ELSEVIER

Journal of Chromatography B, 685 (1996) 171–175

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Assay for taurine conjugates of bile acids in serum by reversed-phase high-performance liquid chromatography

James D. Paauw*, Laura Van Wyk, Alan T. Davis

Departments of Surgery, Michigan State University and Butterworth Hospital, 100 Michigan NE, Grand Rapids, MI 495113, USA

Received 19 December 1995; revised 19 March 1996; accepted 28 March 1996

Abstract

The purpose of this study was to develop a new high-performance liquid chromatographic (HPLC) procedure for quantifying taurine conjugates of bile acids in serum. The technique involved three basic steps. The first removed free amino acids via solid-phase extraction of the serum. The second step involved the reaction of the extracted serum with the enzyme choloylglycine hydrolase, which liberated the taurine from the conjugated bile acids. The third step was the reversed-phase HPLC separation of *o*-phthalicdicarboxaldehyde derivatives of taurine. The assay provides a simple technique for determination of the total amount of taurine-conjugated bile acids in serum.

Keywords: Bile acids; Taurine conjugates

1. Introduction

A significant amount of research in recent years has focused on the relationship of bile acids and, specifically, bile acid conjugation patterns, to hepatobiliary disease. A plethora of techniques have been described to assay individual free, glycine-, and taurine-conjugated bile acids in body fluids and especially in serum. Serum bile acid analytical techniques include, among others, thin-layer chromatography [1], gas chromatography [2], radioimmunoassay [3], reversed-phase high-performance liquid chromatography (HPLC) with UV detection [4], HPLC with fluorescent labeling [5–8] and HPLC with post-column enzyme reaction [9]. Most described methodologies have some drawbacks, including expense, instability of reagents (particularly enzymes), and laborious technique. Furthermore, there are instances when only a conjugation pattern, rather than individual bile acid patterns, needs to be identified. In these cases, the more complicated separation techniques, with their inherent drawbacks, may prove to be more complex than necessary.

This paper describes a newly developed HPLC analysis, using a choloylglycine hydrolase reaction, which allows the quantitative analysis of taurine conjugates of bile acids in serum. [^{14}C]Glycocholic acid serves as an internal standard. This technique is advantageous in its simplicity, particularly in avoid-

*Corresponding author.

0378-4347/96/$15.00
PII S0378-4347(96)00166-1

ing the necessity of separation of individual bile acids.

2. Experimental

2.1. Reagents

[^{14}C]Glycocholic acid, taurocholic acid and choloylglycine hydrolase (EC 3.5.1.24) were purchased from Sigma (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were obtained from Baxter Scientific Products (McGaw Park, IL, USA). β-Mercaptoethanol and *o*-phthalicdicarboxaldehyde (sold as fluoraldehyde) were obtained from Pierce (Rockford, IL, USA). All other reagents were of American Chemical Society reagent grade from commercial sources.

