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ASSAYING ERYTHROCYTE HAEM BIOSYNTHETIC ENZYME ACTIVITIES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH THE ADVANCED AUTOMATED SAMPLE PROCESSOR

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SUMMARY

The four cytosolic haem biosynthetic enzymes in erythrocytes were assayed with the Varian advanced automated sample processor (AASP) for rapid sample concentration and clean-up with fast and effective high-performance liquid chromatography systems for separation and quantitation. In the assay for 5-aminolaevulinic acid dehydrase, the porphobilinogen (PBG) formed was extracted on a C_{18} AASP cartridge and separated by reversed-phase ion-pair chromatography with 32% methanol in 0.05 *M* sodium acetate buffer (pH 3.5), containing 5.4 m*M* of 1-heptanesulphonic acid as eluent. PBG was the substrate for the simultaneous assay of hydroxymethylbilane synthase and uroporphyrinogen III synthase. The uroporphyrinogen I and III isomers formed were oxidised to porphyrins, concentrated on a C_2 or C_8 cartridge, and separated by reversed-phase chromatography with 13% acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16) as eluent. Uroporphyrinogen decarboxylase was estimated with pentacarboxylic porphyrinogen III as substrate. The coproporphyrinogen formed was extracted on a C_2 or C_8 cartridge, oxidised to coproporphyrin and separated by reversed-phase chromatography with 30% acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16) as mobile phase.

INTRODUCTION

There are four cytosolic haem biosynthetic enzymes present in red blood cells. 5-Aminolaevulinic acid dehydrase (5-ALA-D) catalyses the conversion of two molecules of 5-aminolaevulinic acid (5-ALA) to porphobilinogen (PBG). Four molecules of PBG are then condensed, by a two-step reaction catalysed by two enzymes, into uroporphyrinogen III. The first step is the conversion of PBG to the unstable intermediate hydroxymethylbilane[1] and is catalysed by hydroxymethylbilane synthase (HMB-S). This is followed[1] by the rapid conversion of hydroxymethylbilane to uroporphyrinogen III, catalysed by uroporphyrinogen III synthase (Uro III-S). Spontaneous non-enzymic cyclisation of hydroxymethylbilane to uroporphyrinogen I occurs in the absence of Uro III-S, and a small amount of uroporphyrinogen I is always produced by this reaction. The uroporphyrinogens are decarboxylated stepwise,

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through hepta-, hexa- and pentacarboxylic porphyrinogens to the coproporphyrinogens[2]. The reactions are catalysed by uroporphyrinogen decarboxylase (Urogen-D).

The assay of haem biosynthetic enzyme activites is important for the differential diagnosis and the detection of carriers in diseases of abnormal haem synthesis[3–6]. Existing procedures are often tedious, insensitive, and non-specific, due to poor recovery, inadequate purification, and insufficient separation of enzyme substrates and products from interfering compounds. In an attempt to overcome these problems, we have developed semi-automatic methods, based on rapid and effective sample preparation with the Varian advanced automated sample processor (AASP) and fast and efficient separation by high-performance liquid chromatography (HPLC), for determining the activities of the four erythrocyte haem biosynthetic enzymes.

EXPERIMENTAL

Materials and methods

5-ALA, PBG, copro- and uroporphyrins, Triton X-100, and 1-heptanesulphonic acid were from Sigma (Poole, U.K.). Pentacarboxylic porphyrin III was isolated as methyl esters from the faeces of hexachlorobenzene-treated rats by preparative thin-layer chromatography[7]. The methyl esters were hydrolysed, and the pentacarboxylic porphyrin isomers were further separated by HPLC, as previously described by us[8]. The pentacarboxylic porphyrin III, with the ring A, B and D acetic acid groups decarboxylated, was used as enzyme substrate. Ammonium acetate, glacial acetic acid, EDTA, dipotassium hydrogen orthophosphate, potassium dihydrogen orthophosphate, dithiothreitol (DTT), concentrated hydrochloric acid, trichloroacetic acid (TCA), Tris, $MgCl_2$, NaCl, I_2 and Na/Hg (5:95) amalgam were AnalaR-grade from BDH (Poole, U.K.). Acetonitrile and methanol were HPLC-grade from Rathburn (Walkerburn, U.K.).

Preparation of red blood cells

Blood was collected in heparinised tubes and was centrifuged (2000 *g*) for 15 min at 4°C. The plasma and leucocytes were discarded. The erythrocytes were washed twice with cold 0.15 *M* sodium chloride solution with centrifugation for 10 min. The packed red cells were assayed immediately or kept at −20°C until used.

