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Separation of Conjugated and Free Bile Acids by Paper Chromatography. Bile Acids and Steroids 12

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In connection with work on bile acid metabolism it has become necessary to have a rapid method for separating bile acids directly as they appear in the bile, *i.e.* in conjugated form. Ahrens and Craig [1] have used countercurrent distribution and Norman and Bergström [2] partition chromatography for this purpose. In an investigation of bile acid metabolism where it is necessary to take bile samples at short intervals these methods, however, require too much substance and too much time. Several methods of separating bile acids using paper chromatography have recently been developed [3-6,13]. In the work described in this paper the method of Sjövall has been further investigated and developed also for the separation of conjugated bile acids.

A. SEPARATION OF FREE BILE ACIDS

Experimental

Filter paper Whatman No. 1, 4 and 3 MM can all be used. Whatman 3 MM has a greater capacity and 50—100 γ of an acid gives a well defined spot without tailing. It has earlier been used by Haslewood [6] for the separation of bile acids and their ethyl esters. Whatman 1 is mostly used when smaller amounts, 1—10 γ, are to be analysed, and when radioactive acids are run.

The paper is cut in strips 9 × 45 cm. When radioactivity is to be counted these strips are cut so that each sample runs on its own strip 1.5 cm broad, separated from the next strip by a 1 cm broad space where the paper is removed, leaving a small margin at both ends of the paper. Thus a 9 cm broad strip contains 4 such 1.5 cm strips all held together with intact paper at both ends.

For the separation of free acids the following solvents have been used.

Moving phase: *iso*propyl ether : heptane 60 : 40 v/v.

Stationary phase: 70 % acetic acid.

For the separation of desoxycholic acid and chenodesoxycholic acid isopropyl ether: heptane 20 : 80 is used as moving phase and 70 % acetic acid as stationary phase.

Equal parts of the phases are equilibrated in a separating funnel before use, at the same temperature as for the chromatography.

The cut papers are soaked in the stationary phase and dried at 100° C until they just seem dry. The acids or their sodium salts are put on the starting point 6 cm from one end of the paper. This is then hung in the chromatographic chamber overnight for a complete equilibration with both phases.

We have used both ascending and descending chromatography. The descending technique is used only for long runs, *i. e.* 16 hours and more when slow running components are to be separated. For both techniques cylindrical glass vessels 14 × 60 cm are used, their inner walls coated with filter paper wetted with moving phase. On the bottom of the vessel is placed a beaker containing stationary phase in which a strip of filter paper is hung. The stationary phase ascends on this strip and a more complete phase saturation is obtained. The vessels are closed with glass plates tightened with stopcock grease.

For the ascending chromatography the running phase is on the bottom of the vessel. During the equilibration period the chromatograms must not come in contact with the moving phase. The papers are hung with their lower edges 1 cm above the surface of the moving phase. When equilibrium has been obtained, this surface is brought up to the papers by filling more moving phase through a glass tube introduced through a hole in the cover. In this way opening of the chromatographic chamber is avoided. Another and more simple method [6] is to tilt the vessel so that the moving phase is not in contact with the paper and then, after equilibration, tilt it until the solvent reaches the paper.

When the front of the moving phase is to run off the paper, descending chromatography has to be used. The arrangements for the phase saturation are the same as for the ascending technique but the papers hang in a trough and after equilibration the trough is filled with moving phase through a small hole otherwise closed with a cork in the covering glass plate.

After equilibration for 12—16 hours the chromatograms are developed for a suitable time. The chromatography has been carried out at constant temperature (23° C).

The spots are revealed by spraying with a 10 % solution of phosphomolybdic acid in absolute alcohol and subsequent drying at 80—100° C [7]. This reagent is not specific but gives a blue colour with all the common bile acids and their conjugates with glycine and taurine. In a previous paper [4] it was stated that lithocholic acid did not give any colour. This has been found to be due to an impure phosphomolybdic acid. Cholic, desoxycholic and chenodesoxycholic acid and their conjugates can be detected after chromatography of 1—3 γ or even less, while lithocholic acid and its conjugates require 10—20 γ.

