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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BILE PIGMENTS

APPLICATION TO THE DETERMINATION OF UNCONJUGATED BILIRUBIN IN SERUM

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SUMMARY

A rapid, sensitive and specific method for the determination of unconjugated bilirubin in serum is described. Reversed-phase chromatography on short-chain alkylsilica (C_2) or octadecylsilica (C_{18}) with acetonitrile–dimethylsulphoxide–water as eluent is used for the separation of bilirubin. The method is applied to the analysis of bilirubin in patients with disorders associated with jaundice.

INTRODUCTION

Bile pigments are tetrapyrroles derived from oxidative fission of porphyrin macrocycles[1]. Most natural bile pigments have the IXα configuration of side chains, arising by fission at the α-bridge of protoporphyrin IX. The compounds are classified primarily according to the extent of conjugation, ranging from the green or blue biliverdins to the colourless urobilinogens with no conjugation. The most important bile pigment is the yellow pigment bilirubin IXα.

The estimation of unconjugated bilirubin concentration in serum is important in the diagnosis, investigation and control of treatment of haemolytic anaemias or prehepatic jaundice[2,3], although in these conditions concentrations of total bilirubin are adequate. However, in haemolytic anaemia, if increased haemopoiesis fails to maintain adequate concentrations of haemoglobin, the anaemia may become sufficiently severe that anoxia causes liver damage and an increased concentration of conjugated bilirubin in the serum.

In posthepatic (obstructive) jaundice, serum bilirubin is almost wholly conjugated, as it may also be in the cholestatic jaundice, common in many patients with severe hepatitis. In hepatitis with less cholestasis, increased concentrations of unconjugated bilirubin appear to accompany the conjugated bilirubin although this may be due to the inadequacy of existing methods for the estimations. Although the proportions of conjugated and unconjugated bilirubin are probably of little significance in hepatitis, their estimation is necessary to distinguish the Dubin–Johnson and

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Rotor's syndrome from the pure unconjugated hyperbilirubinaemia of Gilbert's syndrome due to a defect in transport of unconjugated bilirubin and of the Crigler–Najjar syndrome in which there is a defect in the enzymic mechanism of conjugation. In the first two conditions the serum contains both conjugated and unconjugated bilirubin, the Dubin–Johnson syndrome being due to a defect in the excretion of conjugated bilirubin with no impairment of the conjugation mechanism while in Rotor's syndrome the abnormality is failure of uptake and storage of unconjugated bilirubin[2,3]. In the Dubin–Johnson syndrome haem granules are deposited in the liver.

In neonates, unconjugated hyperbilirubinaemia must not become high enough to permit entry of unconjugated bilirubin into the basal ganglia causing kernicterus. The need for urgent treatment must depend upon the concentration of non-protein bound unconjugated bilirubin in the serum. Although methods have been proposed for the approximate measurement of this bilirubin fraction, an accurate method for the estimation of unconjugated bilirubin with an estimation of the albumin concentration could be of great importance in assessing the need for urgent treatment.

Existing methods of estimating unconjugated bilirubin depend either upon the extent of the direct and indirect diazo reaction under specific conditions[4,5] or on solvent partition by which the unconjugated bilirubin passes into an organic solvent leaving the conjugated bilirubin in an aqueous layer[6]. These methods give conflicting results[7] and a more specific and reliable method is therefore needed.

Although high-performance liquid chromatography (HPLC) separations of bilirubin have been reported[8–10] its application to the analysis of bilirubin in serum has not been described. This paper describes a rapid, sensitive and specific method for the determination of unconjugated bilirubin in serum.

EXPERIMENTAL

Materials and reagents

Bilirubin was from Sigma (London, Great Britain). Acetonitrile was HPLC grade from Rathburn (Walkerburn, Great Britain). Tetraethylenepentamine (TEPA) was technical grade and dimethylsulphoxide (DMSO) was AnalaR grade from BDH (Poole, Great Britain).

Apparatus

Hypersil, Hypersil-SAS and Hypersil-ODS (Shandon Southern Products, Runcorn, Great Britain) were slurry packed. Except for the Hypersil column which was 250 mm × 5 mm I.D., the column dimensions were 100 m × 5 mm I.D. A Pye Unicam (Cambridge, Great Britain) LC3-XP liquid chromatograph with a LC3-UV detector was used. Injection was via a loop-value injector (Rheodyne 7210) fitted with a 20-μl loop.

HPLC

The mobile phases were thoroughly de-gassed (ultrasonication) before use. The separation of bilirubin isomers was performed on Hypersil-SAS with acetonitrile–DMSO–water (30:30:40) as the mobile phase and on Hypersil eluted with acetonitrile–water–TEPA (90:10:0.05). The IXα isomer was collected and used for the construction of the calibration curve.

The analysis of bilirubin in serum was carried out on Hypersil-ODS with acetonitrile–DMSO–water (40:40:20) as the eluent. A small pre-column packed with Hypersil-ODS was used to protect the analytical column. The flow-rate for all the separations was set at 1 ml/min and detection was at 450 nm. Serum (20 μl) or standard bilirubin solution in DMSO were injected directly. Due to the instability of bilirubin to oxygen and light, standard solutions (1–10 mg/dl) were freshly prepared and stored in the dark.