Incubation and sample preparation procedures

5-ALA-dehydrase. Red cells, 3 μl, were thoroughly mixed with 100 μl 0.2% Triton X-100 in water, 100 μl of 50 m*M* DTT in 0.2 *M* potassium phosphate buffer (pH 6.8), and 100 μl of 20 m*M* 5-ALA in water in a 1.5-ml plastic-stoppered centrifuge tube. The mixture was incubated for 1 h at 37°C in a shaking water-bath. The reaction was terminated with 200 μl of cold 10% TCA, vortex-mixed, and centrifuged at 2000 *g* for 5 min. The supernatant was mixed with 0.5 ml of 5.4 m*M* 1-heptanesulphonic acid in 0.05 *M* sodium buffer (pH 3.5, adjusted with hydrochloric acid) and loaded onto a C_{18} AASP cartridge, which has previously been washed successively with 0.5 ml of methanol and 1 ml of 1-heptanesulphonic acid solution. The cartridge was washed with 1.5 ml of 1-heptanesulphonic acid solution and then inserted into the AASP cassette-processing station for automatic injection and HPLC separation and quantitation.

Hydroxymethylbilane synthase and uroporphyrinogen III synthase. The incubation procedure was essentially as described previously[6]: 30 μl of red cells were thoroughly mixed with 1.40 ml of the incubation medium, containing 1.5 g of magnesium chloride and 1 ml of Triton X-100 in 1 l of 0.05 *M* Tris–HCl buffer (pH 8.25). The haemolysate was heated for 1 h at 56°C in a water-bath in the dark. After cooling to room temperature, a further 3 μl of red cells were added. The mixture was pre-incubated for 5 min at 37°C, and 50 μl (83 μg) of PBG solution was added. The reaction was carried out for 30 min and then terminated by adding 1.50 ml of 10% TCA, containing 0.5% (w/v) I_2, vortex-mixed, and centrifuged at 2000 *g* for 10 min. The sample extraction was as follows: the C_2 or C_8 cartridge ws conditioned by washing with 0.5 ml of methanol and 1 ml of 1 *M* ammonium acetate buffer (pH 5.16). Then, 0.5 ml of the supernatant were loaded onto the cartridge, followed by washing with 1.5 ml of 1 *M* ammonium acetate buffer (pH 5.16). The cartridge was then placed into the AASP for injection and HPLC separation.

Uroporphyrinogen decarboxylase. Red cells (5 μl) were added to 100 μl of 0.1 *M* phosphate buffer (pH 6.8), containing 150 μM of EDTA, 0.1% (v/v) of Triton X-100, and 20 m*M* DTT. The mixture was pre-incubated at 37°C for 5 min, and 5 μl of pentacarboxylic porphyrinogen (20 μM), prepared by reducing pentacarboxylic porphyrin with Na/Hg (5:95) amalgam, was added. The tube was flushed with nitrogen, stoppered, and incubated at 37°C in a water-bath for 30 min. The reaction was terminated by cooling on ice. The solution was diluted with 0.5 ml of 0.1 *M* ammonium acetate buffer (pH 5.16) and then loaded onto a C_2 or a C_8 catridge, which has been conditioned by washing with 0.5 ml of methanol and 1 ml of 1 *M* ammonium acetate buffer (pH 5.16). The cartridge was then washed successively with 1 ml of 5% methanol in 1 *M* ammonium acetate (pH 5.16), 1 ml of 10% TCA containing 0.5% (w/v) I_2, and 1 ml of 10% methanol in 1 *M* ammonium acetate (pH 5.16), before loading it into the AASP for injection and HPLC separation.

High-performance liquid chromatography

A Varian Assoc. (Walnut Creek, CA, U.S.A.) Model 5000 liquid chromatograph, coupled to the AASP and a Varian UV-100 detector were used. AASP cassettes were from Analytichem International (Harbor City, CA, U.S.A.). Each cassette contains ten extraction cartridges. The HPLC column was 25 cm × 5 mm ODS-Hypersil (5 μm particle size, 100 Å pore size) from Shandon Southern Products (Runcorn, U.K.). The mobile phase for separating PBG was 32% methanol in 0.05 *M* sodium acetate buffer (pH 3.5, adjusted with hydrochloric acid), containing 5.4 m*M* of 1-heptanesulphonic acid as ion-pairing agent. The eluents for uro- and coproporphyrins were 13 and 30% acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16), respectively. The flow-rate for all separations was 1.5 ml/min. PBG was detected at 240 nm, and uro- and coproporphyrins were detected at 400 nm. The AASP injection and the HPLC separations were fully automated. The retention times of PBG and the porphyrins were established with the AASP injection using pure standards. The recoveries were virtually 100%.

