THE PREPARATION OF AMINO BILE ACID DERIVATIVES

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Received 12-1-80

ABSTRACT

Bile acid derivatives have been prepared by reaction of their mixed anhydrides with diaminoethane. The new compounds have side chains modified by amide bond formation which extends the side chain by two carbon atoms and terminates in a primary amino group. Important properties of the new compounds are their solubility in acid, and their ability to act as nucleophiles in the carbodiimide reaction or mixed anhydride reaction. The derivatives have been used to prepare high molar ratio immunogens with bovine serum albumin and to prepare ^{125}I labelled ligands for radioimmunoassay

INTRODUCTION

Serum bile acid levels are a sensitive index of hepatobiliary disease (1,2,3), an observation which has stimulated the development of radioimmunoassays (RIA) for individual bile acids (4,5). Two fundamental requirements of such assays are the immunogen, against which an antiserum is raised, and a radioactive tracer.

Bile acids are not immunogenic, per se, but become so when conjugated to large molecular weight proteins (4). Such conjugations have always been carried out by formation of amide bonds between the bile acid carboxylic acid group and free amino groups of lysine residues in the protein. Introducing a free amino group at the end of the bile acid side chain would make it possible to covalently bond the molecule to free carboxylic acid groups of aspartate and glutamate residues in the protein. This approach would allow the carbodiimide reaction to be carried out in aqueous solution at the optimum pH of 4.8 (6), leading to a higher molar ratio of bile salt to protein.

Direct iodination of bile acids is not possible but on reaction of the amino derivatives with the mixed anhydride of 4-hydroxybenzoic acid, in organic solution, a site suitable for iodination with ^{125}I would be introduced.

This paper describes the synthesis of amino bile acid derivatives, their conjugation to bovine serum albumin and their reaction with

4-hydroxybenzoic acid.

MATERIALS AND METHODS

Radioactive chemicals were from the Radiochemical Centre, Amersham. [24-^{14}C] -3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid (59.5 mCi/mmol) and [24-^{14}C] -3α,7α-dihydroxy-5β-cholan-24-oic acid (55 mCi/mmol) were diluted in 2% ethanol to give stock solutions of 10 μCi/ml. Na ^{125}I was supplied as 1 mCi aliquots in dilute NaOH. Electrophoretically pure bovine serum albumin (BSA) was from Hoechst (U.K.) Ltd.

All melting points were determined with a Gallenkamp Melting Point Apparatus and are uncorrected. I.R. spectra were recorded as KBr discs in a Pye Unicam SP1000 I.R. Spectrophotometer. Mass spectra were recorded by direct probe in a Micromass 7070F High Resolution Double Focus Mass Spectrometer. Ultrafiltration was carried out in an Amicon Ultrafiltration Cell 8MC through an Amicon PM 30 membrane. Thin layer chromatography was performed in Shandon tanks on precoated Merck silica gel 60F$_{254}$ plates purchased from B.D.H., in the systems:-

Solvent System I. Ethyl acetate: methanol: toluene: triethylamine = 50:25:25:10

Solvent System II. Ethyl acetate: methanol: toluene: glacial acetic acid = 50:25:25:10

EXPERIMENTAL

Preparation of N-(2-aminoethyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide

3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid (2 mmol) and tri-n-butylamine (2 mmol) were dissolved in 10 ml of dioxane and cooled to 10°C before addition of isobutylchloroformate (2 mmol). After 15-20 minutes, 1,2-diaminoethane (35 mmol) in 10 ml of water was added with vigorous stirring and the mixture stirred for one hour before evaporation to dryness in vacuo at 40°C. The oily residue was made alkaline by addition of 10 ml of 0.1M NaOH and extracted with diethyl ether (3x20 ml) which was discarded. The aqueous layer was extracted with butanol (2x10 ml) and the combined extracts concentrated in vacuo at 40°C before recovery of the crude product by precipitation with diethyl ether. The precipitate was dissolved in 10 ml of M HCl and extracted with ethyl acetate (5x10 ml) before being made alkaline with 5M NaOH. The product was extracted with butanol (2x10 ml) and again recovered by evaporation of the solvent in vacuo and precipitation with diethyl ether. YIELD 72%. The analytical sample was recrystallised from boiling water. MP. 192-193°C. I.R. 1650 cm^{-1} (AMIDE I) 1560 cm^{-1} (AMIDE II). MASS SPECTRUM m/e 450 M^+, 432 M-H_2O, 417 M-(CH_3+H_2O), 399 M-(CH_3+2H_2O) 271 M-(side chain +2H_2O), 253 M-(side chain + 3H_2O).

