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NOVEL METHOD FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AZO DERIVATIVES OF CONJUGATED AND UNCONJUGATED BILIRUBIN

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SUMMARY

A method for the separation and quantitation of ethyl anthranilate or *p*-iodoaniline azo derivatives of bile pigments was developed using reversed-phase high-performance liquid chromatography. A convenient separation was achieved in 15 min, permitting the quantitation of the unconjugated azodipyrrole (α_0) and its glucuronide (δ), xyloside (α_2) and glucoside (α_3) conjugates. The pathological β- and γ-azo pigments, derived from bilirubin glucuronide isomers that occur in cholestatic bile or plasma, are also detected in this system. The results of this method as applied to bile from 25 healthy dogs were in excellent agreement with the values obtained by reversed-phase chromatography of bilirubin and its mono- and dimethyl esters produced from the corresponding conjugates by alkaline methanolysis. This system permits the sensitive and convenient determination of bilirubin and its conjugation pattern in biological fluids.

INTRODUCTION

Bilirubin is produced by the catabolism of several haemoproteins. Unconjugated bilirubin (UCB) is cleared from the plasma by the liver and excreted into the bile almost exclusively in the conjugated form [1]. In normal conditions the hepatic conjugation by the microsomal UDP-glycosyl transferases is the rate-limiting step [1–3]. In humans and in dogs, ca. 20% of the conjugates are produced as monoconjugates (MCB) and 80% as diconjugates (DCB) [2]. The con-

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jugates formed in humans and in rats are primarily glucuronides with small amounts of other conjugating sugars [2,4]. In dogs large fractions of glucoside and xyloside conjugates are also produced [5,6]. A variety of conjugation patterns has been found in several other species [2,5,7]. As the bile pigment conjugates are mainly formed in the liver, hepatic disease might alter the conjugation pattern. The influence of pathological conditions in several species on the bilirubin conjugation pattern has been partially investigated.

The detection of the different conjugates depends largely on the separation of the azodipyrroles derived from treatment with diazotized ethylanthranilate (EA) or *p*-iodoaniline (PIA) [2,4,7]. So far, the separation has been primarily performed by thin-layer chromatography (TLC). However, TLC gives semiquantitative rather than quantitative results, and smaller fractions may not be detected. It is also very hard to prevent some decomposition of the unstable bilirubins during the TLC procedure. High-performance liquid chromatography (HPLC) seems to have distinct advantages over TLC. It permits relatively short separation times, the complete exclusion of light and molecular oxygen, and the quantitation of smaller peaks. There is no routinely applicable HPLC procedure available for the measurement of both physiological and pathological EA or PIA azo pigments. This paper describes the conditions for a convenient HPLC procedure.

EXPERIMENTAL

Animals

Twelve male and thirteen female healthy mature dogs were investigated. The hepatobiliary system was jugded to be normal by the absence of deviations of serum alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase and γ-glutamyl transpeptidase, using standard methods. Total serum protein and albumin were within the normal ranges. Laparoscopy and histological examination of hematoxylin and eosin-stained paraffin-embedded liver biopsies revealed no abnormalities.

Collection and preservation of samples

Bile was obtained during laparoscopy. The gall bladder was punctured with a Chiba cannula (Paes, Zoeterwoude, The Netherlands) and 2 ml of bile were aspirated and immediately stored in four separate aliquots under argon in stoppered tubes in the dark at $-80\,^{\circ}C$ until analysis.

HPLC apparatus and reagents

Pigments were separated on an HPLC apparatus (Beckman Instruments, Berkeley, CA, U.S.A.) consisting of a Model 340 injector, a Model 421 solvent programmer, two Type 112 pumps and a Type 160 absorbance detector. Separations were performed on a 250×4.6 mm I.D. 5-μm Ultrasphere-ODS column (Beckman Instruments). Reagents were HPLC-grade (Baker Chemical, Phillipsburg, NJ, U.S.A.). Tetrabutylammonium phosphate (Pic A reagent) was manufactured by Waters (Milford, MA, U.S.A.).

