

1

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## PREPARATION OF HYODESOXYCHOLIC ACID

Gloria Catherine Buckley and Peter Ziegler, Toronto, Ontario, Canada, assignors to Canada Packers, Limited, Toronto, Ontario, Canada

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The present invention relates to the preparation of hyodesoxycholic acid and more particularly to recovery of hyodesoxycholic acid from fresh or inspissated hog bile.

Because of the therapeutic value of the bile acids research workers have for a long time been engaged in their recovery from animal biles, which are available in large quantities at a low price as by-products of the slaughter houses. The bile acids are readily obtained from most animal biles by saponification with strong bases, such as sodium hydroxide followed by acidification to precipitate the bile acids in forms which lend themselves to further purification steps in an economical manner.

This procedure, however, was not found to be applicable to the recovery of hyodesoxycholic acid, the principal bile acid of hog bile, which has been reported in the literature to have a melting point of 195° to 197° C. On acidification of saponified hog bile, the precipitate of crude bile acids was found to be so highly contaminated with gummy impurities that no commercially feasible method could be devised for isolating relatively pure hyodesoxycholic acid.

Accordingly, it is a primary object of the present invention to provide a process for obtaining hyodesoxycholic acid of high purity from hog bile in greater yields than has heretofore been accomplished.

A further object is to provide a process for recovering hyodesoxycholic acid from hog bile, which may be carried out in a very economical manner.

A still further object of the present invention is to provide a process for preparing hyodesoxycholic acid in which may be employed either fresh bile or inspissated bile as the starting material.

Other and further objects of the present invention will be apparent from the following detailed description of the process.

In carrying out the present invention, fresh bile or inspissated bile, which has been diluted so that the total solids content (11% to 12%) approximates that of fresh bile, is saponified by refluxing with approximately 10% caustic, such as sodium hydroxide. The saponified mixture is then brought to pH 8 with a mineral acid, such as hydrochloric acid, which is added in concentrated form to maintain the water content as low as possible.

While maintaining the saponified bile at an elevated temperature, there is added a bleaching agent, such as sodium hydrosulfite, and an organic solvent, which must and exert a preferential solvent action for hyodesoxycholic acid with respect to the gummy impurities present in the bile. It is important to maintain the proper temperature range to prevent local precipitation at lower temperatures and significant losses of solvent at higher temperatures because of volatilization. With the preferred solvent, ethyl acetate, a temperature range of 50° to 55° C. is most satisfactory.

Only a small amount of bleaching agent is added to the saponified bile to lighten the water solution to aid in the subsequent separation of aqueous and solvent layers

2

and not enough to have any appreciable decolorizing effect on the solvent solution of bile acid. Approximately .5% of sodium hydrosulfite based on the weight of the starting bile has been found adequate for this purpose.

The addition of approximately 40% of organic solvent based on the volume of the starting bile has been found to effect an advantageous initial separation of bile acid and gummy impurities.

After addition of the bleaching agent and solvent, addition of concentrated hydrochloric acid is continued until approximately pH 5 is reached. At this point the solvent and aqueous layers are separated and the aqueous and interface layers, containing the major proportion of the impurities, are rejected. The solvent layer is then preferably treated by filtration with a decolorizing agent, such as bleaching earth.

The solvent solution is then distilled to about half its original volume, the distillate being collected for reuse. The remaining solvent solution is then allowed to stand at room temperature for about a day, during which time the crude bile acids precipitate from the solution as a brown, granular solid, which may be separated by conventional methods, such as filtering or centrifuging, the solvent again being recovered for reuse. The separated, crude bile acids are then washed thoroughly with limited quantities of fresh solvent, which causes the product to be greatly lightened and assume a beige color. The washed solids are dried and ground so as to be ready for further purification, which will be described hereinafter.

By the above procedure, there are obtained excellent yields of a true solid, which melts over a comparatively limited range and, as indicated, may be dried and ground in the usual manner. Considerable purification is obtained in the liquid stage so that the first solid product which results may be easily and economically handled.

