HPLC Analysis of Bile Acids Using a New Technique of Post Column Reaction

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SUMMARY Many trials have been made with HPLC, on bile acids determination, and introduced, the enzymatic method using immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) as an effective method to detect of bile acids and its conjugates, for post column reaction technique, in this paper.

DuPont Zorbax C8 were used as the LC column which was housed in column oven to keep at 55°C, and used the alcohol-phosphate buffer mixture as the mobile phase.

The enzyme were immobilized on aminated porous glass beads with glutaraldehyde, and packed in stainless steel column. The reaction reagent containing NAD and DTT (dithiothreitol) in phosphate buffer was mixed just before the immobilized enzyme column.

The fluorescence intensity of the NADH thus produced were measured, in the presence of bile acids as substrate.

Some results are described on some conditions for separation and determination in this paper.

Introduction

In the studies of diseases related with the function of bile it has become more and more important to determine the qualitative and quantitative changes of free and conjugated bile acids in body fluids. There has been born a strong demand for a reliable analytical technique that can be used in clinical works.

So for, GC and TLC have been generally used to analyze bile acids and their conjugated compounds. But GC is not useful for direct analysis of conjugated compounds, while TLC cannot provide satisfactory separation or sensitivity. Therefore, many trials have been made with HPLC. The recent methods of HPLC for this application can be classified into two groups. One is to separate bile acids without pretreatment and measure their ultraviolet absorbances in a wavelength range from 200nm to 210nm. The other is the precolumn derivatization method in which bile acids are derivatized into compounds having strong UV absorption or strong fluorescence, and then are separated by the...
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The enzymatic method using 3α-hydroxysteroid dehydrogenase (3α-HSD), has been employed fairly extensively as an effective method to detect bile acids, because of its specific reaction property. This 3α-HSD has also been used as the reagent in the post column method for the HPLC of bile acids, and it is reported that it has proved to give satisfactory sensitivity and ease of operation. The only drawback is that 3α-HSD is too expensive to use in routine analysis.

We have developed a highly sensitive and highly selective method of detection by immobilizing 3α-HSD.

**Experiment**

**Materials**

The 3α-HSD we used was offered by Kyowa Fermentation Inc. in Japan. It was derived from *Pseudomonas putida*.

The 3α-HSD was immobilized, with glutaraldehyde, on aminated porous glass beads, made by Sanyo Kasei Inc. in Japan, about 100 mesh in size, 200m²/g in surface area, and 100Å in pore size. The 3α-HSD was immobilized at a ratio of 46mg/g.

The standard reagent of bile acids and their conjugated compounds are purchased from Sigma Inc. in U.S.A.

**Apparatus**

Fig. 1 shows the flow diagram of our equipment. Shimadzu LC-3A high performance liquid chromatograph was used as

![Fig. 1 Schematic Flow Diagram of the System](image)

![Fig. 2 Principle of Detection](image)
the pump unit which includes the pump 1. DuPont Zorbax C₈ was used as the LC column for the reason which I will describe later. The column was housed in the Shimadzu CTO-2A column oven to keep it at 55°C.

The Shimadzu LC-3A was also used as the pump 2.

The detector was the Shimadzu RF-500LCA fluorescence spectromonitor which has a flow-thru cell with an inner volume of 14μl. The excitation wavelength was set at 350nm and the emission wavelength at 460nm. The enzyme to be immobilized was packed in a stainless steel tube, of 2.1 mm in inner diameter and 50 mm or 100 mm long, by the slurry method, using an ordinary column filling unit for HPLC.

The measurement of peak areas and other data processing were performed by the Shimadzu Chromatopac C-RIA.

**Principle**

Fig. 2 shows the principle of detection. The co-enzyme NAD fed as the reagent is reduced into NADH by the immobilized 3α-HSD, in the presence of bile acids as substrate. The fluorescence intensity of the NADH thus produced is measured.

**Immobilization**

Fig. 3 shows how we immobilized the enzyme. The 3α-HSD was immobilized, with glutaraldehyde, on aminated porous glass beads, in the Weetall method.

The enzyme thus immobilized was packed in stainless steel tubes of various sizes by means of an ordinary column filling device for LC, using the slurry method. The solvent for the slurry and the solvent used to drive the slurry are reagent, that are shown in the Fig. 2. NAD is not contained in the solvent, however.