Measurement of bilirubin and its mono- and diconjugates

Bilirubin and its mono- and diconjugates were measured by HPLC analysis of bilirubin and its mono- and dimethyl esters produced from bilirubin conjugates by alkaline methanolysis with xanthobilirubic acid methyl ester as an internal standard [8]. Bile samples were diluted ten-fold with normal saline, and alkaline methanolysis was then performed on 0.2-ml samples. The internal standard was stored and included in the procedure as described by Muraca and Blanckaert [8]. These and all other manipulations were performed in red light under protection from daylight.

HPLC analyses were performed at a flow-rate of 1.5 ml/min at room temperature. The absorbance was measured at 436 nm. The solvents were (A) methanol–water–Pic A reagent (75:25:0.5, v/v) and (B) methanol–ethanol–water–Pic A reagent (80:15:15:0.5, v/v). Both solvents contained 2 m*M* sodium ascorbate. The separation was performed with a gradient in two linear steps: 0 to 50% B in 3 min, 50 to 100% B in 1 min and finally 100% B for 5 min. The column was regenerated in 10 min at 100% A.

HPLC of azodipyrroles

Bile samples were treated either with diazotized PIA or EA [10]. Before treatment the bile samples were diluted twenty-fold with normal saline, and the absorbance of the organic extracts was measured at 546 nm. The extracts were subsequently evaporated to dryness under nitrogen and redissolved in a small volume of methanol, and 20 μl were injected in the liquid chromatograph. The separation was performed at room temperature at a flow-rate of 1.5 ml/min. The absorbance was measured at 546 nm. The solvents were (A) acetonitrile–methanol–buffer (50:10:40, v/v) and (B) acetonitrile–methanol–buffer (80:10:10, v/v). The buffer was 0.1 *M* sodium acetate–acetic acid (pH 4.0). The best separation was achieved with a linear gradient in 15 min from 0 to 100% B and re-equilibration with 100% A for 8 min.

HPLC revealed four pairs of peaks in the chromatogram. The relation of these peaks to the known α_0, α_2, α_3 and δ pigments corresponding to azodipyrrole and the xyloside, glucoside and glucuronide ester, respectively [10], was assessed in three ways. The organic extracts used for HPLC were also applied on pre-coated silica gel TLC plates with a concentrating zone (Merck, Darmstadt, F.R.G., Type 11845). The plates were developed with three solvents successively as described by Heirwegh et al. [10]. The individual bands were scraped from the plates and redissolved in 1.0 ml of methanol. The combined pigment extracts from several plates were evaporated under nitrogen to a small volume, and the obtained reference pigments α_0, α_2, α_3 and δ were injected in the HPLC system. The azodipyrrole peak in the chromatogram was independently identified by treatment of chloroform-dissolved manufactured UCB (Merck) with diazotized EA and PIA [10] and injection of the azo pigments into the chromatograph. Analysis by HPLC [11,12] of the purchased UCB revealed the pigment to consist of 91% bilirubin IX_α, 3% III_α and 6% $XIII_\alpha$. The pigments δ and α_0 were further identified by treatment of δ pigment with β-glucuronidase from bovine liver (Sigma, St. Louis, MO, U.S.A.) for 4 h. The δ pigment was purified by TLC as described before;

incubation with β-glucuronidase was performed as described by Fevery et al. [13]. Parallel controls were made by omission of the enzyme. The conversion of δ pigment (glucuronide) into α_0 was evaluated by HPLC of the pigment and the controls before and after incubation with the enzyme.

In addition to the above-mentioned azo pigments, the abnormal β and γ azo pigments may be encountered, especially in bile or serum of patients with cholestatic disease [13,14]. They are the azo derivatives of isomers of bilirubin glucuronides formed in vivo or in vitro by an acyl shift of the glucuronic acid residue [14]. The localization of β and γ azo pigments in the chromatogram was assessed by HPLC of the isolated β and γ bands from TLC plates obtained from the bile of a dog with a bile duct obstruction caused by a pancreatic carcinoma.

