Rapid Analysis of Four Bilirubins in Domestic Animal Sera Using High-Performance Liquid Chromatography

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ABSTRACT. A rapid method was developed to analyze δ-bilirubin (Bδ), conjugated bilirubin (DCB), MCB, and unconjugated bilirubin (Bu) by direct injection of sera using high-performance liquid chromatography (HPLC) with an internal-surface reversed-phase silica support (ISRP) column. Sharp bilirubin peaks were obtained using a simple mobile phase of acetonitrile: 0.5 M Tris-HCl buffer (20:80, v/v, pH 7.2). A variable-wavelength detector set at 450 nm, 0.01 absorbance unit full scale (AUFS), and a recorder set at 4 nm/min were used for detection. Peaks for Bδ, DCB, MCB, and Bu appeared at 4.4, 6.4, 9.2, and 14.5 min, respectively, in human serum from subject with obstructive jaundice which was used as a bilirubin standard throughout this experiment. The mean recovery rate after direct addition of Bu in swine serum was 91.9% and that of DCB was 95.9%. When sera from icteric cattle, pigs, and horses were analyzed using the direct injection technique, four bilirubin peaks were obtained and there was reliable correlation between the sum of the bilirubin peak heights observed on HPLC and the total bilirubin value measured by a standard reference procedure. — KEY words: bilirubin, HPLC, internal-surface reversed-phase column, jaundice.

Conventional reversed-phase (RP) silica support columns have been used for high-performance liquid chromatographic (HPLC) analysis of bilirubins in samples such as serum [1, 6–9, 11, 14, 15, 17]. The pretreatment of samples for this technique is tedious, requires large volumes of organic solvent, and many repetitions may be necessary to achieve a satisfactory result. Direct injection of proteinaceous samples onto a conventional RP column leads to the accumulation of proteins denatured by the organic solvents (e.g., methanol, acetonitrile) in the mobile phase. This results in clogging of the interparticulate spaces of the column and damages the column. Conventional extraction procedures also introduce the possibility of losing the substances to be analyzed, therefore, 100% of bilirubin in the serum can not be recovered. Inevitably the recoveries of the bilirubins from the serum samples decrease. Thus, it is necessary to develop a rapid and easy pretreatment procedure for bilirubins.

Hagestam and Pinkerton [3, 4] developed an internal-surface reversed-phase (ISRP) silica support column that can be used for the determination of drugs in serum without extraction or deproteinization. The internal surface of the ISRP has a hydrophobic partition phase (glycyl-L-phenylalanine) and its external surface has a hydrophilic phase (a glycercylpropyl group and a glycine residue). Since the pores of the supports are intentionally kept small, proteins can only reach the non-adsorptive external surface and elute in the external void fluid with very high recoveries. Drugs, on the other hand, being small molecules, penetrate the supports and partition into the internal polyepitide phase.

To date, a conventional RP column has been used in the analysis of many kinds of drugs and substances such as bilirubin, and there are few published reports concerning the use of the ISRP column [12, 13]. This paper describes the conditions used for HPLC analysis on the ISRP column using direct injection technique and presents chromatograms of four bilirubins in serum samples from jaundiced animals.

MATERIALS AND METHODS

Standard bilirubins: Pure unconjugated bilirubin (Bu), Nihon Shoji, Co., Ltd., Tokyo, Japan) and conjugated bilirubin (DCB, Funakoshi, Co., Ltd., Tokyo) were purchased from commercial sources, however δ-bilirubin (Bδ) and unconjugated bilirubin (MCB) are not commercially available at a purity level suitable for use as a standard. Serum obtained from a patient with obstructive jaundice was provided by human hospital, which was shown to contain four bilirubins by an HPLC analysis. This serum was used as the bilirubin standard throughout this experiment, it has been shown by Okuyama et al. [11] that on HPLC the elution order of bilirubins in the serum obtained from patients with obstructive jaundice is Bδ, DCB, MCB, and Bu.

Chromatography: The liquid chromatograph consisted of a Waters Model 600E Multisolvent Delivery System, a U6K injector (Millipore Corp., Milford, MA, U.S.A.), and a SPD-10A UV-VIS detector (Shimadzu Seisakusyo Co., Ltd., Kyoto, Japan). The chromatograph was recorded with a Caromatopac (Shimadzu). The column used was a Pinkerton ISRP Column (GFF-SS-80, 4.5 x 150 mm, 5 μm, Regis, Morton Grove, IL, U.S.A.). This column was used with a guard column packed with the same packing material. Other analysis conditions were as follows: UV wavelength, 450 nm; flow rate, 1.2 ml/min; column temperature, 25°C;
sensitivity, 0.01 absorbance unit full scale (AUFS); chart speed, 4 mm/min; injection volume, 20 μl. The detection wavelength for the HPLC analysis was examined to determine the maximum absorption wavelength of bilirubin.

