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Glycyrrhizin and glycyrrhetinic acid determination from formalin-fixed tissue

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Summary. Glycyrrhetinic acid (GA), the main metabolic product of glycyrrhizin (GLY), could be detected in formalin-fixed tissue from a man who died 6 hours after therapeutic administration of a GLY-containing agent. GA was extracted from homogenized formalin-fixed liver tissue and 3 ng GA/g could be detected by HPLC. The extraction from formalin-fixed liver tissue gave the same retention time peak as the GLY control. GA could also be detected by mass spectrometry in the blood sample. This confirms that the man had received a GLY-containing agent for therapeutic use prior to his death and that GA can be determined from formalin-fixed tissue.

Key words: Glycyrrhizin – Glycyrrhetinic acid – Formalin-fixed tissue

Zusammenfassung. Glycyrrhizin-Säure (GA), der Hauptmetabolit von Glycyrrhizin (GLY), konnte im formalinfixierten Gewebe eines Mannes nachgewiesen werden, der 6 Stunden nach therapeutischer Verabreichung eines Glycyrrhizin-haltigen Medikaments verstarb. Glycyrrhizin-Säure wurde aus homogenisiertem, formalinfixiertem Lebergewebe extrahiert und 3 Nanogramm Glycyrrhizin-Säure pro Gramm konnten mit Hilfe von HPLC nachgewiesen werden. Die Extraktion von formalinfixiertem Lebergewebe ergab dieselben Retentionszeiten wie die Glycyrrhizin-Kontrolle. Glycyrrhizin-Säure konnte auch mit Hilfe der Massenspektrometrie in der Blutprobe nachgewiesen werden. Dieser Befund bestätigt, daß der Mann ein Glycyrrizin-haltiges Medikament für den therapeutischen Gebrauch vor seinem Tod erhalten hatte und daß Glycyrrhizin-Säure aus formalinfixiertem Gewebe nachgewiesen werden kann.

Schlüsselwörter: Glycyrrhizin – Glycyrrhizin-Säure – formalinfixiertes Gewebe

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Introduction

Glycyrrhizin (GLY) is a natural sweetener contained in plants belonging to the genus *glycyrrhiza,* such as *glycyrrhiza uralesis Fisher* and *glycyrrhiza glabra L.* This substance is also known to be an effective pharmaceutical compound, possessing various pharmacological effects such as anti-allergic and anti-inflammatory properties and is used in the treatment of hepatodisease (Japanese Pharmacopeia IX 1976). Structurally, GLY consists of one molecule of GA as aglycone and two molecules of glucuronic acid. GLY is broken down by beta-glucuronidase into one molecule of GA and two molecules of glucuronic acid mainly in the liver (Fig. 1).

GLY and GA can be extracted and detected from fresh organs. Nakano et al. (1980) performed pharmacokinetic studies on GLY and GA in serum and reported that the peak level of GLY was approximately 10–15 ng/ml, which appeared immediately after an intravenous administration of 80 mg GLY and then declined rapidly. GA appeared in the blood 6 hours after administration and reached its peak level of 120 ng/ml after 24 hours.

It is sometimes necessary to detect drugs and poisons retrospectively in formalin-fixed tissues. Several investigators have reported that they could detect poisons (Curry 1988, Kuo and Kuo 1988, Minakata et al. 1989, Tomono et al. 1989) in such tissue but there have been no reports concerning the detection of GLY and GA in formalin-fixed tissue.

In this paper, we describe a method to determine GLY and GA in formalin-fixed tissue from the cadaver of a man who was known to have received a GLY-containing agent for therapeutic purposes prior to his death.

Case report

A 62-year-old man suffering from shock died 6 hours after being administered 40 mg of a GLY-containing agent for treatment of urticaria. The autopsy was performed 3 days after death; the liver was dissected and immediately preserved in neutral 10% formalin solution and was stored for one year. Whole blood was also collected from the right atrium and stored at −20°C.

