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Research Paper

Analysis of cholyglycine acid as a biomarker for the early diagnosis of liver disease by fluorescence polarization immunoassay

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Abbreviations:
CG, Cholyglycine
FPIA, fluorescence polarization immunoassay
EMIT, enzyme multiplied immunoassay technique
HCC, hepatocellular carcinoma
EDF, fluoresceinthiocarbamyl ethylenediamine
FP, fluorescence polarization
LOD, limit of detection
CR, cross-reactivity
IQR, interquartile ranges
CHBV, chronic hepatitis B virus
LC, liver cirrhosis
DC, decompensated cirrhosis
HC, healthy control
ALT, alanine aminotransferase
AST, aspartate transaminase
AFP, alpha fetoprotein

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ABSTRACT

Trace concentrations of cholyglycine acid (CG) are useful to reflect the damage degree in liver cells, and useful in the diagnosis and prognosis of liver diseases. Therefore, developing an effective strategy to detect the concentration of CG is of great importance. A homogeneous fluorescence polarization immunoassay (FPIA) was developed for determination of CG concentrations. The effects of tracer concentration, antibody dilution and incubation time on FPIA performance were studied. The FPIA was used to detect CG in sera from 522 patients with liver diseases and 449 healthy controls. The results were compared with those obtained through enzyme multiplied immunoassay technique (EMIT). The FPIA showed a detection limit of 50.9 ng/mL for CG and the cross-reactivity of antibodies with nine structurally and functionally related analogs were negligible. Concentrations of CG in patients with chronic hepatitis B virus, liver cirrhosis and decompensated cirrhosis were 5.4, 7.9 and 6.5-fold higher than those in controls, respectively, and hence were significantly different from controls ($P < 0.001$). The FPIA developed in this study is a rapid, convenient, and simple method, suitable to be used as a screening tool for homogeneous detection of CG in serum. The detection results of FPIA demonstrate that concentrations of CG were much higher in liver patients than in controls. Therefore, CG may be a good biomarker for the diagnosis of liver diseases.

1. Introduction

Liver diseases that endanger human health are one of the biggest killers. It is reported that there are approximately 400 million patients with chronic hepatitis B and approximately 200 million with chronic hepatitis C viruses [1,2]. Epidemiological studies have shown that most cases of hepatocellular carcinoma are associated with chronic infection of hepatitis B virus or hepatitis C virus [3], and chronic infection of hepatitis B virus is the main cause of liver cancer [4]. Chronic hepatitis B induces regular activation of the host immune system to defend virus-infected liver cells [5]. This activation process can-not remove the virus alone, but usually develops into liver fibrosis, cirrhosis, and even hepatocellular carcinoma

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[6,7]. Studies have shown that hepatitis B virus proto-oncogene can directly or indirectly affect the occurrence of hepatocellular carcinoma [8–11]. Over the course of liver diseases, protein changes in the body are still a new area of urgency to be studied [12]. Alcoholic liver disease in the United States is the leading cause of liver transplantation [13–15]. Hepatocellular carcinoma is the third leading cause of human death, a serious threat as one of the most malignant tumors [16]. In recent years, with the use of histology and proteomics, the prognosis determination of patients with liver cancer from the molecular level, liver cancer etiology, pathogenesis, biological characteristics and new treatment methods have become the hot topics of oncology currently [17–20]. The studies have confirmed that up regulation of CG is associated with hepatocellular carcinoma (HCC) [21]. Therefore, accurate and quantitative detection of CG in patients with suspected liver diseases is greatly needed.

Cholyglycine acid (CG), a combination of bile acid and glycine, is one of the main components of bile acids. The studies have shown that the inhibitory effects of cholic acid on chronic and acute inflammation and the inhibition of arachidonic acid metabolism are an important mechanism behind the anti-inflammatory effects of CG [22,23]. CG concentrations are usually abnormal in most hepatobiliary diseases, such as viral hepatitis, alcoholic liver disease, cirrhosis, and pediatric liver diseases [24,25]. The measurement of postprandial CG by radioimmunoassay is a very sensitive test for the detection of hepatic alterations [26,27] and has been proposed for early detection of hepatic injury from industrial products and drugs [28]. A study performed by Manners confirmed that of the relevant indicators, CG is the best prognostic indicator of cirrhosis [29]. Ferracis reported that CG is superior to conventional liver function test in the diagnosis of severe liver diseases and plays a decisive role in clinical practice. Jara reported that CG is also important for predicting the recurrence or prognosis of hepatitis [30].

Fluorescence Polarization Immunoassay (FPIA), being popular for detecting small molecules, is based on the following principle [31–33]. The fluorophore-labeled antigen conjugate has a low FP value. When it is combined with the antibody in the system, it has a high FP value. Because the labeled antigen and native antigen in the sample have the same binding sites of the antibody, they have a competitive binding for the antibody. With the increase of native antigen in the sample concentration, the binding of the native antigen and the antibody increases, thereby reducing FP value of the system. Based on that, a FPIA method was developed to detect CG serum concentrations of liver patients, the results show that CG is a good biomarker for the diagnosis and prognosis of liver diseases.