RESULTS AND DISCUSSION

The successful application of HPLC to clinical analysis depended largely on

a good sample preparation procedure and an efficient HPLC separation. This is especially true in clinical enzymology of disorders of haem metabolism, where the enzyme reaction products are often unstable and are present in low concentrations. Pre-column concentration and rapid sample processing are therefore necessary. This can be achieved by using the AASP, which has many advantages over conventional solid-phase extraction procedures[9]. The potentials of this technique in enzyme assay are demonstrated by the development of simple, rapid, sensitive and specific methods for assaying the activities of the four cytosolic red cell haem biosynthetic enzymes.

Determination of 5-ALA-D in erythrocytes

Fig. 1 shows the separation of PBG in the incubation mixture for the determination of 5-ALA-D. The substrate 5-ALA and many early-eluted compounds were removed during the AASP extraction and the washing steps. Furthermore, late-eluting peaks were trapped on the cartridge by setting the AASP valve reset function to remove it from the solvent flow 1 min after sample injection. The result is a fast, clean HPLC separation. This, coupled with the ability to extract ten samples simultaneously with the AASP cassette greatly improves the sample throughput. Rapid extraction of PBG is particularly important, because it is unstable in the acid solution condensing to form porphyrins[10]. However, once PBG has been immobilised on the solid phase, it is stabilised for at least 24 h. This is an obvious advantage, as up to 100 samples may be processed as a batch and then run automatically overnight without sample deterioration.

The mean 5-ALA-D activity in normal subjects (n = 100) was 29.7 nmol PBG per ml erythrocytes per min at 37°C. The standard deviation was 5.9 and the 95% reference range was 17.6 to 41.8.

Determination of HMB-S and Uro III-S in erythrocytes

HMB-S and Uro III-S can be determined simultaneously. This is based on the observation that HMB-S is heat-stable, while Uro III-S is heat-labile and can be inactivated by heating[11]. In practice, 30 μl of red cells are heated at 56°C for 1 h to inhibit Uro III-S, while retaining the activity of HMB-S. After cooling it to room temperature, 3 μl of fresh red cells, containing both HMB-S and Uro III-S, were added to the incubation medium. The total uroporphyrins (I + III) formed is a measure of HMB-S activity in 33 μl of red cells, and the uroporphyrin III produced represents Uro III-S activity in 3 μl of red cells[6].

Fig. 2 shows the separation of uroporphyrin I and III isomers in the incubation mixture for the simultaneous determination of HMB-S and Uro III-S. The porphyrins were effectively extracted into a C_2 or a C_8 cartridge and were stable for overnight operation. To prevent acid-catalysed condensation of PBG to porphyrins, the excess substrate was rapidly removed by washing the cartridge with 5% methanol in 1 *M* ammonium acetate (pH 5.16).

The mean HMB-S activity in 100 normal subjects was 29.2 nmol uroporphyrin per ml erythrocytes per h at 37°C. The standard deviation was 5.6 and the 95% reference range was 18.0 to 40.5. The mean uro III-S activity was 235 nmol uroporphyrin III per ml erythrocytes per h. The standard deviation was 45 and the 95% reference range was 140 to 330.