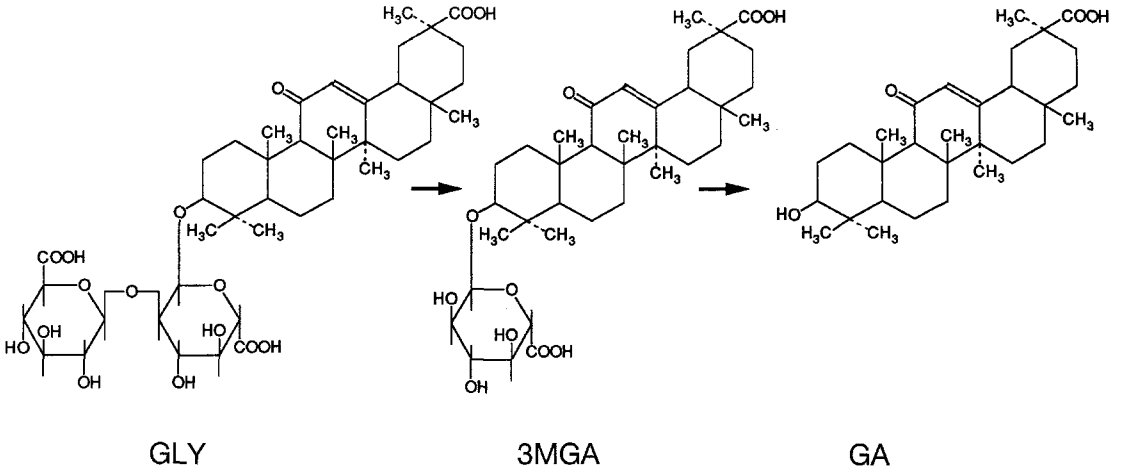

Fig. 1. Metabolic pathway to glycyrrhizin metabolites. Abbreviations: *GLY*, glycyrrhizin, *GA*, glychyrrhetinic acid, *3-MGA*, 3-monoglucuronyl glycyrrhetinic acid

Materials and methods

Reagents. GLY (reagent for the herbal medicinal test) and GA (reagent for the food additive test) were purchased from Wako Pure Chemicals (Osaka, Japan). Acetonitrile used for HPLC and of HPLC grade has purchased from Nacalaitesque (Kyoto, Japan). All other reagents used were analytical grade.

GLY extraction procedure for formalin-fixed tissue. Formalin-fixed tissue was finely minced with 2 vol. saline and homogenized with a polytron and teflon glass homogenizer. Equal volumes of 0.5 N NH_3 in 85% ethanol and indomethacin (IMC, 1 µg) as an internal standard were added to the homogenate, which was stirred for 15 min and then filtered. After evaporation, the residue was dissolved in 20 ml 1N HCl and extracted 3 times with chloroform. After evaporation of the chloroform layer, the residue was dissolved in distilled water and passed through a Sep-pak C_{18} cartridge (Waters Associates, Milford, MA) pre-activated according to the manufactures instructions. The cartridge was washed with 20 ml distilled water and the adsorptive component was eluted with 20 ml ethanol. After evaporating the eluate, the residue was dissolved in 2 ml ethanol and 50 µl aliquots of this solution were used for the HPLC analysis after being filtered through a 0.45 µm teflon filter.

HPLC analysis for GLY. HPLC analysis was performed with a Shimadzu LC-5A model. The 15 cm stainless steel tube column with a 6 mm inside diameter (i.d.) was packed with Shim-pack CLC-ODS (octadesyl silica) and the column temperature was 40°C. The mobile phase was 99% acetonitrile:1% acetic acid (40:60) and the flow rate was 1.0 ml/min. GLY was detected by a Shimadzu SPD-2A UV detector adjusted to a wavelength of 254 nm and attenuation was 0.16. The GLY concentration was obtained by calculating the ratio of peak area to 1 µg of IMC (I.S.) and relating this to a previously constructed calibration curve using a Shimadzu chromatopac C-R3A. This was corrected using the recovery ratio 0.685, obtained when GLY was added to the normal formalin-fixed liver homogenate and then analysed.

GA extraction procedure for formalin-fixed tissue. The extraction method was based on the method of Gonmori et al. (1988) with minor modifications. Formalin-fixed tissue was finely minced with the addition of 2 vol. saline and homogenized with a polytron and teflon glass homogenizer. An equal volume of methanol was added to the homogenate, which was stirred for 15 min and then filtered through filter paper. After evaporating the extract, the residue was dissolved in 20 ml of saturated tartaric acid and passed through a Sep-pak C_{18} cartridge. The cartridge was washed with 20 ml distilled water and the adsorptive component was eluted with 20 ml chloroform. After evaporating the eluate, the residue was dissolved in 1 ml methanol and 20 µl aliquots of this solution were used for the HPLC analysis after being filtered through a 0.45 µm teflon filter.

HPLC analysis for GA. HPLC analysis for GA was performed using the same system as GLY except as follows: the mobile phase was 99% acetonitrile:10 mM sodium phosphate buffer (pH 2.6) (65:35). The flow rate was 0.8 ml/min. GA was detected by a Shimadzu SPD-2A UV detector adjusted to a wavelength of 250 nm and attenuation was 0.04. The GA content was calculated by comparing the peak area on the chromatogram with that of the GA standard using a Shimadzu chromatopac C-R3A and corrected by the factor 0.657, a recovery rate, obtained when GA was added to the normal formalin-fixed liver homogenate and then analysed.