After grinding, the crude bile acids are readily treated so as to isolate substantially pure hyodesoxycholic acid. This may be accomplished by recrystallization from acetone, which is a relatively inexpensive, readily available organic solvent and provides high yields of pure hyodesoxycholic acid. The crude bile acids are dissolved by refluxing in 25 cc. of acetone per gram of dried product in the presence of activated charcoal. The charcoal is removed by filtration through a bed of one of the adsorptive earths and the acetone solution is distilled to about two-fifths the original volume. The reduced volume of solution is allowed to stand for about a day at 0° C. The precipitate which forms is separated by filtration or centrifuging, washed in cold acetone and dried. The white crystalline product which results is substantially pure hyodesoxycholic acid.

The above description of the use of acetone is to be considered merely as exemplary. A number of additional organic solvents have been employed with varying degrees of success to purify the crude bile acids by recrystallization. Recrystallization from methyl Cellosolve acetate has produced results which compare very favorably with recrystallization from acetone. Ethyl acetate and methyl ethyl ketone have also been employed with fair success but the product, as shown by the melting point, is not nearly so pure as when the crude bile acids are recrystallized from acetone. They may, however, be used as an intermediate for further purification.

The above-outlined process furnishes excellent yields of hyodesoxycholic acid. Usually the yield will average better than 2% based on the weight of whole bile. This is recrystallized from crude bile acids, the yields of crude bile acids averaging above 4%.

In the process detailed above, the time of saponification of fresh hog bile should be longer than that employed in the isolation of bile acids from beef bile. With beef bile, it is customary to saponify overnight,

but in the present process of obtaining hyodesoxycholic acid from fresh hog bile, it has been found necessary to employ a time of saponification at reflux temperature of at least 20 to 24 hours in order to secure a good yield.

With inspissated bile, the longer saponification times have not been found to be necessary. Uniformly good yields are achieved by refluxing overnight under saponification conditions. This may be due to the fact that sodium hydroxide is often added to the bile as it is collected to act as a preservative. If the inspissating is carried out in the presence of caustic, the bile may already be partially saponified, and this may account for the shorter saponification time subsequently required.

While ethyl acetate was indicated earlier as the preferred solvent for extracting the crude bile acids from the saponified and subsequently acidified mixture, it has been found that other organic solvents may be employed with at least fair results. In general alkyl esters of acetic acid, the group to which ethyl acetate belongs, may be employed with some degree of success. Other solvents such as acetonitrile and methyl Cellosolve acetate, the acetate of the monomethyl ether of ethylene glycol, have been employed with fair results.

The following table sets forth the average yield from a number of experiments to show the comparative results obtained by carrying out the present process with a number of different selective solvents:

TABLE

| Solvent                        | Average percent Yield of Crude Bile Acids Based on the Weight of Fresh Bile | Average Melting Point of Crude Bile Acids in ° C. |
|--------------------------------|-----------------------------------------------------------------------------|---------------------------------------------------|
| Ethyl Acetate.....             | 4.40                                                                        | 160-167                                           |
| Methyl Cellosolve Acetate..... | 2.91                                                                        | 168-176                                           |
| Isoamyl Acetate.....           | 2.32                                                                        | 165-173                                           |
| Isopropyl Acetate.....         | 1.76                                                                        | 170-175                                           |
| N-butyl Acetate.....           | 1.01                                                                        | 180-184                                           |
| Acetonitrile.....              | 1.00                                                                        | 189-192                                           |
| Methylamyl Acetate.....        | 0.51                                                                        | 170-176                                           |
| Ethylene Dichloride.....       | 0.12                                                                        | 165.5-172                                         |
| Chloroform.....                | Trace                                                                       | 155-168.5                                         |
| Carbon Tetrachloride.....      | Trace                                                                       | 166-173                                           |

Even when using the preferred organic solvent, care must be employed in its selection. In the first experiments, a reagent grade of ethyl acetate was used, which was 99.8% pure and the results were uniformly good. However, when attempts were made to use a cheaper grade (U. S. P.) assaying 90% ethyl acetate and produced by esterifying acetic acid with ethyl alcohol, quite low yields were encountered.

