Simultaneous determination of hydroxymethylbilane synthase and uroporphyrinogen III synthase in erythrocytes by high-performance liquid chromatography

Dennis J. WRIGHT and Chang Kee LIM*

Division of Clinical Chemistry, M.R.C. Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, U.K.

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A high-performance-liquid-chromatographic method is developed for the simultaneous determination of hydroxymethylbilane synthase and uroporphyrinogen III synthase activity in erythrocytes. Effective separation of uroporphyrin I and III isomers allows the accurate quantification of individual isomers and the total uroporphyrin concentration. Total uroporphyrin production is used to calculate hydroxymethylbilane synthase activity, and the amount of uroporphyrin III formed represents the activity of uroporphyrinogen III synthase. Normal ranges are established for the two enzymes.

Uroporphyrinogen III, the natural precursor for haem, chlorophyll and vitamin B₁₂, is derived from the condensation of four molecules of PBG catalysed by two enzymes in a two-step reaction. The first step is the conversion of PBG into the unstable intermediate hydroxymethylbilane (Battersby *et al.*, 1982) and is catalysed by HMB-S. This is followed by the conversion of hydroxymethylbilane into uroporphyrinogen III catalysed by UroIII-S. In the absence of UroIII-S, hydroxymethylbilane is spontaneously cyclized into uroporphyrinogen I (Battersby *et al.*, 1982).

The estimation of HMB-S activity in erythrocytes is widely used for the diagnosis of acute intermittent porphyria where the abnormality of this enzyme is the primary genetic defect (Strand *et al.*, 1970). Current spectrophotometric and fluorimetric methods for measuring HMB-S activity lack specificity, since in a complex incubation mixture interference is likely to occur.

There is no simple and reliable procedure for the determination of UroIII-S activity, mainly because efficient and reproducible methods for the separation of uroporphyrin I and III isomers were not available. Most methods required the isolation and decarboxylation of uroporphyrins followed by separation and estimation of the resulting coproporphyrin isomers as tetramethyl esters by t.l.c. or

as the free porphyrins by paper chromatography (Romeo *et al.*, 1970; Levin, 1971; Deybach *et al.*, 1981). Apart from being complicated, tedious and time-consuming, the precision and accuracy of these methods were unsatisfactory. Jordan *et al.* (1980) described a direct spectrophotometric method for measuring UroIII-S activity using a short incubation time to limit the production of uroporphyrin I. This method is unlikely to be useful in cases where UroIII-S is defective, as a mixture of isomers will be formed.

We have successfully developed a simple, rapid and effective h.p.l.c. system for the separation of uroporphyrin I and III isomers (Rideout *et al.*, 1983). The present paper describes the application of the separation to the development of a simple procedure for the simultaneous determination of the activities of the enzymes HMB-S and UroIII-S in erythrocytes. The normal ranges of the enzymes were established.

Experimental

Materials and reagents

Porphobilinogen, uroporphyrin I and III octamethyl esters and Triton X-100 were from Sigma Chemical Co. (Poole, Dorset, U.K.). Ammonium acetate, acetic acid, trichloroacetic acid, MgCl₂, NaCl, I₂, conc. HCl and Tris were AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.). Acetonitrile was h.p.l.c.-grade from Rathburn Chemicals (Walkerburn, Peebleshire, U.K.).

Abbreviations used: h.p.l.c., high-performance liquid chromatography; PBG, porphobilinogen; HMB-S, hydroxymethylbilane synthase (EC 4.3.1.8); UroIII-S, uroporphyrinogen III synthase (EC 4.2.1.75).

* To whom reprint requests should be sent.

H.p.l.c.

A Pye-Unicam (Cambridge, U.K.) model PU4010 solvent delivery system and a Perkin-Elmer (Beaconsfield, Bucks., U.K.) LS-3 fluorescence detector set at an excitation and an emission wavelength of 404 nm and 618 nm respectively was used. Injection was by a Rheodyne 7125 injection valve fitted with a 100 μ l loop. The separation was carried out on a 25 cm $\times$ 5 mm ODS-Hypersil column (Shandon Southern, Runcorn, Cheshire, U.K.) with 13% (v/v) acetonitrile in 1 M-ammonium acetate buffer, pH 5.16, as the mobile phase. The flow rate was 1 ml/min.

