Radioassay of UDP-glucuronyltransferase-catalysed formation of bilirubin monoglucuronides and bilirubin diglucuronide in liver microsomes

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1. A radioassay for specific determination of the rates of UDP-glucuronic acid-dependent conversion of bilirubin into the two isomeric (C-8, C-12) bilirubin monoglucuronides and bilirubin diglucuronide is described and illustrated by its application to rat liver microsomes. 2. The method is based on measurement of the relative amounts of radiolabel in unesterified bilirubin and its mono- and di-esterified reaction products after incubation with [^{14}C]bilirubin as substrate. This analysis is performed by the alkaline-methanolysis procedure, combined with one of two t.l.c. systems developed in order to enhance the sensitivity, accuracy and precision of the radioassay. 3. Results for rates of total bilirubin glucuronide formation obtained with the new assay and the standard enzyme assay based on the ethyl anthranilate diazo-method were identical. However, the sensitivity of the latter technique is approx. 10-fold lower than that of the radioassay.

In most mammals, conjugation of bilirubin occurs predominantly with glucuronic acid and to a minor extent with glucose and xylose (Blanckaert & Schmid, 1982). Some species, including man, rat, dog and cat, excrete these conjugated bilirubins in bile in both mono- and di-esterified forms (Fevery *et al.*, 1977; Gordon *et al.*, 1977), and the natural monoconjugated pigment comprises two isomeric forms, i.e., the C-8 and C-12 isomers, esterified at the propionic acid side chain attached at C-8 or C-12 respectively of the dis-symmetrical bilirubin molecule (Jansen & Billing, 1971; Blanckaert, 1980). Formation of the two monoglucuronides (BMG) as well as of the diglucuronide (BDG) is catalysed by a hepatic microsomal UDP-glucuronyltransferase system (Blanckaert *et al.*, 1979; Gordon & Goresky, 1980), but detailed kinetic analysis of this enzyme system has not been carried out, largely because a specific and sensitive assay for each of the three reaction products was unavailable.

We now describe a radioassay of glucuronida-

tion of bilirubin by liver microsomes (microsomal fractions) employing radiolabelled bilirubin substrate and the previously described alkaline-methanolysis method combined with t.l.c. for analysis of the reaction products (Blanckaert, 1980). This assay permits measurements of enzymic formation of the two individual isomers of BMG and of BDG. In order to further the accuracy, sensitivity and precision of this method, improved t.l.c. systems were developed for analysis of bilirubin and its mono- and di-methyl esters, which virtually eliminate a problem experienced with the previous t.l.c. systems, i.e., contamination of the bilirubin methyl ester bands with radioactivity from unconjugated [^{14}C]bilirubin migrating in front of the esterified pigments. The new radioassay is at least one order of magnitude more sensitive than the previous standard ethyl anthranilate diazo-method (Heirwegh *et al.*, 1972) for measurement of bilirubin UDP-glucuronyltransferase activity.

Experimental

Materials and general procedures

The following chemicals were used: UDP-glucuronic acid, UDP-*N*-acetylglucosamine, Hepes and bilirubin (Sigma, St. Louis, MO,

Abbreviations used: BMG, bilirubin monoglucuronide; BDG, bilirubin diglucuronide; h.p.l.c., high-pressure liquid chromatography.

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U.S.A.); NAD⁺ (Boehringer, Mannheim, Germany); bovine serum albumin, fraction V (Miles Laboratories, Elkhart, IN, U.S.A.); glucaro-1,4-lactone (Calbiochem, La Jolla, CA, U.S.A.). Except when noted otherwise, chloroform (Merck, Darmstadt, Germany) contained 0.5% (v/v) ethanol. The following reference materials were prepared as previously described: mono- and dimethyl esters of bilirubin-III α and bilirubin-XIII α , C-8 and C-12 monomethyl esters and dimethyl ester of bilirubin-IX α (Blanckaert, 1980); [¹⁴C]bilirubin with sp. radioactivity 24570 d.p.m./nmol (Ostrow *et al.*, 1961); [¹⁴C]bilirubin mono- and dimethyl esters with sp. radioactivities of 4000 d.p.m./nmol and 14000 d.p.m./nmol respectively (Muraca & Blanckaert, 1983), except that additional purification was performed by individual rechromatography of the isolated pigments in t.l.c. solvent system B (see below); bilirubin mono- and di-amides (Jansen & Billing, 1971).