For counting of radioactivity the paper was taped on a ruler that could be moved ½ cm at a time past the GM-tube. A slit in a metal plate between the paper and the mica window allowed the counting of ½ or 1 cm of the paper each time.

Results

When pure synthetic bile acids are run the spots appear well defined with no tailing. The R_F values are, however, variable and reference acids always have to be run parallel with the mixture to be analysed. This is probably dependent upon the difficulty of keeping the concentration of acetic acid in the paper constant.

If the acids are put on the paper as sodium salts they run at the same speed as when put on as free acids, since they are set free by the acid solvents.

Sometimes it is advisable to change the moving phase. An increase of heptane in the moving phase causes a reduction of the moving rate of all the acids. It has been found for the acids with substituents at 3 α, 7 α and 12 α that this reduction for the glycine conjugates is proportionally larger than that for free cholic acid whose reduction is larger than that of dihydroxy acids. Thus if an elongated spot is suspected to contain more than one acid the heptane can be increased and a descending chromatogram developed, letting the solvent drip off the lower edge of the paper for a suitable time.

Fig. 1 a shows the separation of cholic, hyodesoxycholic, desoxycholic and lithocholic acid. With the oxygen-containing substituents in α-position at carbon atoms 3, 7 and 12 the acids separate in groups depending on the number of oxygen substituents. Desoxycholic and chenodesoxycholic acid for example

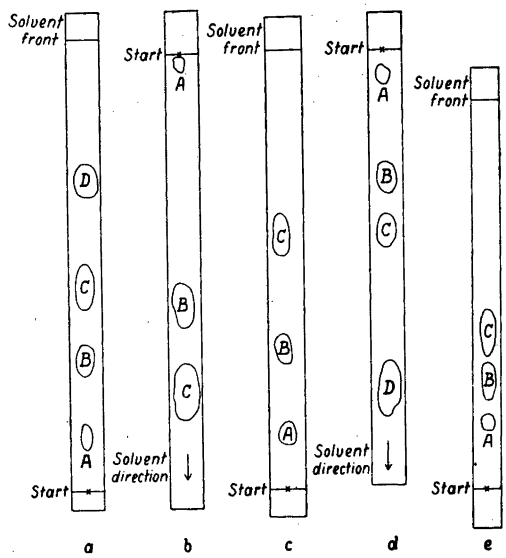

Fig. 1. Separation of different bile acids (see text).

a. Ascending, 4 hours.
Moving phase: Isopropyl ether:heptane 60 : 40 v/v.
Stationary phase: 70 % acetic acid.
Acids: Cholic (A), hyodesoxycholic (B), desoxycholic (C) and lithocholic (D).
b. Descending, 18 hours.
Moving phase: Isopropyl ether:heptane 20 : 80 v/v.
Stationary phase: As 1 a.
Acids: Impurity in the chenodesoxycholic acid (A), chenodesoxycholic (B) and desoxycholic (C).
c. Ascending, 4 hours.
Moving phase: Isopropyl ether:heptane 85 : 15 v/v.
Stationary phase: As 1 a.
Acids: Glycocholic (A), glycochenodesoxycholic (B) and glycolithocholic (C).
d. Descending, 18 hours.
Moving and stationary phase as 1 a.
Acids: Glycocholic (A), glycochenodesoxycholic (B), glycodesoxycholic (C) and cholic (D).
e. Descending, 12 hours.
Moving phase: n-Butanol saturated with 3 % acetic acid/water v/v.
Stationary phase: 70 % acetic acid.
Acids: Taurocholic (A), taurodesoxycholic (B) and taurolithocholic (C).

do not separate, neither do the ketonic acids separate from the hydroxylic acids with the same number of oxygen atoms on the skeleton. There is only a very small difference in the travelling rate. Hyodesoxycholic acid with the two hydroxyl groups at 3 α and 6 α separates clearly from the other two dihydroxy acids, desoxycholie and chenodesoxycholic acid. Acids with hydroxyl groups in β-position have not yet been tested but there are strong reasons to believe that ursodesoxycholic acid (3 α 7 β) separates from chenodesoxycholic acid (3 α 7 α) (Haslewood).

