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Note

Separation and identification of isomeric deuterobiliverdins and mesobiliverdins

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In a previous paper[1], we described thin-layer chromatographic (TLC) methods for the separation and identification of the four isomeric forms (α, β, γ and δ) of both protobiliverdin and mesobiliverdin derived by random cleavage of protohaem IX and mesohaem IX, respectively, at the four carbon bridge positions. The isomeric biliverdins were separated as the dimethyl esters, but complete resolution of the mesobiliverdin mixture was rather difficult and tedious, requiring up to eightfold development of the plates with the solvent.

Here we report a more rapid and convenient method for the separation of the mesobiliverdin isomers as the *n*-propyl or isopropyl esters, and a method for the convenient separation and identification of the four isomeric deuterobiliverdins derivable by similar cleavage of deuterohaem IX.

MATERIALS AND METHODS

Crystalline protohaemin and bovine haemoglobin were obtained from Sigma (London, Great Britain). Mesohaemin was prepared by catalytic hydrogenation of protohaemin followed by re-insertion of iron[2]. Deuterohaemin was prepared from protohaemin by the resorcinol melt method[2].

The mixtures of mesobiliverdin and deuterobiliverdin isomers were prepared from the products of ascorbate-coupled oxidation of the respective haemins as the pyridine haemochromes. The procedure for the coupled oxidation of mesohaemin and the processing of the product up to the point of esterification was as previously described[1]. Coupled oxidation of deuterohaemin was carried out following the general procedure described by Levin for protohaemin[3]. The processing of the product followed the procedures used for the products derived from the other haemins[1] except that the greater hydrophilicity of the deutero product made it more convenient to use ethyl acetate rather than diethyl ether in the first extraction step, and, after subsequent extraction of the product into aqueous HCl, it was found necessary to adjust the pH to between 3 and 4 with NaOH to facilitate efficient extraction from the aqueous phase into chloroform.

Replacement of the protohaem groups of haemoglobin by deuterohaem was

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achieved by removal of the haem by the method of Teale[4] and insertion of deuterohaem following the general method of Smith and Gibson[5]. Coupled oxidation of deuterohaemoglobin was carried out following the method described by Kench and Gardikas[6] for protohaemoglobin and the deuterobiliverdin products were isolated following the procedure described above for the products derived from pyridine deuterohaemochrome.

The deuterobiliverdins were converted to the dimethyl ester form as described previously for the proto and meso isomers[1]. Conversion of the mesobiliverdins to the *n*-propyl and isopropyl diesters was achieved by refluxing the pigment for 2 h in 1–3 ml of *n*-propanol or isopropanol containing 5% H_2SO_4. (Esterification with the propanols was very much slower than with methanol and required the elevated temperature.) The esters were extracted into chloroform, washed completely free of acid, evaporated to dryness and redissolved in a minimum of acetone for application to the TLC plates. Chromatography was carried out on 20-cm plates coated with silica gel G (Merck, Darmstadt, G.F.R.), as described previously[1].

Chromatography on a quantitative or preparative scale was achieved by applying the isomer mixture as a band rather than as a spot. After chromatography, the individual isomer bands were scraped off the plate, eluted into acetone and the silica particles were removed completely by centrifugation. The relative proportions of the different isomers were determined as previously described[1].

Conversion of the separated biliverdin diesters to the free diacids was achieved by solubilising the diesters in 1 ml of acetone and hydrolysing with 2 ml of 2 *N* HCl at 100° for 15 min. After hydrolysis, the solution was cooled to 0° in an ice-water bath. Glacial acetic acid (0.5 ml) and 2 *N* NaOH (2 ml) were added and the solution was extracted twice with 2 ml aliquots of a 1:1 mixture of chloroform and methyl ethyl ketone. The pooled extracts were evaporated to dryness under nitrogen.

Owing to the instability of the biliverdins, it is necessary during all the above procedures to observe the handling precautions outlined previously[1].

