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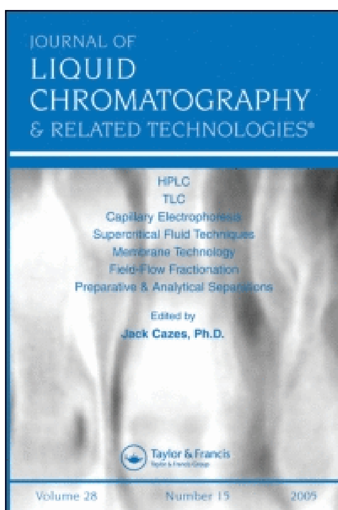

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Henri Saxerholt[a]
[a] Department of Germfree, Research Karolinska Institute, Stockholm, Sweden

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SEPARATION OF ETHYL ANTHRANILATE AZOPIGMENTS FROM GERM-FREE RAT FECES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Henri Saxerholt

Department of Germfree Research
Karolinska Institute
Box 60 400
S-10401 Stockholm, Sweden

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ABSTRACT

A simple method was developed for the separation of ethyl anthranilate azopigments derived from conjugated bilirubins prepared from germfree (GF) rat feces. The δ and α_0 azopigments were separated and these two azopigments were also separated into their endo-vinyl- and exo-vinyl isomers, respectively. The reverse-phase, high-performance liquid chromatographic (HPLC) separation was achieved by using a μBondapak C_{18} column and a mixture of acetonitrile, distilled water and sodium acetate as the mobile phase.

INTRODUCTION

Previous findings have revealed that GF rats excrete conjugated bilirubin by the fecal route, whereas conventional (CONV) rats lack conjugated bilirubin in

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their feces (1,2). In the CONV rat, the bilirubin conjugates are deconjugated by the intestinal microflora (3). The presence of conjugated bilirubin in GF rat feces is confirmed by coupling the fecal samples with diazotized ethyl anthranilate, thus forming the dipyrrolic azopigment derivatives of the tetrapyrroles (2,4, 5). The ethyl anthranilate azopigments are then separated by thin layer chromatography (tlc) (2). The pattern of separated azopigments is similar to the pattern obtained from bile of CONV rats. Azopigment δ (azodipyrrole-ß-D-monoglucuronide) and azopigment α_0 (azodipyrrole) are the predominant azopigments obtained from GF rat feces, whereas no azopigments are obtained from CONV rat feces (1,2,3). Besides these two predominant azopigments, traces of several other azopigment spots are detected by tlc. Most of these other azopigment spots are probably azopigments of the so called γ- and ß-groups with yet unknown structure (4,5). Since it is yet unknown which bacteria are capable of deconjugating bilirubin conjugates in the lower parts of the intestine, it is important that more sophisticated methods are developed for the separation of bilirubin tetrapyrroles and their azopigment derivatives preparable from feces. Recently, Onishi et al (6) have described a new HPLC method for the separation of ethyl anthranilate azopigments from dog bile. This group of investigators have separated the δ, α_3, α_2, and α_0 azopigments and also have succeeded in separating each of these azopigments into their endo-vinyl and exo-vinyl isomers, respectively.

This paper describes a reliable method for the separation and isolation of ethyl anthranilate azopigments prepared from GF rat feces and CONV rat bile by reversed-phase HPLC.

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MATERIALS AND METHODS

Collection of feces and bile. The GF and CONV rats were of the AGUS strain (1) and were reared according to Gustafsson (7,8). Feces was obtained from adult GF rats of both sexes, fed with a commercial pelleted diet (autoclaved at 120°C for 30 min) and water ad lib. The samples were taken by rectal stimulation, pooled and transferred out from the GF isolators. Bile was taken from one male CONV rat by bile duct cannulation during pentobarbital aenesthesia (1). The biological samples were protected from light and frozen directly at -20°C before analysis.

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Formation of ethyl anthranilate azopigments. The pooled feces sample was thawed and mixed to a homogenous paste with a steel spatula. Several 4 g samples of mixed feces were taken and 50 ml of methanol was added to each sample followed by homogenization using an Ultra-Turrax homogenizer. The homogenates were centrifuged at 4000 x g for 20 min followed by evaporation of the supernatants in a rotary flash evaporator at 35°C. The residue was dissolved in 20 ml of distilled water and conjugated bilirubin was prepared according to method C described previously (2). The bilirubin conjugate preparations were then coupled with ethyl anthranilate diazo reagent at pH 2.7 (2,5,9) and the resulting azopigments obtained as described previously (2). From the thawed bile sample, a number of 0.1 ml aliquots were withdrawn and diluted five-fold with distilled water. The samples were then directly coupled with ethyl anthranilate diazo reagent at pH 2.7 and the resulting azopigments were obtained (5,9).