Since the 10% impurity in this product was largely ethyl alcohol, it was suspected that this compound might be causing the decrease in efficiency. Accordingly, another inexpensive technical grade solvent manufactured from acetaldehyde and containing only about 1% ethyl alcohol was tried with the attainment of uniformly good yields of hyodesoxycholic acid.

The final pH reached during extraction of the crude bile acids with organic solvent is an important factor in the present process. In the preparation of beef bile acids, the usual practice is to acidify to pH 3 or lower to make sure that all acids are precipitated. With hog bile, these conditions force out of solution all the pigments and gummy substances which have always caused trouble in obtaining crystalline hog bile acids. A number of experiments have been carried out with the final pH in a range between 3 and 6. Crude acids may be obtained with lower pH's but below approximately 4.2, the crude acids, when recrystallized, give a very poor yield of hyodesoxycholic acid. At pH's materially over 5, much of the desired product is lost. Hence, the pH should be maintained as close to 5 as is practicable.

The treatment of the organic solvent solution with bleaching earth to remove the characteristic green and red colors of fresh bile followed by filtration is not a necessary step in the present process. While the earths are quite efficient in removing the pigments which yield the red coloration, their action in removing those which cause the green coloration is much less efficient. However, filtration of the solvent solution at some point between separation from the aqueous layer and precipitation of the crude bile acids is very desirable to remove any traces of gum, which may be present. Filtration with an earth aside from a bleaching action is effective in removing any small traces of interface gum which may have been retained in the solvent layer.

In the case of inspissated bile where the pigments have already turned brown in the concentration process, treatment with bleaching earths has little or no effect in so far as color removal is concerned.

In the earlier description of the present process, it was stated that the solvent solution, after treatment with bleaching earth followed by filtration, is distilled to about half its volume so that the crude bile acid will precipitate on cooling and standing. It is to be understood, however, that this merely represents the average of a number of experiments in most of which the total volume of solvent extraction and washings amounted to about one-half the original volume of starting bile, and that the proportion distilled can vary greatly in accordance with reaction conditions. In some cases it was found that material was precipitating under distillation conditions before the volume of solvent solution had been reduced by one-half and, in these instances, distillation was discontinued, with good yields of crude acids being obtained on cooling and standing.

Results of the limited number of pilot plant runs, which have been made, indicate that on a commercial scale it may be well to limit the reduction in volume of solvent solution prior to precipitation of crude acid. When reduced by one-half a precipitate was obtained which had poor filtering and washing characteristics. Thus it is indicated that for large scale operations it will prove better to remove a smaller proportion of solvent and obtain a lower yield of crude acid but one from which a greater percentage of pure hyodesoxycholic acid can be isolated.

Thorough washing of the precipitated and separated crude bile acid with the extracting solvent removes much of the remaining gummy impurities. The amount of washing varies with the nature of the precipitate but, in all cases, the washed product should be light in color and granular in consistency. As the product becomes less contaminated with gums, it becomes less soluble in solvents such as ethyl acetate and acetone and thus it is desirable to remove as much impurity at this stage as possible.

A thoroughly washed, dried and ground product may be further washed with hot water or toluene to yield an odorless product, since in some cases a characteristic rancid fat odor will persist even after acetone recrystallization. In the case of beef bile acid, the first precipitated product may be initially washed with hot water, but if this is attempted with hog bile, a gummy solid will result.

A thoroughly washed crude acid product produced in accordance with the present process may be readily air dried without deterioration. Temperatures as high as 100° C. may be employed to speed the drying operation without deleterious effect.

After drying, the product may be ground by conventional procedures and there is obtained a material highly suitable for further treatment to obtain substantially pure hyodesoxycholic acid by simple recrystallization procedures.

5

The following examples will serve to illustrate specific embodiments of the present invention:

#### Example 1

##### PREPARATION OF CRUDE HOG BILE ACIDS

1500 cc. of fresh hog bile and 150 gms. of NaOH were stirred together in a 3 necked, 3 litre flask equipped with reflux condenser. The mixture was heated carefully to reflux temperature with the addition of Dow-Corning Antifoam A (a polymeric silicone derivative with anti-foaming properties) before 80° C. to prevent excessive frothing. Refluxing was continued for 25 hours. The mixture was then cooled to 50° C.