The differences in the composition of the solvent used during development of the gradient could possibly alter the absorbance of the successive azo pigments in the chromatogram. This was investigated as follows. The individual azo pigment peaks were isolated by TLC as described above. The isolated azo pigments were dissolved in small volumes of methanol and separatedly mixed with solvent B. The absorbance curve was monitored during the gradient; it should be linear when the absorbance is not influenced by the composition of the solvent mixture. The possible influence of the solvent composition on the absorbance was further investigated by measuring the absorbance at 546 nm of equimolar solutions of the pigments in solvent A, solvent B and a 1:1 mixture of A and B.

The total bile pigment concentration in bile was independently measured by the method of Brückner [15].

Statistics

The distribution of some of the parameters was asymmetric, so the results are given as median values and the central 90% confidence intervals of the 2.5th and the 97.5th percentiles. Differences between values from different methods were examined with the Wilcoxon–Mann–Whitney test, and correlations were investigated with Spearman's rank correlation test [16]. Significance was considered to exist when $p < 0.05$.

RESULTS

Separation by HPLC produced similar chromatograms for EA and PIA azo derivatives. The retention times of the EA azo pigments were somewhat longer than the times for the corresponding azo pigments obtained by treatment with diazotized PIA (Fig. 1). The results of HPLC of the individual isolated azo pigments δ, α_3, α_2 and α_0 are depicted in Fig. 2A–D. The chromatogram of the azo pigments derived from commercial bilirubin was identical with that of the isolated azo pigment α_0. Incubation of azo pigment δ with β-glucuronidase produced a shift in the chromatogram from a single δ peak to a predominant α_0 peak, when compared with the control. The localization in the chromatogram of the peaks corresponding to the azo pigments γ and β is depicted in Fig. 3A and B, respectively. The gradually changing solvent composition during gradient elution did not influence the absorbance of any of the individual azo pigments. There was a

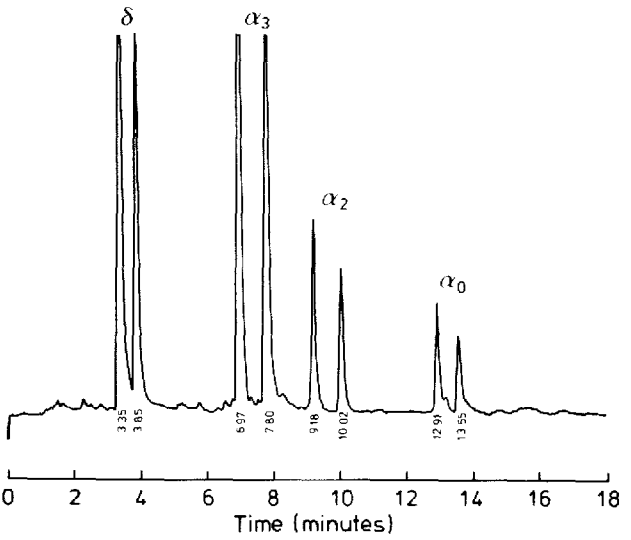

Fig. 1. HPLC profile of PIA azo pigments obtained from bile of a healthy dog. The separation conditions and the meanings of the symbols are explained under Experimental. Separation of EA azo pigments produced a similar chromatogram with somewhat higher retention times ($\delta = 3.90$ and 4.45; $\alpha_3 = 7.52$ and 8.39; $\alpha_2 = 9.62$ and 10.55; $\alpha_0 = 13.15$ and 14.05 min).

linear increase of the absorbance during a linear gradient with the azo pigments dissolved in solvent B. This is exemplified for azopigment δ in Fig. 4. The measured absorbance at 546 nm for each of the azo pigments was identical when equal amounts were dissolved in solvent A, solvent B or a 1:1 mixture of A and B.

