected by the pH of the eluent. By manipulating the pH of the buffer the retention times of ALA and PBG can be precisely controlled. Thus using acetate buffer pH 4.6 ALA is eluted before PBG (Fig. 3a) and by adjusting the pH of the buffer to 3.6, the order of elution is reversed (Fig. 3b).



ALA PBG

Fig. 1. Structures of 5-aminolaevulinic acid (ALA) and porphobilinogen (PBG).

Ion-exchange chromatography is ideal for employing the coupled column technique for the simultaneous separation of acids and bases. The buffer solutions used are also compatible with both columns. It is also possible to couple strong anion-exchange to weak cation-exchange, strong cation-exchange to weak anion-

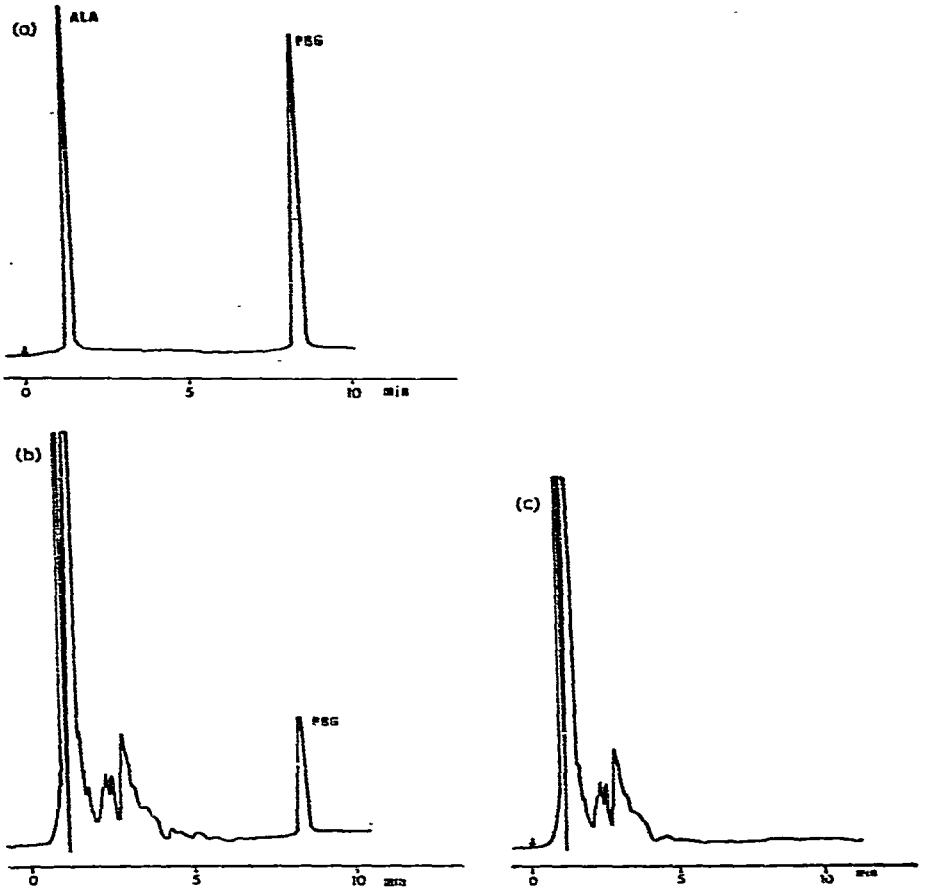


Fig. 2. Separation of ALA and PBG. (a) Standard mixture; (b) incubation mixture in the determination of ALA-D; (c) incubation mixture (blank). Column, Hypersil-APS; mobile phase, acetate buffer pH 4.6; flow-rate, 1 ml/min; detection, 240 nm.

exchange, and weak anion-exchange to weak cation-exchange columns in order to achieve the desired selectivity for a particular separation. The extra column dead volume does not seem to lead to excessive band broadening.