**HPLC mobile phase:** The mobile phase contained 20% acetonitrile (Wako Pure Chemicals Co., Ltd., Tokyo) and 80% 0.5 M tris hydroxymethyl aminomethane (Tris, Wako Pure Chemicals) HCl (Nacalai Tesque, Co., Ltd., Kyoto) buffer. A 1 M Tris HCl buffer was prepared as follows: 121.1 g Tris was added to 900 ml Milli-Q water in a 1,000 ml volumetric flask, then 75 ml HCl was added to adjust the solution to pH 7.2 and the preparation was mixed by magnetic stirrer for 30 min. Finally, the solution was made up to 1,000 ml with Milli-Q water. This solution was stored in the dark. When used in the experiments, it was diluted in half with Milli-Q water. Milli-Q water is a purified water using a Milli-Q Labo (Millipore) until a resistivity of 18 MΩ cm was achieved.

**Recoveries of Bu and DCB in serum samples:** The mobile phase was tested to determine whether it was practically useful for the recovery test. Swine serum was used after it was confirmed to be bilirubin-free by a routine biochemical procedure and HPLC analysis. One milliliter of serum sample was mixed with 5 μg/ml Bu or DCB. These samples were injected directly in an injection volume of 20 μl at 4 times into the HPLC apparatus including the Pinkerton column after centrifugation at 3,500 × g for 6 min.

**Samples:** Each 5 of normal bovine, equine, and swine serum samples was collected at a meat inspection site. On the other hand serum samples from domestic animals with jaundice were collected, which included 6 from cattle, 5 from horse and 5 from pig. The samples were injected directly into the HPLC apparatus in an injection volume of 20 μl after centrifugation at 3,500 × g for 6 min. Twenty μl of standard Bu and DCB solutions diluted with Milli-Q water were injected into the HPLC system without any pretreatment. These sample tubes were covered by aluminum foil, and were handled in a dark room throughout in this experiment.

**Measuring the total bilirubin value:** Reflotron (Boehringer Manheim Co., Ltd., Tokyo, Japan) was used to measure the total bilirubin concentration (mg/dL). This analysis is based on the principles of bilirubin determination by the diazo reaction (diazotization) using the dry chemistry system [2].

**Statistical analysis:** A reliable correlation between the total bilirubin concentration (mg/dL) by Reflotron and the sum of the bilirubin peak heights obtained on HPLC was measured using the StatView software (Abacus Concepts, Inc., U.S.A.) and significant differences between them were analyzed using T-test.

**RESULTS**

**HPLC analysis of standard bilirubins:** Using the most suitable analytical conditions, four bilirubins from human serum were analyzed simultaneously as a standard. Their individual retention times were 4.4, 6.4, 9.2 and 14.5 min (Fig. 1). The retention times of pure DCB and Bu were 6.5 and 14.4 min, respectively. The minimum detectable concentrations of Bu and DCB were 0.6 and 1.2 μg/ml, respectively. Standard curves for both bilirubins were calculated from the bilirubin concentration (μg/ml) and peak heights (μV). The calibration curves for both bilirubins were linear, and their correlation coefficients were more than 0.99 (Table 1).

**Recovery of Bu and DCB in serum samples after direct injection:** Recoveries after direct injection were 87.5 to 96.5% for Bu and 94.8 to 105.6% for DCB. Mean recoveries and standard deviations for Bu and DCB are shown in Table 2.

**Analysis of normal and samples from jaundiced animals using the ISRP support:** Figure 2 shows the chromatograms of normal bovine, equine, and swine serum samples. In normal equine samples, small B8 and Bu peaks were recognized and traces of these peaks also appeared in bovine samples, however, no peaks appeared in swine samples. Figure 3 shows chromatograms of samples obtained from jaundiced animals. Sharp bilirubin peaks appeared in all jaundiced samples. In the swine sample, MCB
Table 1. Minimum detectable concentrations, standard lines and correlation coefficients of unconjugated bilirubin (Bu) and diconjugated bilirubin (DCB)

<table>
<thead>
<tr>
<th>Bilirubins</th>
<th>Minimum detectable concentration (μg/ml)</th>
<th>Standard line</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bu</td>
<td>0.6</td>
<td>Y=540.820 x -543.347</td>
<td>r=0.996**</td>
</tr>
<tr>
<td>DCB</td>
<td>1.2</td>
<td>Y=98.295 x -251.920</td>
<td>r=0.998**</td>
</tr>
</tbody>
</table>

n=5, **: p≤0.01.

predominated, while in bovine and equine samples Bu was predominant (Table 3). The peak heights in normal and jaundiced samples are shown in this Table 3, including total bilirubin concentration measured by Reflotron.