Addition of concentrated hydrochloric acid was carried out with continued stirring and temperature maintained at approximately 55° C. ( $\pm 3^\circ$  C.). At pH 8, 7.5 gms. of sodium hydrosulphite were added and 600 cc. ethyl acetate. The reaction mixture was further acidified with constant stirring at 55° C. to pH 5.0. At this point, 2 layers, a yellowish lower aqueous layer and an upper brownish layer of ethyl acetate formed, separated by a gummy brown interface. The ethyl acetate layer was completely removed with as little of the interface as possible by allowing the mixture to stand for an hour and the ethyl acetate siphoned off.

After separation the ethyl acetate layer was slurried with 25 gms. of bleaching earth, e. g. superfiltrol, then filtered by suction.

The decolorized ethyl acetate was distilled at ordinary pressure to approximately 425 cc., then seeded, and stirred slowly till precipitation started.

The precipitate was allowed to stand 15 hours at room temperature, then filtered by suction and washed with ethyl acetate till the washings were pale yellow.

The washed precipitate was air dried at room temperature to furnish 46 gms. of a white-beige amorphous solid having a melting point of 187° to 190.6° C. This represents a yield of 3.07% based on the weight of the starting bile.

The crude bile acids were ground for recrystallization.

##### RECRYSTALLIZATION OF CRUDE BILE ACIDS

20 gms. of the powdered crude hog bile acids were refluxed half an hour with 25 cc./gm. of acetone and 5% activated charcoal, Norit C. F. Extra. The charcoal was removed by filtration while hot through supercel.

The filtrate and washings were then distilled down to 10 cc./gm. of solvent mixture, seeded and cooled. Scratching the walls of the flask or stirring gently while cooling prevents excessive formation of crystals on sides of flask. After cooling to room temperature, the mixture was refrigerated at 0° C. for 15 hours, then filtered and washed free of colored mother liquor with ice-cold acetone. On drying there was obtained 13.6 gms. of a white, crystalline product having a melting point of 196.7° to 197.8° C., which represents a yield of 68% of the weight of the crude acids recrystallized and an overall yield of 2.09% based on the weight of the fresh bile.

#### Example 2

##### PREPARATION OF CRUDE HOG BILE ACIDS

1500 cc. of fresh hog bile were saponified for 20 hours by refluxing with 150 gms. NaOH with constant stirring, D. C. Antifoam A added. The mixture was water cooled to 50°, then brought to pH 8 with concentrated HCl, with the temperature maintained at 55–63° C. The mixture was cooled to 53° C. and 7.5 gms. sodium hydrosulphite added with 600 cc. ethyl acetate and constant stirring. Acidification was continued to pH 4.95 (later test of aqueous layer after separation—pH 5.02). 50 gms. of supercel were added and the mixture stirred, then filtered by suction. The supercel cake was washed with ethyl acetate. The volume of ethyl acetate used was half the volume of the starting bile with approximately  $\frac{1}{2}$  added to the reaction mixture and the remainder employed for the various washings.

6

The solvent and aqueous layers were separated in a separatory funnel. The ethyl acetate layer was treated with 25 gms. of bleaching earth (special Filtrol), filtered, and washed with ethyl acetate.

The solvent was distilled down to 400 cc. by ordinary distillation. At this point precipitate was seen so distillation was stopped and the mixture stirred occasionally as it cooled.

The precipitate was allowed to stand overnight, then filtered, washed with ethyl acetate, dried and ground to provide 69 grams of a crude acid product, which was beige to red in color. This represents a yield of 4.6% based on the weight of the fresh bile.

##### RECRYSTALLIZATION OF CRUDE HOG BILE ACIDS

20 gms. of the above product were dissolved in 600 cc. acetone and treated with 1 gm. of activated charcoal, Norit C. F. Extra, then filtered with supercel and superfiltrol as described in Example 1. The filtrate was distilled down to 225 cc. and white crystals precipitated quickly. When filtered after cooling overnight at 0° C. and washed with cold acetone, there was obtained 13.06 grams of a white, crystalline product having a melting point of 195–198.2° C. This represents a yield of 65.3% of the crude acids recrystallized or an overall yield of 3.0% based on the weight of the fresh bile.