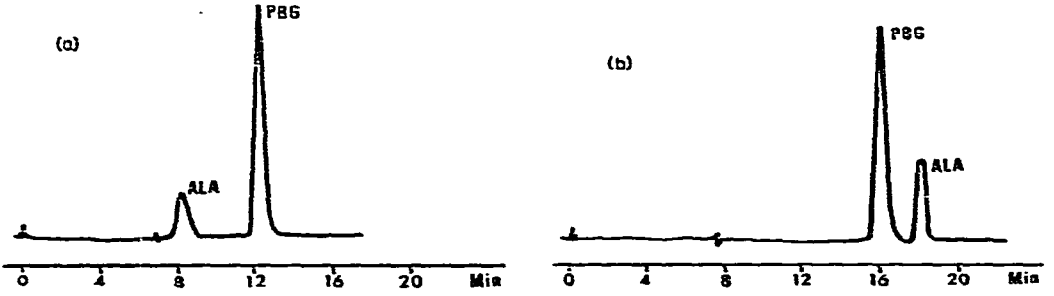


Fig. 3. Separation of ALA and PBG. Column, Partisil-10 SAX coupled to Partisil-10 SCX; mobile phase, (a) acetate buffer pH 4.6; (b) acetate buffer pH 3.6; flow-rate, 1 ml/min; detection 240 nm.

An alternative approach to ion-exchange chromatography is reversed-phase ion-pair chromatography. Since both ALA and PBG contain an amino group they can be separated on an ODS column using methanol–water in the presence of the counter ion 1-heptanesulphonic acid as the mobile phase (Figs. 4a, 5a).

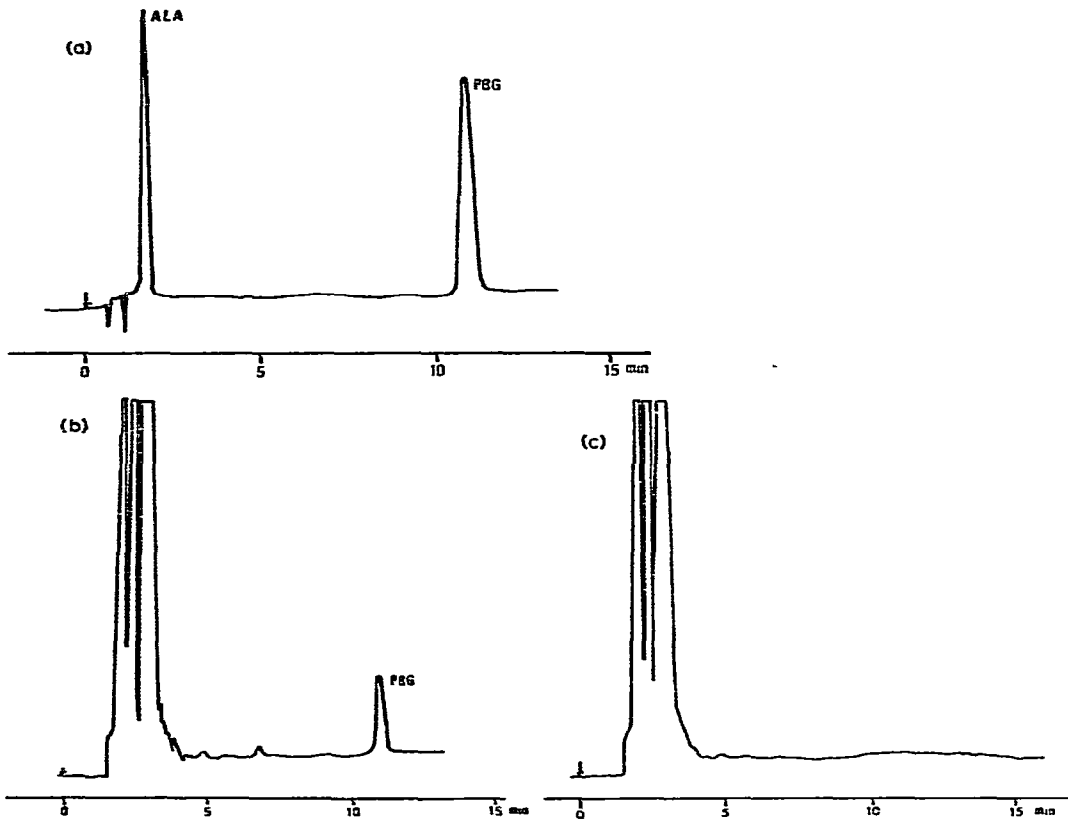


Fig. 4. Separation of ALA and PBG. (a) Standard mixture; (b) incubation mixture in the determination of ALA-D; (c) incubation mixture (blank). Column, Hypersil-ODS; mobile phase, PIC-B7 in water–methanol (97:3); flow-rate, 1 ml/min; detection, 240 nm.