Calibration curves

The calibration curves for the determination of uroporphyrin I and III were constructed by plotting the h.p.l.c. peak heights against concentrations and were linear in the range 10–430 nmol/l (Fig. 1). Porphyrin solutions of known concentrations were prepared by hydrolysing the octamethyl esters in 25% (w/v) HCl at room temperature in the dark for 96 h. The concentrations were then determined spectrophotometrically by the method of Falk (1964). Corrections were made for porphyrin solutions containing small quantities of isomeric impurities.

Fig. 1. Calibration curves for the determination of HMB-S and UroIII-S in erythrocytes

Uroporphyrin I and III concentrations are measured from the peak heights obtained by h.p.l.c. (see Fig. 2 for example) and are used for calculating the enzyme activities.

Preparation of red blood cell haemolysate

Blood was collected in heparinized tubes and was centrifuged at 2000 *g* for 15 min at 4°C. The plasma and leucocytes were discarded. The erythrocytes were washed twice with cold iso-osmotic saline (0.9% NaCl) and were centrifuged at 2000 *g* for 10 min after each washing. The erythrocytes were used immediately or kept frozen at –20°C until used. The enzymes were stable for several months at –20°C. The red cells were effectively and totally haemolysed by Triton X-100 present in the incubation medium.

Incubation medium

This was made by mixing 1.5 g of MgCl₂ and 1 ml of Triton X-100 in 1 litre of 0.05 M-Tris/HCl buffer, pH 8.25.

Incubation procedure

Washed red cells (30 μ l) were accurately pipetted with a volume-adjustable (10–30 μ l) micropipette (Scientific Manufacturing Industries, Emeryville, CA, U.S.A.) and was added 1.40 ml of incubation medium. After mixing, the haemolysate was heated for 1 h at 56°C in a water bath in the dark and then allowed to cool down to room temperature. Red cells (3 μ l) were accurately pipetted with a fixed-volume micropipette and were added to the solution and thoroughly mixed. The mixture was pre-incubated for 5 min at 37°C, and 50 μ l (83 μ g) of PBG substrate was added. The incubation was then carried out for exactly 30 min at 37°C and the enzyme reaction was terminated by adding 1.50 ml of cold 10% (w/v) trichloroacetic acid containing 0.5% (w/v) I₂. After centrifugation at 2000 *g* for 15 min at 4°C, 2 $\times$ 100 μ l of the clear supernatant were used for duplicated analysis by h.p.l.c. Two blanks were analysed in parallel; the first contained PBG and the enzyme solution but the reaction was terminated at zero time; the second contained the enzyme solution without PBG.

Calculation

The enzyme activities were expressed as nmol of uroporphyrin/ml of red blood cells per h and were calculated as follows:

$$\text{HMB-S} = (U_I + U_{III}) \times \frac{2.983}{1000} \times \frac{1000}{33} \times \frac{60}{30}$$

$$\text{UroIII-S} = U_{III} \times \frac{2.983}{1000} \times \frac{1000}{3} \times \frac{60}{30}$$

where U_I and U_{III} are the concentrations (nmol/l) of uroporphyrin I and III formed respectively and 2.983/1000 is the dilution factor.

Results and discussion

It has been shown that HMB-S and UroIII-S are two enzymes acting independently and sequentially (Battersby *et al.*, 1982); whereas HMB-S is heat-stable, UroIII-S can be completely deactivated by heating (Levin, 1971). These facts, coupled with an effective h.p.l.c. separation of uroporphyrin I and III isomers, made possible the development of a simple procedure for the simultaneous determination of the activities of these enzymes.

In preliminary experiments it was concluded that 33 μ l of red blood cells heated for 1 h at 56°C before enzyme assay produced exclusively uroporphyrin I the amount of which was identical with the total porphyrin (uroporphyrin I and uroporphyrin III) formed by 30 μ l of heated red blood cells plus 3 μ l of unheated red blood cells. The uroporphyrin III formed is a result of UroIII-S activity in 3 μ l of red blood cells, whereas the total porphyrin produced is due to the activity of HMB-S in 33 μ l of red blood cells. Thus the two enzymes can be assayed simultaneously.