Extraction procedure for frozen whole blood. After thawing, 10 ml blood was added to an equal volume of distilled water and extracted with the Sep-pak C_{18} cartridge. The cartridge was washed with 20 ml distilled water and the adsorptive component was eluted with 20 ml chloroform. After evaporating the eluate, the residue was dissolved in the same volume of methanol. The methanol was evaporated and the residue dissolved in 0.5 ml methanol and 20 µl aliquots of this solution were used for the HPLC separation after being filtered through a 0.45 µm teflon filter.

Mass spectrometry (MS) analysis for GA. The GA fraction separated by HPLC was confirmed by MS analysis. HPLC separation was repeated and the HPLC/GA fractions were collected. The pooled sample was evaporated and dissolved in 500 µl methanol. An aliquot (2 µl) was analysed by the direct inlet-electron impact ionization method using a Shimadzu GCMS-QP1000. The acceleration temperature was 5°C/min up to 300°C, and the separator and ion source temperature were 280°C and 300°C, respectively. The ionization voltage was 70 eV. The scan range for the mass spectrum was set at m/z 100–650.

Results and discussion

GA was detected in blood by GC/MS confirming the administration of a GLY-containing agent to the victim. Figure 2 shows the mass spectrum of the GA control at 118°C using the direct inlet method with electronimpact (EI) ionization. The spectrum of the GA standard (2 ng/

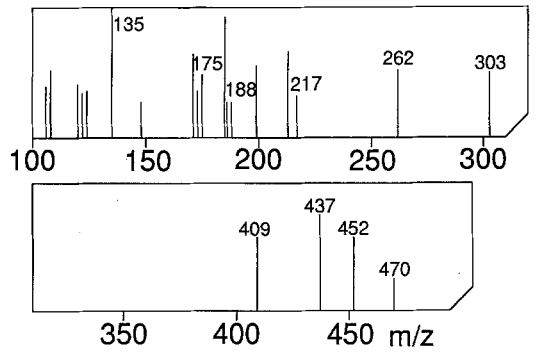

Fig. 2. Mass spectrum of glycyrrhetinic acid *(GA)* control at 118°C using the direct inlet method with an accelerated temperature

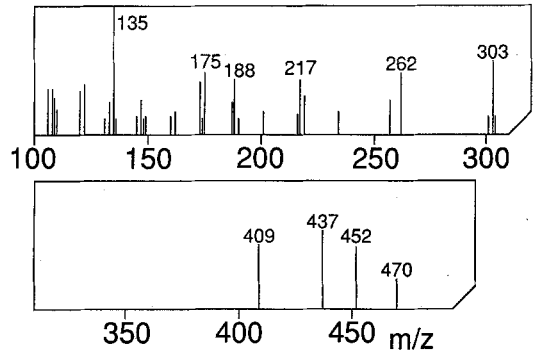

Fig. 3. Mass spectrum of the HPLC separated fraction of glycyrrhetinic acid *(GA)*

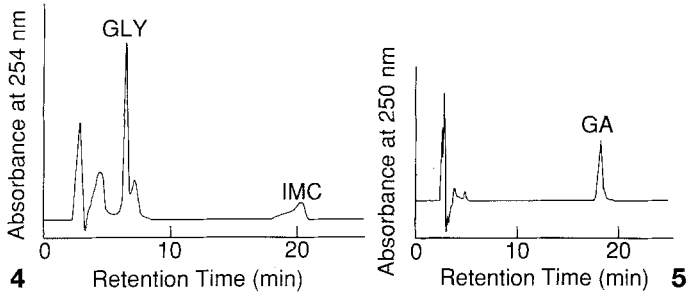

Fig. 4. HPLC profiles of glycyrrhizin control. Abbreviations: *GLY*, glycyrrhizin, *IMC*, indomethacin

Fig. 5. HPLC profiles of glycyrrhetinic acid (*GA*) control

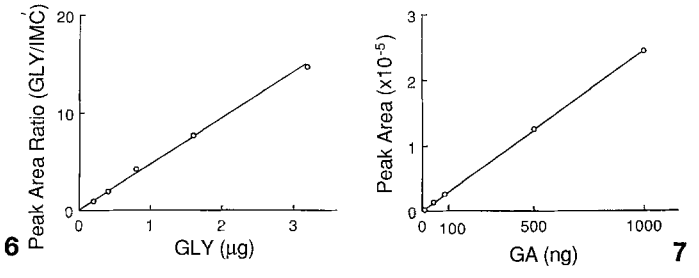

Fig. 6. Calibration curve of GLY. Abbreviations: *GLY*, glycyrrhizin, *IMC*, indomethacin

Fig. 7. Calibration curve of glycyrrhetinic acid (*GA*)