RESULTS AND DISCUSSION

Fig. 1 illustrates the separations achieved. The solvent system for the deuterobiliverdin isomers is particularly effective, the separation being very clear-cut and rapid. The systems for the *n*-propyl and isopropyl diesters of the mesobiliverdin isomers require up to fourfold development to achieve separation of the two middle spots (the α and β isomers). However, this represents a considerable improvement over the system previously described[1] for the dimethyl esters which requires up to eightfold development to achieve separation of the α and β isomers.

The indicated identities of the isomers (Fig. 1) were established as follows: The isomeric mesobiliverdins were identified by converting the dimethyl esters (already identified[1]) to the di-*n*-propyl and diisopropyl derivatives which were then used as chromatographic standards. The α and γ deuterobiliverdin isomers were identified by dichromate degradation[7,8] as previously described[1]. No direct method could be devised to distinguish the β and δ deuterobiliverdin isomers. Attempts were made to correlate the deutero isomers with the already identified protobiliverdin isomers by interconversion of the two forms (*i.e.*, by removal of the vinyl groups from the protobiliverdins or by addition of vinyl groups to the deutero forms). However, the chemical

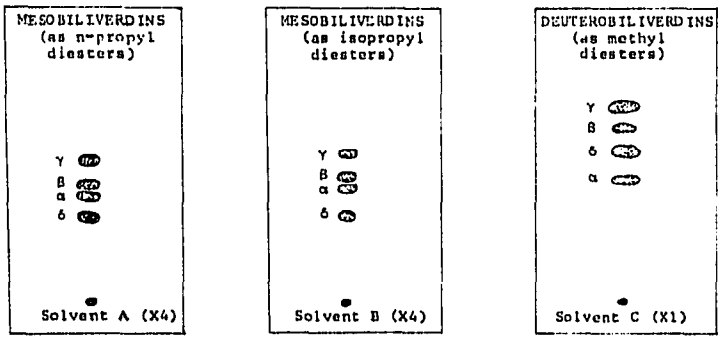

Fig. 1. Separation of isomeric meso- and deuterobiliverdins of the IX series on thin layers of silica gel G. The gypsum "binder" in the silica gel is necessary for the separations. Solvents: (A) *n*-heptane–ethyl methyl ketone (2:1), fourfold irrigation; (B) *n*-heptane–ethyl methyl ketone (2.5:1), fourfold irrigation; (C) cyclohexane–ethyl methyl ketone (2:3), single irrigation. The mesobiliverdin spots are blue-green in colour while the deutorobiliverdins are either royal blue (β and δ isomers) or sky blue (α and γ isomers).

procedures used for such interconversions of protohaem and deuterohaem proved unsuitable for the more labile open tetrapyrroles.

The β and δ deuterobiliverdins were finally identified by taking advantage of the bridge specificity imposed on the cleavage by the apoprotein of haemoglobin. Previous experiments had shown that the apoprotein globin directs coupled oxidation of the haem groups specifically to the α and β methine bridges, resulting in the production of only α and β protobiliverdin isomers from native haemoglobin[9]. The production of only the α and β mesobiliverdin isomers during coupled oxidation of mesohaemoglobin indicated that this specificity was retained when the protohaem groups were replaced by mesohaem. Coupled oxidation of deuterohaemoglobin (formed by replacing the protohaem groups of native haemoglobin with deuterohaem) also yielded only two of the four deuterobiliverdin isomers. One of these corresponded with the already identified α isomer and the other was therefore identifiable as the β isomer. Since the γ isomer was already identified by the dichromate method, this allowed the identification of the fourth deuterobiliverdin spot as the δ isomer (Fig. 1C).

The TLC methods described here and in a previous publication[1] allow the separation and identification of the isomeric proto, meso and deuterobiliverdins and have been useful in studies related to haem catabolism[9,10]. The separations can be applied both as micro-scale methods for quantitative isomer analysis of biliverdin preparations, and as preparative-scale methods for the isolation of the isomers as the diesters. These can then be converted to the free diacids by hydrolysis in HCl. The hydrolysis procedure outlined above in Materials and methods is designed to overcome problems of solubility and stability.

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