Bile yielded predominantly the azo pigments δ and α_3, with less of the α_2 and α_0 azo pigments (Table I). No β or γ azo pigments were detected in the bile of healthy dogs. The total bilirubin concentration in bile measured after treatment with diazotized EA was significantly lower than the values of the PIA azo pigments ($p < 0.01$). There was no significant difference between the concentrations of total bilirubin measured by a conventional diazo method [15], by alkaline methanolysis HPLC or by treatment with diazotized PIA. The ratios between the total pigment concentrations obtained with the several methods were PIA/alkaline methanolysis = 0.99 (0.85–1.15); PIA/Brückner = 0.98 (0.87–1.11) and PIA/EA = 1.35 (1.08–1.63).

The results of alkaline methanolysis HPLC and chromatography of the PIA and EA azo pigments were further compared by calculating the expected percentage of azodipyrrole (α_0) from the fractions UCB, MCB and DCB in bile. In these calculations the EA azo derivatives were assumed to be exclusively derived from bilirubin conjugates with the PIA azo pigments corresponding to all bilirubins [10,17]. There was no significant difference between the expected and the measured PIA and EA α_0 fractions. The ratios between the measured and the expected α_0 fractions were 1.00 (0.74–1.36) and 0.95 (0.73–1.16) for PIA and EA azodipyrrole, respectively. The percentage of MCB in bile, calculated [17] as twice the α_0 fraction measured after treatment with diazotized EA, was 20 (14–29),

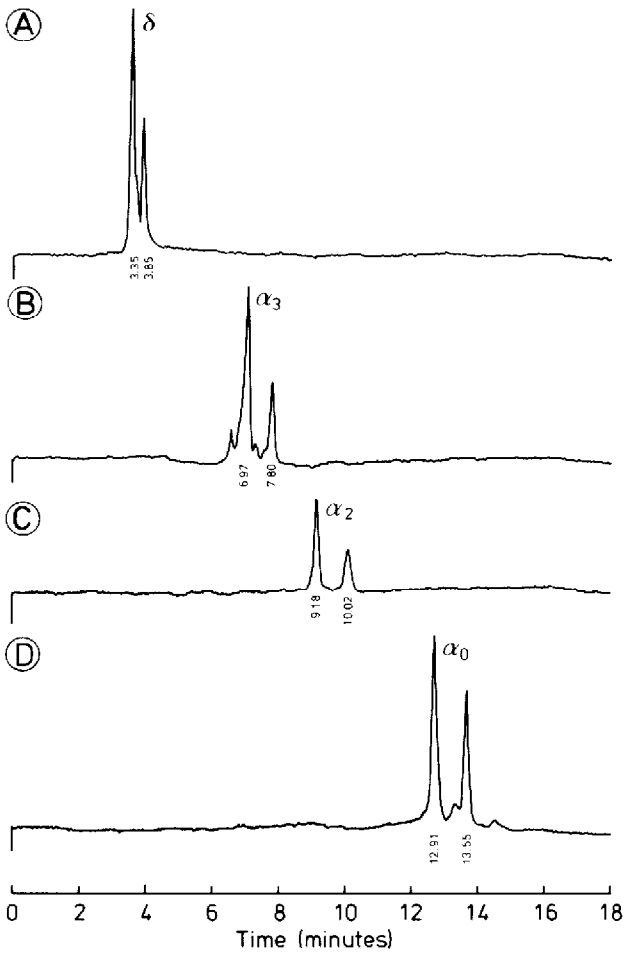

Fig. 2. HPLC profiles of the individual PIA azo pigments isolated by TLC. The chromatograms of azo pigments δ, α_3, α_2 and α_0 are given in A, B, C and D, respectively.