Rat liver microsomes were prepared as previously reported (Blanckaert *et al.*, 1979). The h.p.l.c. methods described by Blanckaert *et al.* (1980) and Muraca & Blanckaert (1983) were used for analysis of the isomeric composition (III α , IX α , XIII α) of bilirubin mono- and di-methyl esters and separation of bilirubin and its methyl esters respectively. Unconjugated bilirubin-III α , -IX α and -XIII α were measured by the h.p.l.c. method described in the former paper, except that the mobile phase was chloroform (Merck) containing 1% amylene as stabilizer/acetic acid (99:1, v/v). Measurement of total bilirubin glucuronide formation by the ethyl anthranilate diazo-assay was performed as described by Heirwegh *et al.* (1972). Protein and total bilirubin were measured respectively by the methods of Bradford (1976) and Novros *et al.* (1979). Glass plates precoated with silica gel (5763/0025 from Merck) were used for t.l.c.

Assay of formation of BMG and BDG

Incubation mixtures contained the following components. Rat microsomes resuspended in 0.25 M-sucrose containing 1 mM-EDTA (150 μ l per 600 μ l of total incubation mixture; 2–3 mg of protein/ml); Hepes buffer, pH 7.7 (0.140 M); MgCl₂ (6.25 mM); NAD⁺ (10 mM); UDP-*N*-acetylglucosamine (2 mM); glucaro-1,4-lactone (1 mM); UDP-glucuronic acid (0 or 5 mM); [¹⁴C]bilirubin and albumin (50 μ M each). [¹⁴C]Bilirubin of appropriate sp. radioactivity (to give approx. 25000 d.p.m. per 600 μ l of final incubation mixture) was dissolved in 0.05 M-NaOH containing 1 mM-EDTA (2 μ l per μ g of pigment) and added to albumin dissolved in water. The incubation mixture was made up at 0°C and blanketed with an argon atmosphere, the bilirubin-albumin complex was added

last, and the mixture was pre-incubated for 3 min at 37°C. Thereafter the glucuronidation reaction was started by addition of UDP-*N*-acetylglucosamine solution (30 μ l for each 600 μ l of total incubation mixture), and incubation at 37°C was continued for up to 10 min. Blanks were incubated under identical conditions, but water was added instead of the UDP-*N*-acetylglucosamine solution. Total bilirubin concentration remained unchanged during incubation for up to 10 min. A 600 μ l portion was withdrawn at appropriate time intervals and transferred to 6 ml of methanol kept at 18–22°C and containing approx. 2 mg of EDTA (disodium salt), approx. 10 mg of sodium ascorbate and approx. 10 nmol of unlabelled carrier bilirubins. The latter pigment consisted of 5–20 μ l of 'enriched' rat bile (Blanckaert *et al.*, 1979) and contained unconjugated bilirubin, BMG and BDG in approximately equal proportions.

Determination of the relative amounts of [¹⁴C]bilirubin, [¹⁴C]BMG and [¹⁴C]BDG formed during incubation was performed by the alkaline-methanolysis procedure, using chloroform/methanol/acetic acid (97:2:1; by vol.; solvent A) for separation of the pigments by t.l.c. (Blanckaert, 1980). Alternatively, development of the chromatogram over about 15 cm was carried out with a new solvent system (solvent B) consisting of chloroform/methanol/NH₃ (1000:150:7; by vol.). Chromatography with solvent B was performed on a t.l.c. plate (5 cm $\times$ 20 cm) evenly sprayed with 4 ml of a freshly prepared sodium ascorbate solution (1 g of ascorbate was first dissolved in 2.5 ml of water and 48 ml of methanol was added). After spraying, the plates were, in darkness, dried by exposure to ambient air (18–22°C) for at least 30 min and were used within 2 h.