2.2. Assay

The taurine-conjugated bile acids were determined as follows. A stock solution of [^{14}C]glycocholic acid was prepared in distilled water to 8510 Bq/ml. A 1-ml volume of human serum, 1 ml of 0.5 *M* sodium phosphate (pH 7.0), 2.65 ml of distilled water and 0.1 ml of the [^{14}C]glycocholic acid stock (as an internal standard) were mixed. A blank tube was made up simultaneously, mixing 1 ml of sodium phosphate (pH 7.0), 3.65 ml of distilled water and 0.1 ml of the [^{14}C]glycocholic acid stock. A 10-μl aliquot was taken from each sample mixture, to determine a baseline count of the internal standard by a liquid scintillation counter (Tri-Carb Model 4530, Packard Instrument, Downer's Grove, IL, USA). The sample, standards and blank mixtures were then each placed on Sep-Pak C_{18} cartridges (Millipore, Milford, MA, USA) which had been previously activated with 5 ml of acetonitrile and 10 ml of distilled water. This step is a variation of the procedure of Scalia and Guarneri [10], in order to remove free amino acids from the bile acid eluate. After successive washing with 5 ml of 40% methanol in 0.05 *M* sodium acetate (pH 4.3) and 2 ml of distilled water, bile acids were eluted with 95% ethanol (3 ml). The effluents were evaporated in a centrifugal evaporator (Speed Vac Model 100H, Savant Instruments, Hicksville, NY, USA) and the residues were dissolved in 1 ml of 0.025 *M* sodium acetate (pH 5.6). The remainder of these solutions were combined with a premixed and prewarmed (37°C) solution of 1 ml 0.025 *M* sodium acetate (pH 5.6), 1 ml of 0.075% β-mercaptoethanol, 1 ml of 0.055 *M* ethylenediaminotetraacetic acid (EDTA) and 0.48 ml of distilled water. The choloylglycine hydrolase was initially prepared as a stock solution of 600 units/ml and kept frozen at −30°C. Immediately prior to beginning the assay, 0.25 ml of this stock was added to 4.75 ml of 0.005 *M* sodium phosphate (pH 7.0) to create a working enzyme stock. To each assay mixture, 0.5 ml of the choloylglycine hydrolase working enzyme stock solution and 20 μl of 1.25 *M* γ-amino-*n*-butyric acid (GABA) were added, and the solutions were placed in a shaking water-bath at 37°C for 2.5 h. The solutions were then centrifugally evaporated, with the residues resolubilized in 0.5 ml of 0.1 *M* sodium acetate (pH 6.7). During this step, the pellets were first manually broken up and then placed in a 45°C shaking bath for 30 min. A 10-μl aliquot was taken from each tube to determine recovery of ^{14}C activity. Part of the remainder was processed for amino acid analysis.

Amino acids were analyzed by use of precolumn *o*-phthalicdicarboxaldehyde derivatization, separation by reversed-phase HPLC and fluorometric detection, in a modification of the method of Jones and Gilligan [11]. A 250-μl sample was added to 63 μl of 12% perchloric acid and 10 μl of distilled water and placed on ice for 30 min. After centrifugation for 10 min at 6000 *g*, the supernatant was withdrawn and neutralized with 63 μl of 2 *M* potassium bicarbonate. After icing for 30 min, this mixture was centrifuged for 10 min at 3000 *g*. The supernatants were then applied to Microfilterfuge tubes (0.45 μm uncharged Nylon-66; Rainin Instruments, Woburn, MA, USA) and centrifuged for 10 min at 2200 *g*. A 100-μl aliquot of the sample was transferred to a 32×12 mm polypropylene autosampler vial (200 μl volume, Sun Brokers, Wilmington, NC, USA) and the vial was capped with a septum and cap.

The standards were prepared by mixing taurine and GABA. Standard concentrations of taurine ranged from 5.0 to 25.0 μM. A 100-μl aliquot in

duplicate was added to autosampler vials as described above. During the development of the assay, taurocholic acid standards were run through the entire procedure, by substituting 1 ml of the standard for the 1 ml of serum. This was done to verify the utility and reproducibility of the assay.

The autosampler was programmed to perform pre-column derivatization. To each sample was added 100 μl of an *o*-phthalicdicarboxaldehyde mixture (50 mg of *o*-phthalicdicarboxaldehyde, 1.25 ml of methanol, 11.25 ml of 0.4 *M* sodium borate, pH 9.5, and 50 μl of β-mercaptoethanol). The solution was mixed and allowed to react for 1 min, and then 100 μl were injected onto the chromatographic system.

2.3. Chromatography

The HPLC system consisted of two Model 110A pumps, a 3 μm Ultrasphere-XL ODS column (70× 4.6 mm I.D.) and guard column (5×4.6 mm I.D.; Beckman Instruments, Fullerton, CA, USA), a Gilson Spectra Glo Filter fluorometer (Gilson Medical Electronics, Middleton, WI, USA), a Kortec K65B automated sample injector (Rainin Instruments, Woburn, MA, USA) and a Maxima 820 chromatography workstation (software version 3.31; Millipore). The workstation utilized a Gateway 2000 386SX computer (Gateway 2000, N. Sioux City, SD, USA) and a Data Translation DT2805 data acquisition board (Data Translation, Marlborough, MA, USA) to control the gradient, acquire the data from the fluorescence detector and determine peak areas.