Preparation of N-(2-aminoethyl)-3α,7α-dihydroxy-5βcholan-24-amide

3α,7α-Dihydroxy-5β-cholan-24-oic acid (2 mmol) was reacted as described in the previous preparation. YIELD 58%. The analytical product was recrystallised from ethanol/ethyl acetate. M.P. 98°C (Decomposes). I.R. 1650 cm^{-1} (AMIDE I) 1580 cm^{-1} (AMIDE II). MASS SPECTRUM m/e 434 M^+, 419 M-H_2O, 401 M-(CH_3+H_2O), 383 M-(CH_3+2H_2O) 273 M-(side chain + H_2O) 255 M-(side chain + 2H_2O).

Preparation of Bovine Serum Albumin Conjugates

The appropriate [24-^{14}C] bile acid derivative (200 mg, specific activity 1 μCi/mmol) was dissolved in 3 ml of water by dropwise addition of 0.5 M HCl until solution was complete. BSA (20 mg) dissolved in 1 ml of water, was added and the pH adjusted to 4.8. 1-Ethyl-3 (3-dimethyl aminopropyl)-carbodiimide (100 mg) was added and the reaction mixture stirred for two hours at room temperature, maintaining the pH at 4.8. A 1% aliquot of the final volume was taken to measure total radioactivity while the remainder was eluted on a Sephadex G-25 column with 0.01M acetate buffer pH 4.8. The conjugated protein fraction was collected at the void volume and reduced to 5 ml by ultrafiltration against phosphate buffer pH 7.4. A 10% aliquot was removed to measure radioactivity bound to albumin.

The following molar ratios were obtained:-

N-(2-aminoethyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide-
B.S.A. = 70 : 1

N-(2-aminoethyl)-3α,7α-dihyroxy-5β-cholan-24-amide-
B.S.A. = 55 : 1

Preparation of 3-[^{125}I]-4-Hydroxybenzoyl Derivative

4-Hydroxybenzoic acid (0.25 mmol) and tri-n-butylamine (0.5 mmol) were dissolved in 5 ml of dioxane and after cooling to 10°C isobutylchloroformate (0.25 mmol) was added. After 15-20 minutes N-(2-aminoethyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (0.25 mmol) or N-(2-aminoethyl)-3α,7α-dihydroxy-5β-cholan-24-amide (0.25 mmol) in 5 ml of ethanol was added to this solution and stirred for two hours. The solution was evaporated to dryness in vacuo, 5 ml of 0.5 M. NaOH added and extracted with diethyl ether (3x10 ml) before acidifying with 2M HCl and extracting the product with ethyl acetate (2x5 ml). The combined extracts were reduced to 0.5-1 ml by evaporation in vacuo and the product chromatographed on silica gel 60F_{254} plates in Solvent System I. The band with lower R_F (visualised by U.V. light) was recovered, eluted with ethanol and further purified in Solvent System II. The band with lower R_F was eluted with ethanol, reduced to about 0.5 ml and the product obtained by precipitation with diethyl ether before dissolution in ethanol to give stock solutions of 2 mg/ml. Aliquots (10 μl) of these stock solutions were added to 5 μl Na^{125}I and 10 μl of 0.5 M phosphate buffer pH 7.4. Chloramine T (5 μl of 1 mg/ml in H_2O) was added and the

reaction stopped after 30 seconds by adding 100 µl of sodium metabisulphite (1 mg/ml in 0.5M phosphate buffer pH 7.4) Sodium iodide (100 µl of 1 mg/ml in 0.5M phosphate buffer pH 7.4) was added and the mixture extracted with ethyl acetate (2x1 ml) and the combined extracts evaporated to dryness in vacuo. The residue was dissolved in 100µl of ethanol and the iodinated compound was separated from the unreacted compound by thin layer chromatography in Solvent System I and eluted with 10 ml of ethanol in which it was stored.

^{3}H and ^{125}I tracers could be interchanged in RIA without altering assay conditions.

DISCUSSION

Diaminoethane derivatives of 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid and 3α,7α-dihydroxy-5β-cholan-24-oic acid were prepared to introduce a free amino group at the end of the side chain. These acid-soluble derivatives can be conjugated to bovine serum albumin via free carboxylic acid groups of aspartate and glutamate at the optimum pH 4.8 of the carbodiimide reaction (6). Using carbodiimide to conjugate these bile acids to BSA via free amino groups of lysine residues requires higher pH which can lead to cross linking of protein molecules (6,7). Further, reaction of the diaminoethane derivatives with 4-hydroxybenzoic acid introduces a site in the molecule suitable for iodination with ^{125}I. These ^{125}I derivatives successfully replaced tritium tracers in the RIA of 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid conjugates by antisera raised in our laboratories (8). Antisera titres were increased, thus reducing their consumption, and since ^{125}I radioactivity can be measured directly, costly scintillation fluid is not required. However, attempts to raise antiserum to the new immunogens of high molar ratios have proved unsuccessful in rabbits.