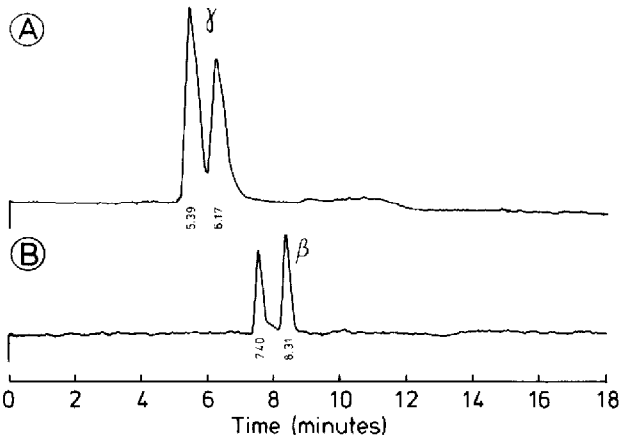

Fig. 3. HPLC profiles of the PIA azo pigments β (B) and γ (A). The pigments were isolated by TLC from bile of a dog with chronic extrahepatic cholestasis.

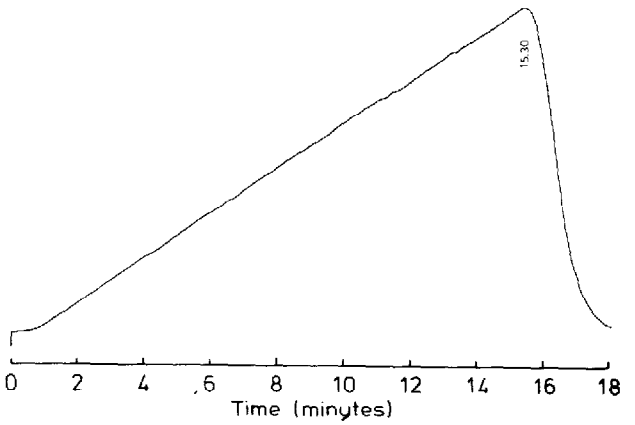

Fig. 4. Absorbance measured at 546 nm of PIA azo pigment δ during a linear gradient from 100% A to 100% B in 15 min. The azo pigment was dissolved in solvent B. Similar linear absorbance curves were obtained for all azo pigments.

TABLE I

CONCENTRATIONS OF BILIRUBIN IN BILE

Values in μmol/l as measured by HPLC after treatment with diazotized *p*-iodoaniline (PIA) and ethyl anthranilate (EA), and after alkaline methanolysis (AM), and fractions of the azo derivatives from the diazo methods.

Method	Concentration (μmol/l)	α_0 (%)	α_2 (%)	α_3 (%)	δ (%)
PIA	1772 (1042–3697)	14 (11–17)	13 (10–17)	34 (30–39)	38 (33–44)
EA	1256 (582–3141)	10 (7–14)	15 (11–17)	36 (33–41)	39 (34–45)
AM	1692 (1098–2692)	–	–	–	–

which was not significantly different from the value obtained by alkaline methanolysis HPLC.

DISCUSSION

The fractions UCB, MCB and DCB in bile as measured with alkaline methanolysis HPLC are in agreement with the results obtained from other methods [2,7]. The percentage of UCB in bile may have been slightly overestimated, because hydrolysis instead of methylation of ca. 0.5% of the conjugates may occur during alkaline methanolysis. This small error could not influence the presented results to any significant extent.

Different methods were compared for the determination of the total bilirubin concentrations in bile. The results of alkaline methanolysis HPLC, treatment with diazotized PIA and a conventional diazo procedure were identical. However, the concentration as measured by the EA azo colour absorbance was significantly lower. As the bile samples were diluted twenty-fold to prevent reaction of UCB

with diazotized EA [17], this difference is partly explained by the fraction of UCB in bile. However, the difference between the EA azo colour and the results of the other methods by far exceeded the small UCB fraction. Therefore, loss of azo colour in the EA procedure is most probably responsible for the observed difference. The different azo pigments were equally affected by this phenomenon, since the azo pigment fractions measured with EA and PIA were not significantly different.