Separation of azopigments by tlc. The azopigment preparations from feces and bile were evaporated to dryness under a stream of nitrogen at 40°C. Half the

number of the azopigment residues were dissolved in 0.1 ml of pentan-2-one:n-butyl acetate (17:3 v/v) and applied to precoated silica gel plates. Chromatography was carried out as described previously (2). The predominant azopigment spots were eluted from the tlc plates with ethanol.

HPLC. An ALC series liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) was used. The instrument was equipped with a Model U6K injector and a Model 440 absorbance detector operating at a fixed wave-length of 546 nm. The reverse-phase column (30 cm x 4 mm ID) was obtained prepacked with μBondapak C_{18} (10μm) from Waters Assoc. A Waters guard column packed with Bondapak C18/Corasil (37-50 μm, Waters Assoc.) was connected prior to the analytical column. The mobile phase was a mixture of acetonitrile, distilled water and sodium acetate. Separation was carried out by using a linear gradient of acetonitrile (50% to 90% (v/v) in 60 min) in 0.1 M acetate buffer (pH 4.0). Peak assignments were performed by using (a) the tlc method described above and (b) comparison with the retention time of reference samples on the HPLC. As references, δ azopigment (azodipyrrole-β-D-monoglucuronide) was prepared from CONV rat bile whereas azopigment α_0 (azodipyrrole) was prepared from CONV rat bile and bilirubin IX_α, respectively (5,9). The azopigment residues prepared from GF rat feces and CONV rat bile were dissolved in ethanol before injection. The predominant peaks were collected in individual test tubes and the excess of acetonitrile was blown off under a stream of nitrogen at 40°C. Then, one vol of glycine-HCl buffer, pH 2.7 (5,9) was added and the azopigment extracted with 2 vol of pentan-2-one:n-butyl acetate (17:3 v/v). The azopigment was then subjected to tlc as described above and its Rf value was compared with the Rf values of the reference azopigments.

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Chemicals. All chemicals used were of analytical quality and came from Merck (Darmstadt, Germany) except ethyl anthranilate which was of pure quality and came from Fluka AG (Buchs, Switzerland). Pentan-2-one was distilled before use.

RESULTS AND DISCUSSION

Fig. 1 gives a schematic representation of the separation of the azopigments on a tlc plate. The azopigment tracks from GF rat feces yielded identical separation patterns and nine azopigment spots were detected. The most polar and least polar spots, respectively, were the predominating ones, while the other spots were seen as more or less faint lines. The azopigment tracks from CONV rat bile yielded identical separation patterns and ten azopigments spots were seen. The most polar spot was greatly predominating while the other spots were seen as more or less faint lines. The most polar spot represents the δ azodipyrrole while the least polar spot represents the α_0 azodipyrrole (4,5, 9,10). The other azopigment spots with intermediate Rf values are most probably azopigments of the so called γ- and β-groups, and also the α_3, α_2 and α_1 azodipyrroles seems to be present in trace amounts according to the data of Heirwegh et al (5,10). By using the HPLC method on the azopigments prepared from GF rat feces and CONV rat bile, the δ and α_0 azodipyrroles were separated (Fig. 2). Both the δ and α_0 bands, separated by tlc, had a pair of peaks as were previously demonstrated for these azopigments prepared from dog bile by Onishi et al (6). Peaks 1 and 2, respectively, had the same Rf values obtained by tlc as the δ azodipyrrole prepared from CONV rat bile. Peaks 3 and 4, respective-

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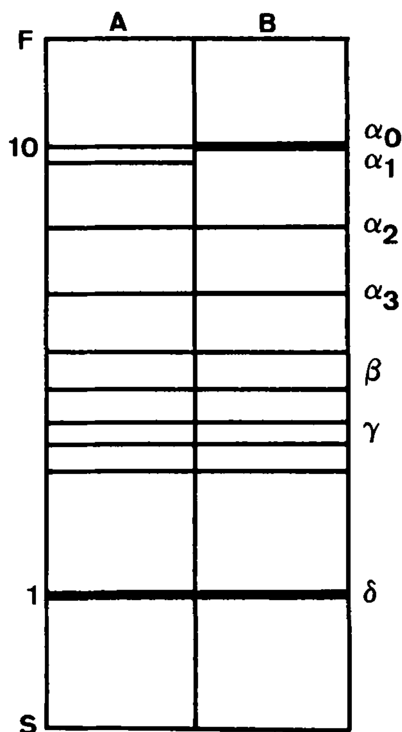

Fig. 1 Schematic representation of a tlc plate showing the separation of azopigments prepared from CONV rat bile (chromatogram A) and GF rat feces (chromatogram B), respectively. The separated azopigment spots are shown as horizontal lines (the predominant ones as heavier lines) numbered from 1 to 10. The Greek letter notations are symbols for the azopigments according to the data of Heirwegh et al (5,10). S and F indicate the starting line and the solvent front, respectively.