The two mobile phases were 0.1 *M* sodium acetate, pH 6.7 (reagent A) and methanol (reagent B). The gradient used for this assay was as follows: 0–0.5 min, linear increase from 16 to 20% methanol; 18.0–19.0 min, linear increase from 20 to 32% methanol; 26.0–30.0 min, linear increase from 32 to 39.5% methanol; 32.0–32.5 min, linear increase from 39.5 to 80% methanol; 40.0–40.5 min, linear decrease to 16% methanol. The column was re-equilibrated for 9.5 min prior to the next injection. The flow-rate was 1.6 ml/min. The chromatography was conducted at room temperature. The fluorometric detector was operated with an excitation filter of 330–380 nm and an emission filter of 430–600 nm.

2.4. Calculations

The quotient of taurine peak area divided by the GABA peak area for the blank was subtracted from the corresponding quotient for each sample. An amount of taurine-conjugated bile acids was then calculated from the standard curve. This amount was then divided by the percent recovery as determined from the dpm determined from the [^{14}C]glycocholic acid. This gave the total quantity of taurine-conjugated bile acids per unit volume of serum. This value was then divided by the total serum bile acids to give the percent of bile acids as taurine conjugates.

2.5. Evaluation of the amino acid removal step

Using pooled serum, we evaporated the 95% ethanol eluate from the Sep-Pak columns and resuspended the samples in 0.5 ml of 0.1 *M* sodium acetate, pH 6.7. The samples were processed for amino acid analysis as described above. The resultant chromatograms showed no peaks in the area where taurine would have been anticipated. We concluded that, for the purposes of this assay, the Sep-Pak columns were effective in removing serum free taurine.

3. Results and discussion

A chromatogram from one of the taurocholic acid standards showing glycine, taurine and GABA, the internal standard, is shown in Fig. 1A. A chromatogram from a sample run of human serum is shown in Fig. 1B. The glycine present in both chromatograms is derived from the [^{14}C]glycocholic acid internal standard. Standard curves from five succesive assays resulted in a linear relationship between concentration of taurine and the area ratio of taurine to GABA, with a mean r^2 for five analyses of 0.999. Slopes of these five replicate standard curves had a relative standard deviation (R.S.D.) of 4.7%. The R.S.D. of eight successive injections of a taurine standard was 4.5%. To verify that taurocholic acid was being hydrolyzed into free measurable taurine, standard curves were prepared from three successive assays using taurocholic acid. For these curves, the taurocholic acid standards (1.0, 2.5 and 5.0 μ*M*)

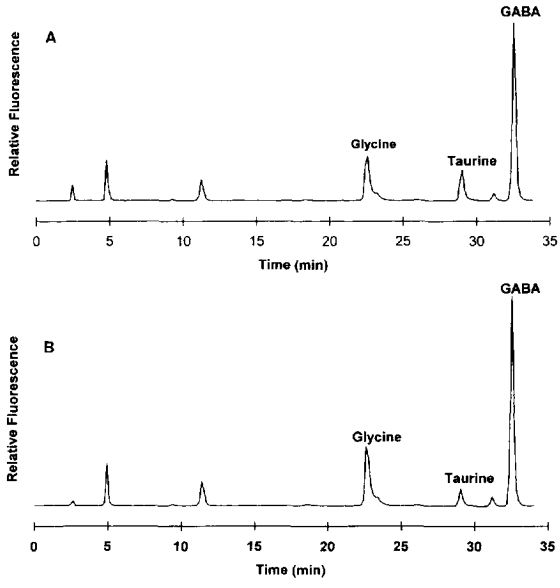

Fig. 1. Chromatograms of taurine-conjugated bile acid assay. (A) Standard, i.e., 5 μM taurocholic acid, (B) human serum. γ-Amino-*n*-butyric acid (GABA) is the internal standard. Chromatographic conditions: 3 μm Ultrasphere ODS column (75×4.6 mm I.D.); gradient elution, 0.1 *M* sodium acetate, pH 6.7, and methanol (gradient described in text); flow-rate, 1.6 ml/min. The fluorometric detector was operated with an excitation filter of 330–380 nm and an emission filter of 430–600 nm.

were processed similarly to the plasma samples. These curves showed that there was a linear relationship between concentration of taurocholic acid and the area ratio of taurine to GABA, with a mean r^2 of 0.994. Slopes of these three replicate standard curves had an R.S.D. of 2.0%.