In the two preceding examples, reagent grade ethyl acetate was used. In the following procedure, a commercial ethyl acetate was used.

#### Example 3

Half of the reaction mixture from a 1520 cc. saponification of fresh bile was treated as described previously to obtain the ethyl acetate extract containing the hyodesoxycholic acid. The ethyl acetate was distilled down to 190–200 cc. and a crude acid precipitate obtained by allowing it to stand at room temperature overnight after seeding. The precipitate was filtered, washed with ethyl acetate, dried and ground to provide 34 grams of beige, lumpy crude acid product having a melting point of 156–170° C. This represents a yield of 4.47% based on the weight of the starting bile.

10 gms. of the above product were recrystallized from acetone as in Example 1 and the precipitated product obtained after cooling overnight was filtered and washed with cold acetone. This furnished 5.4 grams of a white, crystalline product having a melting point of 194.4–195.8° C., which amounts to an overall yield of 2.42% based on the weight of the fresh bile.

#### Example 4

Inspissated bile was diluted with water in a stainless steel tank to a solid content of 12.5%.

One litre of this material was saponified and acidified as described in the previous examples. The ethyl acetate layer was separated, treated with bleaching earth and distilled down to 250 cc.

After standing overnight, a granular product was filtered off, washed and dried as before to yield 48 grams of crude acids having a melting point of 162.8–174° C. This amounts to a yield of 4.8% based on the weight of the made-up bile.

10 gms. of this crude material were charcoaled in 250 cc. of acetone. Little or no color was removed from the solution. The filtrate was distilled down to 100 cc. and a white, crystalline product came out quickly, which when dried was found to weight 5.2 gms. and have a melting point of 193.8–194.8° C. This represents an overall yield of 2.5% based on the weight of the made-up bile.

#### Example 5

44.5 gms. of crude bile acids, prepared as in Example 1, were ground, then refluxed for one-half hour with 250 cc. of ethyl acetate, a considerably lesser proportion of organic solvent than was used heretofore and insufficient to completely dissolve all of the crude bile acids

7

even under reflux conditions. The mixture was cooled at 0° C. for 5 hours, then filtered and washed with cold ethyl acetate. The filtrate was highly colored. The product, when air dried, was found to weigh 37.0 gms. and have a melting point of 190.5–192° C. This represents a yield of 83% of the crude bile acids which were treated. This extraction technique does not yield a final product of as high purity as is obtained by recrystallization but does provide a higher yield of a cruder product which may be quite suitable for many purposes.

#### Example 6

76.7 gms. of crude hog bile acids (melting point 176–182° C.) were dissolved in 284 cc. of methyl Cellosolve acetate (3.7 cc. solvent per gm. of crude acids) and refluxed for twenty minutes in the presence of 3.5 gms. of activated charcoal (Norit C. F. Extra). The mixture was filtered through a bed of adsorptive earth (supercel) to remove the charcoal. The filtrate was stirred slowly until the crystalline material precipitated throughout the solution. The mixture was allowed to stand at room temperature for four hours, filtered, and washed with a small amount of cold methyl Cellosolve acetate. When air dried, 52.5 gms. of a white, crystalline solid having a melting point of 194–195.8° C. were obtained. This represents a yield of 68.4% of the crude bile acids, which were recrystallized.

#### Example 7

120 gms. of crude hog bile acids (melting point 189.8–192° C.) were dissolved at reflux temperature in 360 cc. of methyl Cellosolve acetate (3 cc. of solvent per gram of crude acids). The mixture was stirred slowly while cooling until crystallization was well started. After standing for four hours at room temperature, the solid was filtered and washed with cold methyl Cellosolve acetate. When air dried, 97 gms. of white, crystalline hyodesoxycholic acid were obtained having a melting point of 195.4–197.8° C. This represents a yield of 81% of the starting crude bile acids.