RESULTS AND DISCUSSION

Bilirubin occurs in nature as the IXα isomer and since commercial preparations contain small quantities of IIIα and XIIIα isomers the purification of the IXα isomer before the construction of a calibration curve is essential.

Separations of bilirubin isomers have been reported[9,10] but these required gradient elution and long analysis time. Fig. 1a shows the separation of bilirubin IIIα, IXα and XIIIα isomers by reversed-phase chromatography on Hypersil-SAS with acetonitrile–DMSO–water (30:30:40) as the eluent. The same separation is also obtainable on Hypersil-ODS with acetonitrile–DMSO–water (38:38:24) as the solvent. Alternatively a silica column can be used with acetonitrile–water–TEPA (90:10:0.05) as the mobile phase (Fig. 1b). The separation mechanism here is uncertain, but probably involves adsorption and partition as well as ion exchange. By careful adjustment of the acetonitrile–water ratio, the system is found to be useful for the separation of other bile pigments such as bilirubin conjugates, biliverdins, biliviolins, stercobilins, half-stercobilin and urobilins[11].

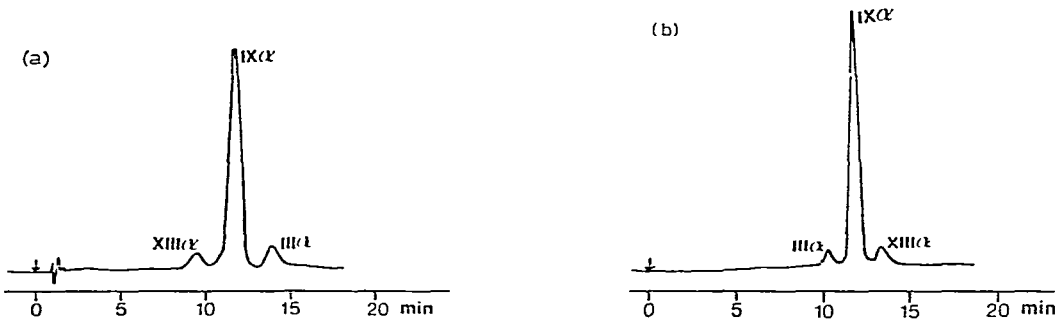

Fig. 1. HPLC separation of bilirubin isomers; (a) Column, Hypersil-SAS; mobile phase, acetonitrile–DMSO–water (30:30:40); flow-rate, 1 ml/min; detection, 450 nm; (b) Column, Hypersil; mobile phase, acetonitrile–water–tetraethylenepentamine (90:10:0.05); flow-rate, 1 ml/min; detection, 450 nm.

For the determination of bilirubin in serum, Hypersil-ODS was used. The mobile phase, acetonitrile–DMSO–water (40:40:20) was chosen to give fast analysis time (5 min) without the loss of resolution. Bilirubin is unstable in solution, being easily oxidised and is sensitive to light. Sample preparation by protein precipitation and solvent extraction was therefore avoided. Serum samples were injected directly and a pre-column was used to protect the analytical column from irreversible adsorption of serum proteins and other impurities.

Fig. 2a and b show the separation of bilirubin from standard solution and from serum of a patient with jaundice due to unconjugated hyperbilirunaemia respectively. Background contamination in the analysis of serum specimens from patients was never a problem as few compounds absorbed at 450 nm, the wavelength used for the detection of bilirubin.

Fig. 2. HPLC separation of bilirubin IXα; (a) Standard; (b) in serum of a patient with unconjugated hyperbilirubinaemia: column, Hypersil-ODS; mobile phase, acetonitrile–DMSO–water (40:40:20); flow-rate, 1 ml/min; detection, 450 nm.

The recovery of bilirubin from a pooled serum sample was calculated by chromatographing the sample before and after adding a known amount of bilirubin. The recoveries (98–99%) were identical for standard bilirubin solutions prepared in DMSO and in a protein matrix[4]. The calibration curve over the range of 1–10 mg/dl was linear. From ten replicate analyses of a serum specimen the coefficient of variation was 2.0%.

The analysis of unconjugated bilirubin in serum of neonates treated with phototherapy[10] by this method always gave results which were lower than the diazo reaction[5] or direct spectrophotometric method. This is not surprising since photobilirubin present in these sera gives positive diazo reaction and spectrophotometry does not differentiate bilirubin from photobilirubin. The HPLC systems described separate bilirubin from photobilirubin.

In sera of patients not undergoing phototherapy and containing both unconjugated and conjugated bilirubin, the HPLC method often gave results for unconjugated bilirubin which were 2–3% higher than the diazo reaction method[5]. Unconjugated bilirubin is known to react with diazotised ethyl anthranilate[7]. This may account for the lower values obtained by the diazo reaction method.

Direct spectrometry measures only total bilirubin. It therefore cannot be used to determine unconjugated bilirubin in samples containing both conjugated and unconjugated bilirubin.

CONCLUSION

The simple reversed-phase procedure described for the rapid and specific determination of unconjugated bilirubin in serum should be of particular value in the

diagnosis and treatment of neonatal jaundice, especially in assessing the effectiveness of phototherapy in reducing serum unconjugated bilirubin.

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