Preparation of samples for measurement of ¹⁴C and data analysis were carried out as described by Blanckaert (1980). Radioactive material used per chromatogram varied between 10000 and 30000 d.p.m. Initial rates of glucuronide formation were computed on the basis of the known starting [¹⁴C]bilirubin concentration and slope of the linear portion of the curve defined by the percentages of each product in the incubation medium as a function of time.

Results and discussion

The objective of the present study was to adapt the alkaline-methanolysis method to the specific requirements of the radioassay of bilirubin UDP-glucuronyltransferase. When the previously reported t.l.c. method is used, radioactive contamination of the bilirubin methyl ester bands caused by smearing of ¹⁴C label over the chromatography path of the [¹⁴C]bilirubin substrate migrating close

to the solvent front is approx. 1–2% in each of the individual monoester bands and the diester band (Blanckaert, 1980), which is of the same order of magnitude as the radioactivity derived from enzymically formed esters. This chromatographic problem rendered the assay inaccurate, imprecise and insensitive, particularly when the percentage of radiolabelled substrate converted into esterified pigment was below 5%. Therefore an important goal of the present study was to improve the t.l.c. system by two approaches: inhibition of breakdown of bilirubins during chromatography to decrease smearing of ^{14}C label over the chromatogram, and development of a new t.l.c. system in which the esterified bilirubin products migrate ahead of the [^{14}C]bilirubin substrate.

Separation of bilirubin and its methyl esters by t.l.c. with solvent B

Bilirubin-III α , -IX α and -XIII α chromatographed as a single band (R_F 0.11) and so did the III α , IX α and XIII α isomers of bilirubin dimethyl ester (R_F 0.84). The monomethyl ester fraction was resolved in two distinct bands, one corresponding with the IX α C-12 isomer (R_F 0.37), the other with a mixture of the IX α C-8, III α and XIII α isomers (R_F 0.34). Bilirubin diamide coincided with the dimethyl ester, and the mixture of bilirubin-IX α C-8 and C-12 monoamides gave two bands (R_F 0.54 and 0.60) separated from, and located between, the dimethyl ester and monomethyl esters. Recoveries of ^{14}C in the various sections of the chromatogram for individually chromatographed pigments are given in Table 1. Unlike in the t.l.c. system with solvent A (Blanckaert, 1980), spill-over of ^{14}C from unesterified bilirubin was negligible. For comparison, t.l.c. of the same [^{14}C]bilirubin preparation with solvent A gave recoveries (% of ^{14}C label applied to the plate; mean $\pm$ S.D.; $n = 5$) of 2.3 ± 0.5 in the monomethyl ester bands and 1.7 ± 0.5 in the dimethyl ester band. The corresponding results ($n = 5$) were 0.46 ± 0.04 and

0.19 ± 0.01 respectively when the antioxidant 2,6-di-*t*-butyl-*p*-cresol (0.5%; w/v) was added to solvent A in order to inhibit breakdown of [^{14}C]bilirubin on the plate. With solvent B, smearing of ^{14}C over the chromatographic track of individually chromatographed mono- and di-methyl esters was limited to about 4% of total ^{14}C on the plate.

Does disproportionation, hydrolysis or ammonolysis occur during t.l.c. with solvent B, thereby invalidating the assay?