The values for the taurocholic acid standards were also used to determine the effectiveness of the enzyme in hydrolyzing taurocholate to liberate free taurine. When compared to a standard curve of free taurine standards, the estimated concentrations from the 1.00, 2.50 and 5.00 μM taurocholic acid standards were 1.02±0.14, 2.50±0.27 and 5.10±0.80, respectively (mean±standard deviation). These values were based upon 5 analyses run on five separate days.

The assay was used on 20 critically injured trauma patients. The serum concentration of taurine-conjugated bile salts in these patients was 0.18±0.04 μM (mean±S.E.M.). These values in injured patients compared to reported values for normal subjects of 0.11 to 0.97 μM [5,7,9,12,13].

Since the hydrolase is virtually the last step (other than dehydration and reconstitution), there is only a minimal risk of loss of free taurine and glycine following their liberation by the hydrolase. Therefore, the measured specific activity of the internal standard from the final step of the deconjugation process, expressed as a percentage of the corresponding count from the initial step, should accurately reflect any loss of conjugated bile acids during the assay, as further loss beyond the hydrolase step is unlikely. This conclusion is substantiated by the close correlation between initial taurocholic acid standards and final post-deconjugation measurement of free taurine.

In response to the need for a reliable, economic assay of serum bile acid conjugation pattern, we have developed a technique which uses precolumn bile acid deconjugation with choloylglycine hydrolase. The C_{18} cartridges resulted in successful removal of free amino acids from the original samples, and [^{14}C]glycocholic acid proved to be a simple but reliable internal standard.

Acknowledgments

This work was supported by a grant from the Butterworth Foundation. The authors wish to thank Geoffrey Sandman and John Skinner for their technical assistance.

References

[1] P. Eneroth, J. Lipid Res., 4 (1963) 11.
[2] H. Jaeger, W. Nebelung, H. Klor and H. Ditschuneit, in H. Jaeger (Editor), Glass Capillary Chromatography in Clinical Medicine and Pharmacology, Marcel Dekker, New York, 1985, Ch. 13, p. 349.
[3] Y.A. Baquir, J. Murison, P.E. Ross and I.A.D. Bouchier, J. Clin. Pathol., 32 (1979) 560.
[4] J. Goto, H. Kato, Y. Saruta and T. Nambara, J. Liq. Chromatogr., 3 (1980) 991.
[5] J. Goto, M. Saito, T. Chikai, N. Goto and T. Nambara, J. Chromatogr., 276 (1983) 289.
[6] G. Wang, N.H. Stacey and J. Earl, Biomed. Chromatogr., 4 (1990) 136.

[7] J. Goto, Y. Saisho and T. Nambara, J. Chromatogr., 567 (1991) 343.
[8] S. Güldütuna, T. You, W. Kiyrts and U. Leuschner, Clin. Chim. Acta, 214 (1993) 195.
[9] S. Okuyama, N. Kokubun, S. Higashidate, H.D. Uemura and Y. Hirata, Chem. Lett., 12 (1979) 1443.
[10] S. Scalia and M. Guarneri, Il Farmaco, 45 (Supplement to No. 6) (1990) 797.
[11] B.N. Jones and J.P. Gilligan, J. Chromatogr., 266 (1983) 471.
[12] S. Baba, R. Uenoyama, K. Suminoe, F. Takeda, S. Hasegawa and Y. Kameno, Kobe J. Med. Sci., 26 (1980) 89.
[13] H. Okuda, H. Obata, T. Nakanishi, T. Hisamitsu, K. Matsubara and K. Watanabe, Hepato-gastroenterol., 31 (1984) 168.