Our own experience with bile acid-BSA immunogens prepared by the mixed anhydride technique suggest that there is a minimum molar ratio below which suitable antiserum cannot be produced (8). Similar observations have been reported for other steroids (9). The results in the present study may indicate a maximum molar ratio above which suitable antiserum cannot be produced. These findings taken together suggest that below the minimum ratio the bile acid nucleus is hydrophobically bound to nonpolar regions of the protein surface with masking by surrounding amino acid residues. Above the maximum ratio the protein

surface becomes saturated with bile acid molecules which stack together due to hydrophobic forces between their nonpolar regions. Antibodies raised to such immunogens would have low or no specificity to a bile salt molecule. Between these limits the protein surface would contain areas where the molecule was completely exposed and able to act as determinant in antibody production.

The theory may explain the failure of other workers to raise antisera to certain immunogens. Walker et al (10) found that 1,3,5(10)-estratriene-3,16α,17β-triol (estriol) or 1,3,5(10)-estratriene -3,17β-diol (estradiol-17β) linked to a synthetic polypeptide failed to produce antisera while BSA conjugates were successful. The synthetic polypeptide was of relatively low molecular weight (20,000) with 28-30 molecules bound while the BSA (M.W. 70,000) had 20-22 molecules bound. The synthetic polypeptide with smaller surface area was probably saturated with steroid molecules at this ratio. Becket et al (11) have compared three different immunogens. The only successful immunogen, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid-BSA, had a molar ratio of 17:1. The other two immunogens, which failed, had glycine or a saturated six carbon chain inserted between 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid and H_2N-lysine-protein with molar ratios of 10:1 and 29:1 respectively. The molar ratio hypothesis would predict that a ratio of 10:1 was too low and although the third immunogen had a ratio of 29:1, the longer, flexible bridge may allow bile acid molecules, bound at greater distances on the protein surface, to interact with each other hydrophobically. Weinberg et al (12) found that a 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid-BSA immunogen with molar ratio 36:1 produced a low titre antisera, an observation in accordance with prediction based on the molar ratio hypothesis. The specificity of antisera raised is therefore probably dependent on the bile acid-BSA ratio. Minder et al (13) have reported these variable specificities and tried to overcome the problem by conjugating 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid to thyroglobulin. Their immunogens had an average molar ratio of 250:1 but the carrier protein was of large molecular weight (700,000) with a surface area large enough to accommodate such numbers without saturation.

Preliminary data indicate that the reaction sequence may be readily applied to glycine conjugates although the dioxane-water solvent system

must be replaced by dimethylformamide due to the lower solubility of the starting material. Alternative solvents are required for crystallisation of the products but this has not yet been fully resolved.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J.D. Baty and Mr. R.G. Willis for production and interpretation of the mass spectra and to Mrs. J. Brown for skilful typing of the manuscript.

REFERENCES

1. Sherlock, S. and Walshe, V. CLIN. SCI. 6, 223 (1948)
2. Rudman, D. and Kendall, F.E. J. CLIN. INVEST. 36, 530 (1957)
3. Makino, I., Nakagawa, S. and Mashimo, K. GASTROENTEROLOGY 56, 1033 (1969)
4. Simmonds, W.J., Korman, M.G., Go, V.L.W. and Hofmann, A.F. GASTROENTEROLOGY 65, 705 (1973)
5. Murphy, G.M., Edkins, S.M., Williams, J.W. and Catty, D. CLIN. CHIM. ACTA. 54, 81 (1974)
6. Carraway, K.L. and Koshland, D.E. METHODS IN ENZYMOLOGY 25, 616 (1972)
7. James, V.H.T. and Jeffcoate, S.L. BRIT. MED. BULL. 30, 50 (1974)
8. Baqir, Y.A., Murison, J., Ross, P.E. and Bouchier, I.A.D. J. CLIN. PATH. 32, 560 (1979)
9. Niswender, G.D. and Midgley, A.R. in IMMUNOLOGIC METHODS IN STEROID DETERMINATION, p.149 (1970) eds. Peron, F.G. and Caldwell, B.U.
10. Walker, C.S., Clark, S.J. and Wotiz, H.H. STEROIDS, 21, 259 (1973)
11. Becket, G.J., Hunter, W.M. and Percy-Robb, I.W. CLIN. CHIM. ACTA. 88, 257 (1978)
12. Weinberg, P.B., Kinkade, J.M. Jr. and Collins, D.C. STEROIDS 30, 637 (1977)
13. Minder, E., Karlaganis, G., Schmied, U., Vitins, P. and Paumgartner, G. CLIN. CHIM. ACTA. 92, 177 (1979)