Absence of significant disproportionation was demonstrated by two approaches. First, the isomeric composition (III α , IX α , XIII α) of each individual pigment fraction recovered from the t.l.c. plate after chromatographic development was identical with that of the applied pigment (Table 2). Second, when equal amounts of [^{14}C]bilirubin and [^{14}C]bilirubin dimethyl ester together with carrier pigment containing unlabelled bilirubin and its mono- and di-methyl esters were chromatographed, only $0.10 \pm 0.02\%$ ($n = 5$) of the total ^{14}C on the plate was recovered in the two monomethyl ester bands. This relative amount was fully accounted for by the smearing of ^{14}C over the whole chromatographic track when the same preparation of [^{14}C]bilirubin dimethyl ester was chromatographed in the absence of [^{14}C]bilirubin, in which case $0.12 \pm 0.05\%$ ($n = 5$) of the total ^{14}C was found in the monomethyl ester area. Possible hydrolysis and/or ammonolysis of the methyl esters in solvent system B were tested in the recovery experiments given in Table 1. For the monomethyl esters, radioactivity in the bilirubin band and the area located between the monomethyl esters and bilirubin accounted for about 3.5% of the total ^{14}C on the plate, which probably was caused predominantly by breakdown of the pigment during chromatography and possibly to a minor extent also by hydrolysis. This interpretation is supported by the results obtained with [^{14}C]bilirubin dimethyl ester, for which hydrolysis appeared to be

Table 1. Recoveries of ^{14}C label from purified ^{14}C -labelled pigments developed with solvent B

^{14}C -labelled esterified bilirubin, its monomethyl ester or its dimethyl ester was mixed with unlabelled carrier pigment containing bilirubin (BR) and its monomethyl ester (BMM), monoamide (BMA) and dimethyl ester (BDM) derivatives, and subjected to t.l.c. with solvent system B. Distribution of ^{14}C label was determined in the various pigment bands and in the areas of the chromatogram between B and BMM (A1), between BMM and BMA (A2), and between BMA and BDM (A3). The values correspond with mean $\pm$ 1 s.d. obtained from five chromatograms for each of the three individually chromatographed ^{14}C -labelled pigments. Abbreviation used: n.t., not tested.

^{14}C pigment applied to plate	Recovery of ^{14}C label in chromatogram (% of applied ^{14}C label)						
	BR	A1	BMM	A2	BMA	A3	BDM
Bilirubin	99.87 ± 0.07	n.t.	0.05 ± 0.04	n.t.	n.t.	n.t.	0.05 ± 0.05
Bilirubin monomethyl ester	1.81 ± 0.42	1.76 ± 0.38	96.02 ± 0.08	0.11 ± 0.10	0.10 ± 0.06	0.05 ± 0.09	0.15 ± 0.01
Bilirubin dimethyl ester	0.45 ± 0.25	0.21 ± 0.04	0.79 ± 0.52	0.39 ± 0.06	0.73 ± 0.07	1.60 ± 0.42	95.82 ± 0.81

Table 2. Comparison of isomeric composition of bilirubins before and after t.l.c. with solvent B
Bilirubin and its mono- and di-methyl esters were individually chromatographed. After development of the chromatogram, the bilirubin band was eluted with chloroform, the methyl esters with chloroform/methanol (1:1, v/v). The III α , IX α , and XIII α isomers were determined by the h.p.l.c. method.

Pigment applied to plate	Isomeric composition [% of total pigment (mean $\pm$ s.d.)]					
	Before application			After chromatography		
	III α	IX α	XIII α	III α	IX α	XIII α
Bilirubin ($n = 3$)	2.84 $\pm$ 0.02	93.67 $\pm$ 0.05	3.49 $\pm$ 0.07	2.86 $\pm$ 0.17	93.52 $\pm$ 0.26	3.62 $\pm$ 0.09
Bilirubin monomethyl ester ($n = 5$)	8.22 $\pm$ 0.21	87.37 $\pm$ 1.98	4.40 $\pm$ 0.18	7.66 $\pm$ 0.14	87.73 $\pm$ 0.63	4.61 $\pm$ 0.15
Bilirubin dimethyl ester ($n = 5$)	12.56 $\pm$ 0.61	72.46 $\pm$ 0.67	14.97 $\pm$ 0.06	11.23 $\pm$ 1.05	73.83 $\pm$ 0.98	14.93 $\pm$ 0.52