**Result and Discussion**

1. Fig. 4 shows the effect of pH of reagent on fluorescence intensity.

2. Fig. 5 shows the effect of temperature in enzymatic reaction on fluorescence intensity.

3. Fig. 6 show the time necessary for the reaction to be completed in the immobilized enzyme column. The time were measured using the flow system shown in the Fig. 1. The separation column was removed from the flow system, and about 3 ml of sample solution

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<table>
<thead>
<tr>
<th>Support material</th>
<th>Surface area</th>
<th>Pore size</th>
<th>Particle size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porous glass (P G)</td>
<td>200 m²/g</td>
<td>100 Å</td>
<td>100 mesh</td>
</tr>
</tbody>
</table>

**Immobilization method**

<table>
<thead>
<tr>
<th>PG - NH₂</th>
<th>Amino Porous Glass</th>
<th>2% Glutaraldehyde soln. 4°C 2h mix.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG - N(CH₂)₃-CHO Aldehyde Porous Glass</td>
<td>Enzyme in Phosphate Buffer 4°C 2h gently mixed.</td>
<td></td>
</tr>
<tr>
<td>PG - N(CH₂)₃-CHO + Enzyme</td>
<td>Ratio of immobilization 69.5%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amounts of immobilization 46mg/g</td>
<td></td>
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</tbody>
</table>

Fig. 3 Preparation of Immobilized Enzyme
was injected into the system to establish a steady state. Then the responses in the plateau area were measured; thus the influence of the band broadening in the flow system was excluded.

The immobilized enzyme reacted very rapidly. The reaction took place in about 10 seconds for CA and CDCA.

When the total flow rate of the carrier liquid and the reagent solution is adjusted to 1.8ml/min., necessary reaction can be obtained by two immobilized enzyme columns, measuring 2.1 mm in inner diameter and 50mm in length, and connected in series.

Even when a longer enzyme column was used, the peak height was not increased because of the broadened band in the immobilized column.

4. Fig. 7 shows the relationship between fluorescence intensities and amounts of various bile acids, obtained when the total flow rate was adjusted to 1.8ml/min. and an immobilized enzyme column, 2.1 mm in inner diameter and 100mm in length, was used. The differences in the slopes of the calibration curves correspond to the differences in the speed of the enzyme reaction.
These curves show linearity up to a 2µg. This shows that this method is sufficiently quantitative. The detection limit under this set of operational conditions is a few nano grams.

5. It is reported that bile acids can be analyzed in either of the two methods. One is to use a mixture of acetonitrile and ammonium carbonate buffer as the mobile phase, its pH value being adjusted to be alkaline. The other method is to use a mixture of methanol and a phosphate buffer, its pH value being adjusted to be acidic.

Of these two methods, the acetonitrile-ammonium carbonate as the mobile phase has been studied in detail by Nanbara and his coworkers⁵, Tohoku Univ. in Japan. And I believe this mobile phase is the most practical for the analysis of bile acids. But since this mobile phase requires a high running cost and shorten the life of the immobilized enzyme column, we studied the reversed-phase chromatography method that uses the alcohol-phosphate buffer mixture as the mobile phase, adjusting it to be acidic. Acetonitrile can be used as mobile phase in this detection system. When this mobile phase was used, it was necessary to make the alcohol content in the mobile phase less than 20%, so that it would not shorten the life of the immobilized enzyme column. However this value is not accurate. Fig. 8 shows the retention behavior for various bile acids when the alcohol content was varied. The column was Zorbax C₈, 4.6 mm in inner diameter and 150mm in length.

In order to elute lithocholic acid, which has the largest k’ value, in a reasonable length of time, it is necessary to adjust the ethanol content to be larger than 60%. So, the ratio of the flow rates between the mobile phase and the reagent
solution was adjusted to 1:2, so that there would be 20% ethanol in the immobilized enzyme column.

If a Zorbax ODS column had been used instead of the Zorbax C8 column, or if methanol mobile phase had been used instead of ethanol, the lithocholic acid would have been retained too long.

6. The pH value of the phosphate buffer buffer also has some influence upon the separation.

Fig. 9 shows how the separation of bile acids changes when the pH of the phosphate buffer buffer is changed. The ethanol content was kept constant at 50%.

Note that the retention times of cholic acid, tauro lithocholic acid, and glico lithocholic acid change greatly.
7. Fig. 10 shows the chromatogram of a mixture authentic sample of free and conjugated bile acids which is analyzed in the step-gradient mode.

The step-gradient program was decided taking the effect of pH value and ethanol concentration into consideration. The detection limit for bile acids is about 10\( \mu \)g.

The stepwise fluctuation of the base line shall be attributable to the impurity, that is alcohol dehydrogenase, contained in the 3\( \alpha \)-HSD. Flat base line shall be able to obtained by purifying the 3\( \alpha \)-HSD in advance.

8. Fig. 11 shows chromatogram of conjugated bile acids, 250\( \mu \)g each.

9. Fig. 12 shows the chromatogram of clinical bile sample (Bile Duct Carcinoma). A 100\( \mu l \) bile and 5\( ml \) methanol were mixed and centrifuged at 3,000rpm for 5 min. Then a 30\( \mu l \) alliquot of supernatant was injected into the column.

We have described on our newly-developed method to analyze bile acids, and on our investigation of the method in this paper.

We found that our method has satisfactory sensitivity, reproducibility, and ease of operation, and we believe this method will be extensively used in the future for analysis of bile acids and related compounds on clinical samples.

Reference

1) S. Baba et al.: Medicine and biology, 97, 219 (1978)