Figs. 2(a) and 2(b) show the h.p.l.c. chromatograms used for the quantitative assay of HMB-S and UroIII-S activities in the erythrocytes of a normal subject and a patient with congenital erythropoietic porphyria respectively. Fig. 2(c) shows a typical blank assay. The increase in uroporphyrin I, and the decrease in uroporphyrin III production (Fig. 2b) to less than half that of the normal subject (Fig. 2a), is typical of congenital erythropoietic porphyria, where UroIII-S is defective (Levin, 1975). The HMB-S activity of this patient, assayed by measurement of the total porphyrin formed, was normal. These results clearly demonstrate the suitability of the present method for the simultaneous determination

of the two enzymes even in situations where one enzyme is abnormal.

Optimal assay conditions

The optimal PBG concentration for the two enzymes was 83 μ g in a total incubation volume of 2.983 ml containing 33 μ l of red blood cells. As HMB-S is the rate-limiting enzyme it is essential to ensure that adequate substrate (hydroxymethylbilane) is provided for the UroIII-S reaction. This was carried out by using a 10-fold excess of red blood cells in which the UroIII-S had been deactivated by heating. In the present assay 30 μ l of heated and 3 μ l of unheated red blood cells were used.

The pH optima of 7.80–7.95 were marginally lower than those previously reported (Magnussen *et al.*, 1974; Battle *et al.*, 1978). A slightly suboptimal pH of 8.25 was used, however, because the activity of uroporphyrinogen III decarboxylase, which catalysed the stepwise conversion of uroporphyrinogens into coproporphyrinogens (Jackson *et al.*, 1976) was negligible at this pH and only traces of decarboxylation products were detected by h.p.l.c.

The enzyme reactions were linear up to 30 min at 37°C and a 30-minute incubation was therefore chosen for the assay.

The addition of MgCl₂ (Battle *et al.*, 1978) enhanced the UroIII-S activity, but slightly inhibited the HMB-S activity. NaCl had no effect on both enzymes and was not included in the assay.

Sensitivity and reproducibility

The h.p.l.c. separation with fluorescence detection is highly sensitive and specific. A smaller sample volume could be used but this was hindered by the difficulty in accurately pipetting small volumes of red blood cells. An U.V. detector, although less sensitive, is also sufficient for the present assay.

To assess reproducibility HMB-S and UroIII-S were measured on 12 separate occasions on a single sample of normal red blood cells. The means were 34.7 nmol of uroporphyrins/ml of red blood cells per h and 313 nmol of uroporphyrin III/ml of red blood cells per h respectively with a coefficient of 6.0 and 7.4%.

Reference ranges

Preliminary reference ranges for HMB-S and UroIII-S were established from specimens taken from 25 apparently healthy subjects. The distribution for both enzymes was non-Gaussian, so a logarithmic transformation was applied to the data.

The mean HMB-S activity was 29.1 nmol of uroporphyrin/ml of red blood cells per h (s.d. range 20.4–41.6). This is well correlated with the values reported by Magnussen *et al.* (1974) and Strand *et al.* (1972).

Fig. 2. H.p.l.c. chromatograms for the simultaneous determination of HMB-S and UroIII-S in erythrocytes. The Figure shows uroporphyrin I and III formed in the incubation mixture of (a) a normal subject, (b) a patient with congenital erythropoietic porphyria and (c) in which the enzyme reaction was terminated at zero time. H.p.l.c. conditions are as described in the Experimental section.

A reference range for UroIII-S has not been reported before. The mean UroIII-S activity in the reference population was 236 nmol of uroporphyrin III/ml of red blood cells per h (2 s.d. range 159–352). There seemed to be no age or sex differences in enzyme activity from these preliminary results. The UroIII-S activity of the patient with congenital erythropoietic porphyria was 131 nmol of uroporphyrin III/ml of red blood cells per h, clearly showing the enzyme abnormality.

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