Correlation between peak height (μV) on HPLC and total bilirubin concentration (mg/dl) measured by a standard reference procedure: A reliable correlation was observed between the sum of the bilirubin peak heights (μV) on HPLC and the total bilirubin concentration (mg/dl) measured by Reflotron in the serum samples obtained from jaundiced animals. A straight regression line was obtained as shown in Fig. 4. The linear regression was expressed as Y=3108.477 x + 3880.586. The correlation coefficient (r) was more than 0.88 (n=16).

DISCUSSION

HPLC analysis using ISRP column is simple, fast, and reliable method that can directly analyze four different bilirubins, B₈, DCB, MCB and Bu. The recovery rates were higher than our preliminary study using conventional RP column [9].

This ISRP column is recommended for mobile phases with a pH ranging from 6.0 to 7.5. However, Haginaka et al. reported that this column could be used with acidic mobile phases (pH 2.5–3.5) in the analysis of hydrophilic drugs [5, 10]. We thought it was desirable to use the recommended pH range to protect the column, taking the column’s life into consideration. In the result, it was

Table 2. Mean recovery rate (%) of unconjugated bilirubin (Bu) and diconjugated bilirubin (DCB) in swine serum (n=4)

<table>
<thead>
<tr>
<th>Bilirubins</th>
<th>Recovery rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bu</td>
<td>91.9 ± 4.6</td>
</tr>
<tr>
<td>DCB</td>
<td>95.9 ± 7.7</td>
</tr>
</tbody>
</table>

(Concentration 5 μg/ml)

Fig. 2. Chromatograms of normal serum from cattle, horse and pig.
Fig. 3. Chromatograms of serum from jaundiced cattle, horse and pig.

Table 3. HPLC peak heights (μV) of bilirubins and total bilirubin concentration (mg/dl) by Reflotron in normal and jaundiced animal sera

<table>
<thead>
<tr>
<th></th>
<th>Normal sera</th>
<th>Jaundiced sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of animals</td>
<td>Number of animals</td>
</tr>
<tr>
<td></td>
<td>Bδ</td>
<td>DCB</td>
</tr>
<tr>
<td>Bovine</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Swine</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Equine</td>
<td>5</td>
<td>4229</td>
</tr>
</tbody>
</table>

Bδ: δ-bilirubin, DCB: Diconjugated bilirubin, MCB: Monoconjugated bilirubin, Bu: Unconjugated bilirubin, T.B: Total bilirubin concentration.

a), b) Peak height and total bilirubin concentration are shown as mean value.

possible to analyze four bilirubins simultaneously using a mobile phase consisting of acetonitrile : 0.5 M Tris HCl buffer=20:80, pH 7.2, and the analytical column maintained with a guard column.

When serum samples were analyzed, large protein peaks never appeared in the chromatograms, and the tails of the small protein peaks never overlapped with the Bδ peak, which eluted comparatively early, so that separation of four bilirubins were carried out without any disturbance of protein peaks.

Bu peak was recognized in normal equine samples and traces of this peak appeared in normal bovine samples. This is because the level of this type of bilirubin is higher in the horse than in other domestic animals [16]. In serum samples obtained from jaundiced animals, which include several different types of jaundice (e.g., conjugated bilirubin predominant or unconjugated bilirubin predominant types), a variety of peak patterns might occur. It could be useful for meat inspectors to determine the type of jaundice present using this method which can fractionate bilirubin into 4 peaks. For instance, when a livestock having severe jaundice is found at ante mortem inspection, meat inspectors have to take the regulatory action of prohibition at the slaughter of the livestock. In these cases, it is important for them to get the information of the critical stage of hepatitis by HPLC peak pattern. In human medicine, it is said that the ratio of Bδ(MCB + DCB) is low in critical stages of hepatitis, however, it becomes higher during the recovery stage [11], so that this method could be helpful to judge the stages of hepatitis with jaundice. It was easily
suggested that it may be applicable to animal serum samples as a routine laboratory test using the same method at even clinical sites.

In conclusion, the ISRP column could be of practical use in the analysis of bilirubins, because four different bilirubins can be analyzed simultaneously, and much time normally spent on pretreatment procedures can be saved.

REFERENCES