The ideal method for the determination of ALA-D activity in human erythrocytes would be to measure the amounts of ALA consumed as well as the PBG formed after the reaction has been terminated. Unfortunately, the UV detector was insufficiently sensitive for the satisfactory measurement of ALA in the incubation mixture. PBG only is therefore measured and the enzyme activity is expressed as micromoles of PBG formed per ml of erythrocytes in 1 h at 38°.

The separation of PBG from the incubation mixture by Hypersil-APS and -ODS is shown in Figs. 2b and 4b, respectively. The PBG peak, clearly separated, is absent in the incubation mixture pretreated with trichloroacetic acid, *i.e.*, the blank (Figs. 2c, 4c).

The reproducibility of the HPLC method was checked by estimating ALA-D activity in a normal subject ten times. The mean was 20.5 ± 1.8 (S.D.) μmol of PBG/

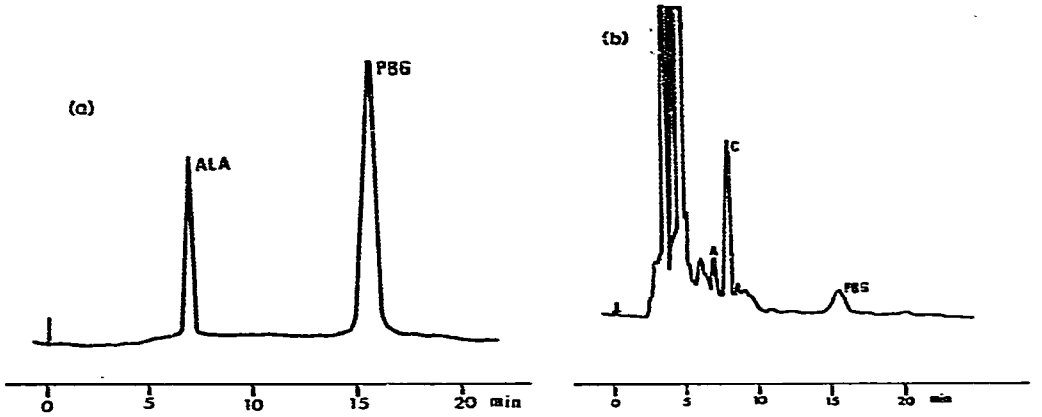


Fig. 5. Separation of ALA and PBG. (a) Standard mixture; (b) urine of patient with acute intermittent porphyria. Column, Partisil-10 ODS; mobile phase, PIC-B7 in water–methanol (95:5); flow-rate, 1 ml/min; detection, 240 nm. A = ALA; C = creatinine.

min/l of erythrocytes. This compared well with the method described by Bonsignore *et al.*[12] the mean of which was 18.8 ± 3.1 (S.D.) μmol of PBG/min/l of erythrocytes.

Elevated levels of ALA and PBG in the urine of patients with acute intermittent porphyria and heavy metal poisoning can also be detected (Fig. 5b). The detection limit for ALA was 50 μg/ml and for PBG was 0.2 μg/ml at 0.005 a.u.f.s. This, although sufficient for the detection of PBG, is not sensitive enough for ALA unless very high levels are present. A possible solution to this problem is to convert ALA into a pyrrole by reacting with ethyl acetoacetate before separation by HPLC.

We have demonstrated the possibility of applying HPLC to the determination of ALA-D activity and to the analysis of urinary ALA and PBG. The methodology is now being elaborated and a comparison with other procedures is in progress.

CONCLUSION

ALA and PBG can be separated simultaneously by HPLC using a strong anion-exchange column coupled to a strong cation-exchange column with acetate buffer as the eluent. The order of elution can be precisely controlled by adjusting the pH of the buffer. Satisfactory resolution of ALA and PBG is also achieved by reversed-phase ion-pair chromatography on an ODS column using 1-heptanesulphonic acid as the counter ion. The separations developed are applicable to the determination of ALA-dehydrase activity and to the analysis of urinary ALA and PBG.

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