The HPLC technique described permitted the rapid and sensitive quantitation of the azo derivatives of bilirubin and the sugar conjugates. The identification of the peaks in the chromatogram was assessed by comparison with the retention of the corresponding azodipyrroles after isolation by TLC. The identification of the α_0 and δ peaks was validated by the shift of the δ azo pigment from the δ to the α_0 position in the chromatogram after incubation with β-glucuronidase. In addition, HPLC of azodipyrrole obtained by treatment of commercial bilirubin with diazotized PIA or EA produced α_0 peaks at the expected location in the chromatogram. The identification of the α_2 and α_3 peaks in the chromatogram has not been validated completely. However, their relative amounts as measured by HPLC and by TLC were of the same order of magnitude. The four azo pigments mentioned were the only predominant bands in canine bile when investigated by TLC, and their R_F values were identical with the previously reported values for structurally identified reference compounds [4,6,10]. Therefore, there is no doubt as to the identification of the α_2 and α_3 peaks in the HPLC profile. The two peaks in the chromatogram observed for each individual azo pigment were probably the *endo-* and *exo*-vinyl isomers reported by Onishi et al. [18].

Because the absorbances of the different HPLC peaks were measured in different solvents, as a logical consequence of gradient elution, the influence of the solvent composition on the absorbance coefficients was investigated. The linear increase of the absorbance for all azo pigments under the conditions of the chromatogram in Fig. 4 indicates that there is no effect from the solvent composition on the measured azo pigment fractions. However, this finding did not exclude a linear change in the absorbance coefficients during gradient elution. The identical absorbance of the azo colour after equal amounts of the pigments had been dissolved in solvent A, solvent B or a 1:1 mixture of A and B demonstrates the absence of any influence from the solvent composition.

The results of HPLC of the bile pigments after treatment with diazotized PIA and EA were further compared with the results of alkaline methanolysis HPLC. Diazotized EA is known to react exclusively with conjugated bilirubins (MCB and DCB), whereas the PIA azo derivatives correspond to all bilirubins (UCB, MCB and DCB) [10]. Therefore, the expected α_0 fraction to be obtained by both methods can easily be calculated from the UCB, MCB and DCB fractions as measured by alkaline methanolysis HPLC. The good agreement between the expected and the calculated α_0 fractions confirms the accuracy of the new technique for HPLC of azodipyrroles. This is supported by the identical percentages of MCB in bile as measured by alkaline methanolysis HPLC and calculated as twice the percentage of α_0 after treatment with diazotized EA.

The results of HPLC separation of the azo derivatives of bilirubin and the sugar

conjugates were comparable with those reported by Onishi et al. [18]. However, the rapidity (15 min instead of 80 min) makes the presented procedure more generally applicable. HPLC of canine PIA azo pigments as described by Little [19] seems to be less sensitive, although the absence of quantitative data prevents adequate comparison.

The fraction α_3 azo pigment in bile of these dogs was higher and the δ fraction lower than previously reported values for canine bile [5]. These differences may be the result of different separation techniques. HPLC seems to have distinct advantages over TLC. HPLC permits relatively short separation times and also the complete exclusion of light and molecular oxygen. Therefore the unstable bilirubins are less likely to decompose during HPLC analysis. The absence of β and γ pigments in bile of these healthy dogs, in contrast to previous reports [5], can be attributed to these differences between HPLC and TLC preventing isomerization of the δ azo pigment. The relation between the α_0 and δ azo pigments, and the absence of β and γ azo pigments in normal canine bile reported here, are in agreement with the chromatograms published by Onishi et al. [18].

The HPLC system described permits the convenient determination of bilirubin azodipyrrole and its glucoside, xyloside and glucuronide esters, and also of the pathological β- and γ-glucuronide isomers. The system has also been applied to the cholestatic serum of dogs with spontaneous hepatobiliary diseases and seems to be equally applicable in those conditions [20]. Therefore, the method may be generally applicable for the determination of bilirubin azo derivatives in biological fluids. The method seems faster and more sensitive than other methods. Because of the advantages of HPLC over TLC this system is a convenient alternative when these measurements are employed.

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