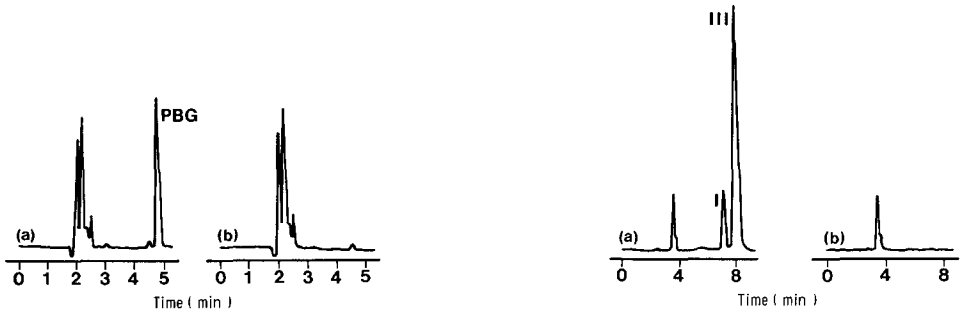

Fig. 1. Separation of PBG in the incubation mixture for the determination of 5-ALA-D in erythrocytes. (a) A normal subject, and (b) a zero-incubation time-blank. Column, ODS-Hypersil; eluent, 32% methanol in 0.05 *M* sodium acetate buffer (pH 3.5), containing 5.4 m*M* of 1-heptanesulphonic acid; flow-rate, 1.5 ml/min; detector, 240 nm, 0.05 a.u.f.s.

Fig. 2. Chromatograms for the simultaneous determination of HMB-S and Uro III-S in erythrocytes. (a) A normal subject, and (b) zero-incubation time-blank. Column, ODS-Hypersil; eluent, 13% acetonitrile in 1 *M* ammonium acetate (pH 5.16); flow-rate, 1.5 ml/min; detector, 400 nm, 0.02 a.u.f.s. I and III are uroporphyrins I and III, respectively.

Determination of Urogen-D in erythrocytes

The stepwise decarboxylation of uroporphyrinogen III to coproporphyrinogen III is catalysed by Urogen-D. The final reaction intermediate, pentacarboxylic porphyrinogen III, was chosen as the substrate, because only coproporphyrin III will be formed, and thus, the separation and quantitation of the products is simplified[3]. With uroporphyrinogen as substrate[12,13], separation and quantitation of all intermediates and of coproporphyrin are necessary.

The combined termination of enzyme reaction and oxidation of porphyrinogen to porphyrin with TCA–I_2, used in the HMB-S and Uro III-S assay, is not suitable

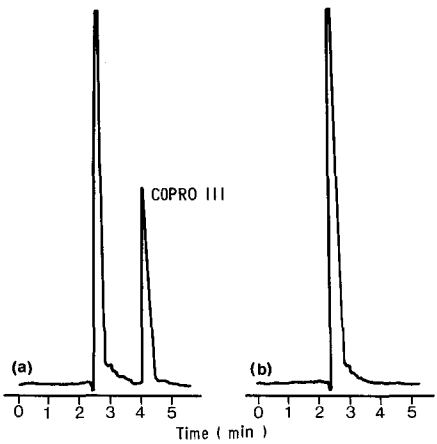

Fig. 3. Separation of coproporphyrin III in the incubation mixture for the determination of Urogen-D in erythrocytes. (a) A normal subject and (b) zero-incubation time-blank. Column, ODS-Hypersil; eluent, 30% acetonitrile in 1 *M* ammonium acetate (pH 5.16); flow-rate, 1.5 ml/min; detector, 400 nm, 0.02 a.u.f.s.

here, since the I_2 will be destroyed by the relatively large amount of DTT present in the incubation mixture. Oxidation by exposure to light is possible, but this is time-consuming. The obvious solution is to remove the DTT prior to I_2 oxidation. The enzyme reaction was therefore terminated by cooling on ice. The incubation mixture was diluted with 0.1 *M* ammonium acetate (pH 5.16), and the solution was loaded onto a C_2 or a C_8 AASP cartridge. The DTT was completely removed from the cartridge by washing it with 5% methanol in 1 *M* ammonium acetate (pH 5.16). The coproporphyrinogen adherent to the cartridge was then oxidised by passing a solution of I_2 in 10% TCA through the cartridge. This on-column oxidation technique illustrates another advantage and the flexibility of the AASP. The separation of coproporphyrin III in the incubation mixture is shown in Fig. 3. A reference range for Urogen-D has not yet been established with this method.

CONCLUSIONS

The combination of an AASP and an automated HPLC system provides a useful technique for the determination of erythrocyte haem biosynthetic enzyme activities. The ability to concentrate selectively the enzyme reaction product for on-line elution greatly improved the sensitivity and the speed of analysis. Assaying the four cytosolic haem enzymes required only 41 μl of red cells. An important advantage of AASP HPLC is that unstable enzyme reaction products can be rapidly immobilised and stabilised on the solid phase, while potential interfering substances are removed by washing the sorbent cartridge with a suitable solvent mixture. Direct derivatization on the sorbent cartridge is also possible by washing with the derivatization reagent. It is expected that this novel approach to clinical enzymology will lead to the development of many other assays for enzymes.

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