2. Experiment

2.1. Reagents and apparatus

Cholic acid, isobutyl chlorocarbonate, triethylamine, tetrahydrofuran (THF), N-hydroxysuccinimide (NHS), dicyclohexyl carbodiimide (DCC) were obtained form Aladdin Co. Ltd. (Shanghai,China). Fluorescence isothiocyanate (FITC) fluorescein was purchased from Invitrogen. Freund's complete adjuvant, incomplete adjuvant and bovine serum albumin (BSA) were acquired from Sigma (St. Louis, MO, USA). N,N-Dimethylformamide (DMF) was obtained from Damao Reagent Company in Tianjin. Methanol was purchased from Merck KGaA (Darmstadt,Germany). In this work, all chemicals were of analytical reagent grade and ultrapure water was used throughout the experiments. CG and polyclonal antibody against CG were prepared. Phosphate buffer saline (PBS): 0.2 g of $NaH_2PO_4{\cdot}2H_2O$, 2.9 g of $Na_2HPO_4{\cdot}12H_2O$, 8.0 g of NaCl in 1 L H_2O, pH = 7.4.

The FPIA analyses were performed on a FPIA Sentry 200 by Ellie (Diachemix, USA) with $10 \times 75\,mm^2$ glass culture tubes. Clinical data were acquired by an automatic biochemical analyzer 7180 (Hitachi, Japan). Nuclear magnetic resonance (NMR) spectra were obtained with a DRX-400 NMR spectrometer (Bruker, Germany-Switzerland).

2.2. Synthesis of immunogen

First, to obtain a complete immunogen for CG, the cholyglycine structure was modified to contain an additional carbon chain in a one-step chemical reaction. Briefly, cholic acid (3.67 mmol) was added to a stirred mixture of tetrahydrofuran (3 mL), triethylamine (509 μL) and isobutyl chlorocarbonate (464 μL). Then, by stirring for 1 h at room temperature (RT), the mixture was clarified, and 379 mg 4-aminobutyric acid (Aladdin Co. Ltd) was added to the reaction mixture. After stirring for 48 h at RT, a small amount of water was added and the solvent was removed under reduced pressure. The extract was adjusted to pH = 2 and added with ethyl acetate. The upper solution was taken and the solvent was removed under reduced pressure. The residue was subjected to silica gel (100–200 mesh) column chromatography resulting in 1.05 g of hapten production. The structure of hapten was confirmed by ^{1}H NMR. ^{1}H NMR (400 MHz, Pyr) δ 4.20 (s, 1H), 4.06 (s, 1H), 3.73 (s, 1H), 3.59 (d, J = 4.7 Hz, 2H), 3.09 (dd, J = 24.9, 12.6 Hz, 1H), 2.88 (t, J = 10.4 Hz, 1H), 2.70 (dd, J = 18.7, 11.5 Hz, 1H), 2.57 (d, J = 20.7 Hz, 2H), 2.49 (d, J = 9.3 Hz, 1H), 2.44–2.34 (m, 1H), 2.33–2.25 (m, 1H), 2.10 (d, J = 12.7 Hz, 5H), 2.00 (d, J = 10.8 Hz, 1H), 1.95–1.72 (m, 7H), 1.60 (d, J = 12.0 Hz, 4H), 1.48 (d, J = 12.8 Hz, 1H), 1.36–1.28 (m, 1H), 1.19 (d, J = 5.2 Hz, 4H), 1.04 (t, J = 11.2 Hz, 1H), 0.97 (s, 3H), 0.78 (s, 3H).

The immunogen was prepared as follows: 10 mg hapten (0.02 mmol), 4.7 μL tributylamine and 3.8 μL isobutyl chloroformate were dissolved in 0.3 mL N,N-dimethyl formamide (Damao Reagent Corp.), the solution was added dropwise to a stirred solution containing 2 mL of 0.1 M (pH 9.6) carbonate buffer, 0.4 mL N,N-dimethyl formamide and 20 mg bovine serum albumin (BSA). The mixture was stirred at 4 °C overnight. Then, the reaction mixture was dialyzed by stirring against PBS (0.01 M pH 7.4) at 4 °C. The reaction proceeded for three days to remove all excess impurities. The purified product was lyophilized and stored at −20 °C.

2.3. Production of polyclonal antibodies

The polyclonal antibodies were obtained through immunization of two female New Zealand white rabbits (Guangdong Medical Laboratory Animal Center) weighing 2000 ± 500 g. The initial immunization injected was 0.5 mg of immunogen in 1 mL of PBS emulsified with 1 mL of Freund's complete adjuvant (Sigma). Subsequent injections containing 0.5 mg of conjugate in 1 mL PBS plus 1 mL of Freund's incomplete adjuvant were performed at 15-day intervals. Ten days after the fifth immunization, blood was collected from the ears of each rabbit and centrifuged. At last, the antiserum was obtained and stored at −20 °C until use.

2.4. Synthesis of fluorescein-labeled cholyglycine tracers

Fluoresceinthiocarbamyl ethylenediamine (EDF) was synthesized [34]. A total of 29 mg FITC (Invitrogen) was dissolved in a mixture of 2.5 mL methanol and 25 μL triethylamine. The solution was added dropwise by stirring to a solution of 50 mg ethylenediamine dihydrochloride, 13 mL methanol and 0.13 mL triethylamine. The reaction mixture was stirred in the dark at room temperature (RT) for 2 h, then kept overnight at RT in the dark without stirring. The orange precipitate, fluoresceinthiocarbamyl ethylenediamine (EDF), was filtered and washed with 5 mL methanol and air dried at RT in the dark.

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