Table 1. Relationship between c/min plated on copper planchets and c/min recovered after paper chromatography.

Acid	Approx. weight γ	Copper planchets c/min	Chromatogram c/min	Recovery %
Cholic	5	390	136	34.9
Desoxycholic	3	867	324	37.4
Chenodesoxycholic	2	797	277	34.8
Lithocholic	30	948	323	34.1
Cholanic	3	432	160	37.0

In order to use the method for biological tracer work some chromatograms were run with C^{14}-labelled bile acids [8]. An estimation was made of the absorption of radioactivity in the filter paper. Table 1 shows the values obtained from five different acids. The same amount as was run on the chromatograms was plated on copper planchets and the activities compared. This was done to make it possible to correlate the curves obtained by column partition chromatography and those obtained by paper chromatography. A comparison between the activity of the spot at the starting point before the chromatogram had been developed and the activity of the spot after development showed that about 80 % was found when the chromatogram had been run. This is in rather good agreement with Rochland *et al.*[9] who recovered 85 $\pm$ 6 % when running glycine C^{14} on Whatman No. 1 paper.

The separation of desoxycholic and chenodesoxycholic acid has always been a great difficulty. The only existing preparative method is a repeated precipitation of the barium salt of chenodesoxycholic acid. As there is a small difference in moving rate with isopropyl ether : heptane as moving phase the heptane content was increased and a descending chromatogram run. Fig. 1 b shows the separation of the two acids after 18 hours descending with isopropyl ether : heptane 20 : 80 as moving phase. Heptane alone was also used as moving phase and gave after 72 hours' descending chromatography a separation of the acids. In this case, however, only 5 γ of desoxycholic acid could be run. When more substance was used the spots became elongated and with 25 γ the acid appeared as a long streak. In order to see whether this was due to a tailing that could not be detected by spraying when only small amounts of acid were used, the same amount of labelled desoxycholic acid was added to varying amounts of inactive acid. With less than 5 γ acid the activity appeared in a narrow symmetrical peak. This is shown in Fig. 2 where 3 γ labelled desoxycholic acid is separated from 2 γ labelled chenodesoxycholic acid. When, however, larger amounts of desoxycholic acid were run the activity spread over a longer area. This did not happen when chenodesoxycholic acid was used. It is possible that desoxycholic acid which easily forms choleic acids has formed a complex with acetic acid or heptane and that this is the cause of the bad capacity of the system. The addition of isopropyl ether to the moving phase largely prevents this elongation of the spots as is seen in Fig. 2 where 25 γ desoxycholic acid was run.

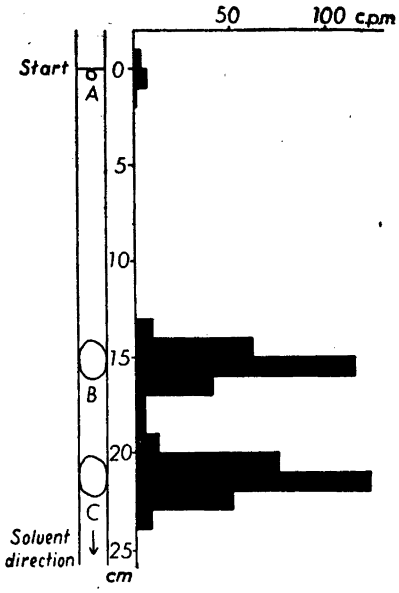

Fig. 2. Separation of labelled dihydroxy acids. The chromatogram is reproduced to the left of the diagram showing the distribution of radioactivity in the paper. A: Impurity in the chenodesoxycholic acid; B: chenodesoxycholic acid; C: desoxycholic acid.