Fig. 1. Radioassay of formation of individual bilirubin glucuronides by rat liver microsomes
[^{14}C]Bilirubin ($50\mu\text{M}$) was incubated with rat liver microsomes (0.67mg of protein/ml) at 37°C and specimens were withdrawn from the incubation mixture at 3 min intervals for analysis by alkaline methanolysis. The organic extract was divided in two portions, one for t.l.c. with solvent A containing antioxidant (a), the other for t.l.c. with solvent B (b). Radioactivity in each pigment band was determined and is shown as a percentage of total ^{14}C in the unesterified bilirubin and various methyl ester bands. Each time point in the two parts of the Figure represents the results of five chromatograms, and mean values $\pm 2\text{s.d.}$ are given. s.d. values are not shown for zero time. The curves show the linear fit by the least-squares method using the 20 individual values. $\bullet$, Dimethyl ester; $\square$, C-12 monomethyl ester; $\blacksquare$, C-8 monomethyl ester in (a) and the sum of C-8, III α and XIII α monomethyl esters in (b).

minimal. Radioactivity in the bilirubin monoamide band was not significantly above the background values in the adjacent areas of the chromatogram when [^{14}C]bilirubin mono- and dimethyl esters were chromatographed, indicating that ammonolysis was insignificant.

Radioassay of enzymic formation of BMG and BDG in rat liver microsomes and comparison with the ethyl anthranilate diazo-method

Progress curves for UDP-*N*-acetylglucosamine-dependent formation of BDG and individual BMG isomers obtained with analysis of the reaction products using either solvent A containing antioxidant or solvent B are shown in Fig. 1. Because contamination of reaction product bands with ^{14}C from [^{14}C]bilirubin was minimal, reproducibility tended to be slightly better for the data points in the progress curves obtained with solvent B, particularly at the first, 3 min, time point corresponding with the smallest conversion of [^{14}C]bilirubin substrate. Consistent with the previously found predominance of C-8 BMG isomer over C-12 BMG isomer in rat bile (Blanckaert, 1980), the rate of C-8 isomer formation exceeded the C-12 isomer formation rate and the ratio, C-8/C-12, was 1.9. A disadvantage of solvent B is that the BMG isomer ratio cannot be correctly assayed because the C-8-containing band also contains the III α and XIII α isomers. Also in agreement with previous findings (Blanckaert *et al.*, 1979), rates of formation of both isomers of BMG largely exceeded the rates of BDG formation when assayed under zero-order kinetic conditions.

grams, and mean values $\pm 2\text{s.d.}$ are given. s.d. values are not shown for zero time. The curves show the linear fit by the least-squares method using the 20 individual values. $\bullet$, Dimethyl ester; $\square$, C-12 monomethyl ester; $\blacksquare$, C-8 monomethyl ester in (a) and the sum of C-8, III α and XIII α monomethyl esters in (b).

Results of the radioassay with solvent B and the standard enzyme assay using the diazo-method were compared by measuring total glucuronide formation in assay mixtures containing 50 μM - ^{14}C bilirubin and variable amounts of rat liver microsomal protein (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/ml). Glucuronide formation was a linear function of protein concentration in both assays, and amounted to 617 nmol of total bilirubin glucuronides/min per mg of protein. Almost identical values were obtained with the two assays, as evidenced by the result of orthogonal regression analysis. When the diazo-method and radioassay results were used as independent and dependent variables respectively, the slope was 0.981, with a positive intercept of 0.033 nmol of total glucuronides/min per mg. The limit of detection of bilirubin UDP-glucuronyltransferase activity with the ethyl anthranilate method is approx. 0.4 nmol of total bilirubin glucuronides, corresponding with 0.005 for the difference between the A_{430}^{cm} values of test and blank. In contrast, the sensitivity of the radioassay is dependent on measurement of a minimal percentage conversion of ^{14}C bilirubin into esterified pigment rather than on detection of an absolute amount of reaction product. Substrate concentrations as low as 1 or 2 μM can be used in the radioassay, and conversion percentages of approx. 1% can be determined with good precision (Fig. 1). Consequently, the sensitivity of the new radioassay is at least one order of magnitude better than that of the diazo-method.

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