#### Example 8

50 gms. of crude bile acids, prepared as in Example 1, were ground, then refluxed for one-half hour with 250 cc. of methyl ethyl ketone. The mixture was cooled at 0° C. for five hours, then filtered and washed with cold methyl ethyl ketone. The product was air dried to furnish 33.0 grams of material having a melting point of 188.8–192° C., which is 66% by weight of the crude acid recrystallized.

Having described our invention, we claim:

1. The process of preparing hyodesoxycholic acid from hog bile containing gummy impurities comprising: treating said bile under saponifying conditions; acidifying said bile to approximately pH 5, while extracting said bile with an organic solvent selected from the group consisting of a lower alkyl ester of acetic acid, methyl Cellosolve acetate, and acetonitrile, so as to dissolve said hyodesoxycholic acid in preference to said gummy impurities; separating said solvent extract from said bile; and precipitating solid matter having a high hyodesoxycholic acid content from solution in said solvent.

2. The process of claim 1 in which said organic solvent comprises a lower alkyl ester of acetic acid.

3. The process of claim 1 in which said organic solvent comprises ethyl acetate.

4. The process of claim 1 in which said organic solvent comprises methyl Cellosolve acetate.

8

5. The process of claim 1 in which said organic solvent comprises acetonitrile.

6. The process of preparing hyodesoxycholic acid from hog bile containing gummy impurities comprising: treating said bile under saponifying conditions; acidifying said bile to approximately pH 5, while extracting said bile with an organic solvent selected from the group consisting of a lower alkyl ester of acetic acid, methyl Cellosolve acetate, and acetonitrile, so as to dissolve said hyodesoxycholic acid in preference to said gummy impurities; separating said solvent extract from said bile; precipitating solid matter having a high hyodesoxycholic acid content from solution in said solvent; and recrystallizing said solid matter from an organic solvent to yield substantially pure hyodesoxycholic acid.

7. The process of claim 6 in which said solvent from which said solid matter is recrystallized comprises acetone.

8. The process of claim 6 in which said solvent from which said solid matter is recrystallized comprises methyl Cellosolve acetate.

9. The process of preparing hyodesoxycholic acid from hog bile containing gummy impurities, comprising: saponifying said hog bile with sodium hydroxide; acidifying said bile to approximately pH 5 while extracting said bile with ethyl acetate so as to dissolve said hyodesoxycholic acid in preference to said gummy impurities; separating said ethyl acetate solution from said bile; distilling a portion of said ethyl acetate solution; allowing said ethyl acetate solution to stand without heating to precipitate solid matter having a high hyodesoxycholic acid content from solution in said ethyl acetate; separating said solid matter from said ethyl acetate; and washing said separated solid matter with additional ethyl acetate.

10. The process of claim 9 wherein said bile is extracted with ethyl acetate while maintaining said bile at a temperature of 50° to 55° C.

11. The process of preparing hyodesoxycholic acid from hog bile containing gummy impurities, comprising: saponifying said hog bile with sodium hydroxide; acidifying said bile to approximately pH 5 while extracting said bile with ethyl acetate so as to dissolve said hyodesoxycholic acid in preference to said gummy impurities; separating said ethyl acetate solution from said bile; distilling a portion of said ethyl acetate solution; allowing said ethyl acetate solution to stand without heating to precipitate solid matter having a high hyodesoxycholic acid content from solution in said ethyl acetate; separating said solid matter from said ethyl acetate; washing said separated solid matter with additional ethyl acetate; and recrystallizing said solid matter from acetone to yield substantially pure hyodesoxycholic acid.

12. The process of preparing hyodesoxycholic acid from hog bile containing gummy impurities, comprising: treating bile under alkaline saponifying conditions; adding an acid to said bile to bring the pH to approximately pH 8; further acidifying said bile to approximately pH 5, while extracting said bile with a lower alkyl ester of acetic acid so as to dissolve said hyodesoxycholic acid in preference of said gummy impurities; separating the solvent extract from said bile; and precipitating solid matter having a high hyodesoxycholic acid content from solution in said solvent.

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