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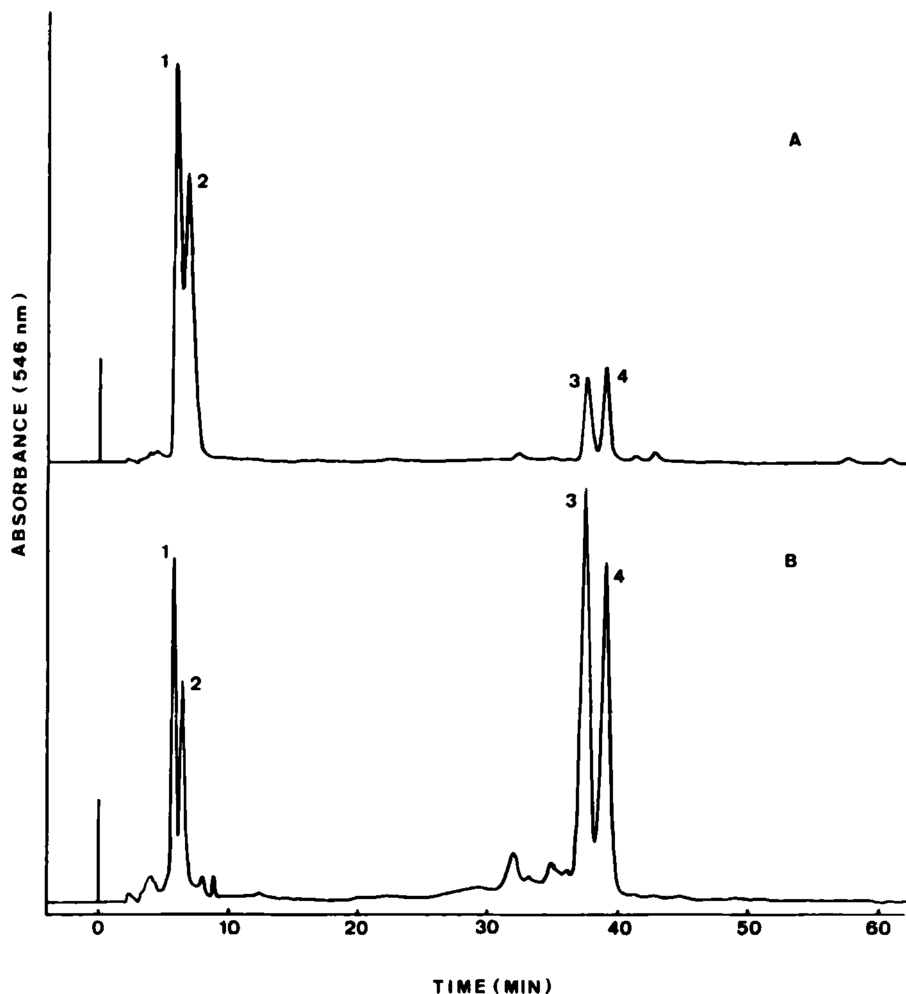

Fig. 2 HPLC separation of azopigments obtained from CONV rat bile (chromatogram A) and GF rat feces (chromatogram B), respectively. Conditions: column, Waters Assoc. µBondapak C_{18}; mobile phase, a linear gradient of acetonitrile (50% to 90% (v/v) in 60 min) in 0.1 M acetate buffer (pH 4.0); flow rate, 1 ml/min; detection, 546 nm, 0.1 a.u.f.s.

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ly, had the same Rf values obtained by tlc as the α_0 azodipyrrole prepared from bilirubin IX_α and CONV rat bile. It was assumed, according to the data of Onishi et al (6), that peaks 1 and 3 represent the endo-vinyl isomers of the δ azodipyrrole and the α_0 azodipyrrole, respectively, while peaks 2 and 4 represent the exo-vinyl isomers of the δ azodipyrrole and the α_0 azodipyrrole, respectively. The other faint azopigments with intermediate Rf values as detected by tlc were difficult to detect by the HPLC method.

This paper with its results describes for the first time separation by HPLC of azopigments prepared from GF rat feces. In a more developed form this method could be used for quantification of azopigments prepared from feces of GF rats infected by single or several strains of bacteria. In that case it would be possible to investigate which bacteria are responsible for deconjugation of the bilirubin conjugates in the lower parts of the intestine.

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ACKNOWLEDGEMENTS

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