B. SEPARATION OF CONJUGATED BILE ACIDS

1. Bile acids conjugated with glycine

Experimental. See A.

Solvent systems. Moving phase: *iso*propyl ether : heptane 80 : 20 v/v or 85 : 15. Stationary phase: 70 % acetic acid.

Spraying reagents. Phosphomolybdic acid has mostly been used. Chlorination with subsequent spraying with potassium iodide-starch [10] gives a colour with conjugated bile acids only and is useful when the question is whether a spot is a free or a conjugated bile acid. The reagent is not very sensitive and 20—50 γ of each acid has to be used for a chromatography. Probably the hydroxyl groups interfere with the reaction because glycocholanic acid gives a colour with much smaller amounts than the hydroxylated acids.

Results.

The acids used were supplied by Bergström and Norman and synthesised according to their method [11].

Fig. 1 c shows the separation of glycocholic, glycochenodesoxycholic and glycolithocholic acid. Glycodesoxycholic acid runs somewhat faster than glycochenodesoxycholic acid but the difference is not large enough for a clear separation. The glycine-conjugated desoxycholic and chenodesoxycholic acids, however, show a larger difference in moving rate than the free acids and are therefore easier to separate. This separation is seen in Fig. 1 d where the two acids are run together with glycocholic and cholic acid. The chromatogram has been developed in a descending direction for 18 hours with isopropyl ether : heptane 60 : 40 as moving phase and 70 % acetic acid as stationary phase.

The conjugation with glycine has approximately the same effect on the moving rate as one hydroxyl group in the free bile acids when isopropyl ether: heptane 80 : 20 is used as moving phase. The same has been found by Norman [12] using reversed phase partition chromatography. If, however, the heptane content is increased and descending chromatography is run for a suitable time, a clear separation is achieved of glycine-conjugated acids from free acids with one hydroxyl group more (see also under Results A). Fig. 1 d for example shows the separation of cholic acid from the glycine-conjugated dihydroxy acids.

As with the free bile acids the glycine-conjugated acids can be put on the starting line as sodium salts. The taurine conjugated acids all run slower than glycocholic acid.

2. Bile acids conjugated with taurine

Experimental.

Solvent systems. Moving phase: Equal parts of *n*-butanol and 3 % acetic acid are equilibrated in a separating funnel. The upper phase is used (butanol).

Stationary phase: 70 % acetic acid.

In this case the two phases are not equilibrated with each other because they mix easily. The equilibrium in the chromatographic chamber is therefore not a true one but it has been found that tailing of the spots is best prevented by using these phases. This is in agreement with the finding for the chromatography of other bile acids that 70 % acetic is the optimal concentration for the papers. Less acetic acid gives tailing of the spots and a higher concentration gives streaking in the direction of the solvent front.

Descending chromatography is mostly used because of the slow ascending rate of the butanol phase.

The same spraying reagents as for glycine conjugates have been used.

Results.

Fig. 1 e shows the separation of taurocholic, taurodesoxycholic and taurolithocholic acid. The acids come close together probably because of the pronounced effect of the taurine part of the molecule which makes the effect of the hydroxyl groups on the partition coefficient less prominent. The taurine conjugates of chenodesoxycholic acid and desoxycholic acid do not separate.

Even the taurine conjugated bile acids can be put on the starting line as sodium salts and still behave as acids in the chromatogram.

The glycine-conjugated acids and the free acids all run faster than taurolithocholic acid and practically with the front.

SUMMARY

A method of separating free and conjugated bile acids using paper chromatography is described.

Desoxycholic acid and chenodesoxycholic acid and their glycine conjugates have been separated.

The method has been applied to work with labelled bile acids.

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