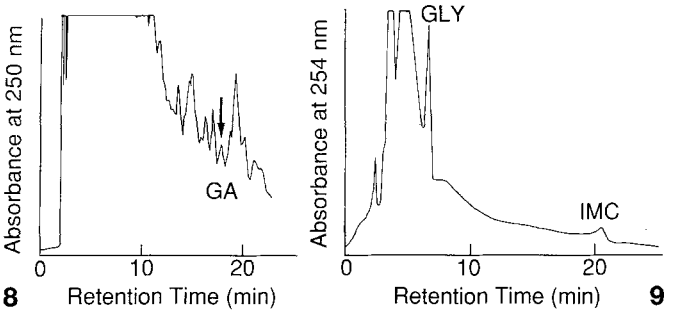

Fig. 8.. HPLC profiles for glycyrrhetinic acid (*GA*) after methanol extraction from formalin-fixed liver

Fig. 9. HPLC profiles for *GLY* after ethanol extraction for *GLY* from formalin-fixed liver. Abbreviations: *GLY*, glycyrrhizin, *IMC*, indomethacin

Table 1. Electron impact ionization data by mass spectrometry of glycyrrhetinic acid (*GA*)

m/z	Relative intensity (%)
470	22.6
452	46.4
437	59.0
409	50.3
303	58.5
262	42.1
217	42.4
188	26.8
175	50.5
135	100.0

μl) can be seen in Table 1 where the relative intensities of the fragment ions are calculated using m/z 135 as 100%. A molecular peak at m/z 470 for GA and 10 other fragment ions were observed. The pooled HPLC GA fraction obtained from blood gave the same spectrum and relative intensities (Fig. 3) as the GA control (Fig. 2).

The conditions for HPLC analysis were also examined. Figures 4 and 5 show the HPLC profiles of GLY and GA controls, respectively. The GLY and IMC peaks appeared 6 min and 20 min respectively, after the injection under the conditions described (Fig. 4). The GA peak appeared 18 min after injection under the other HPLC conditions (Fig. 5).

The quantification limit of GLY was 10 ng and the calibration curve obtained in the range from 200 ng to 3.2 μg by plotting the peak area ratios of GLY to IMC was a straight line through the origin (Fig. 6). The quantification limit of GA was 10 ng and the graph obtained in the range from 10 ng to 1 μg by plotting the peak area was a straight line through the origin (Fig. 7).

Furthermore, GLY and GA recoveries were measured using these extraction methods in formalin-fixed liver tissue from a control patient. When GLY was added to the normal formalin-fixed liver homogenate and then analysed, a 68.5% recovery rate was obtained and for GA, 65.7%. These recovery rates are lower than those from fresh organs. Many investigators have also report-

ed that lower concentrations of drugs and poisons were determined in formalin-fixed tissue or in formalin solution than in fresh organs (Kuo and Kuo 1988; Minakata et al. 1989; Tomono et al. 1989; Tsoukali-Papadopoulou 1987; Winek et al. 1990). This could be due to residual formalin in the homogenate resulting in decreased GLY and GA concentrations in the formalin-fixed liver.

Tsoukali-Papadopoulou (1987) reported that phenobarbital could be detected in formalin solution by permeation of the hypnotic into the solution, however phenobarbital was not detected in formalin-fixed brain tissue. Thus during the period of tissue preservation, phenobarbital in the tissue is thought to be eluted into the formalin solution. It was impossible to determine GA in neutral 10% formalin solution because it contained large amounts of contamination (data not shown). However, it might be that GA in the tissue had not been eluted into the formalin solution during the period of tissue preservation.

The GA concentration was estimated to be 3 ng/g wet weight in the formalin-fixed liver which had been preserved in neutral 10% formalin solution for one year (Fig. 8). The victim had died 6 h after a GLY-containing agent was administered intravenously. The peak GLY concentration in serum was found to be as low as 10–15 ng/ml immediately after intravenous administration of 80 mg GLY. GA appeared in the serum 6 h after administration (Nakano et al. 1980) and could, therefore, be considered acceptable. GLY was estimated to be 708 ng/g wet weight by quantification analysis using a Shimadzu chromatopac C-R3A (Fig. 9). However, this level was less than 1 ng in serum 6 h after intravenous administration as reported by Nakano et al. (1980) and might be a compound possessing the same retention time as that of GLY in the present HPLC system. Kanaoka et al. (1986) reported that GLY was broken down into 3-monoglucuronyl glycyrrhetinic acid (3-MGA) and glucuronic acid and 3-MGA further broken down to GA. Structurally, GLY is diglucuronyl GA (Fig. 1). Consequently, it was considered that the 3-MGA peak appeared close to the GLY peak or it might be possible that both peaks could not be separated using the present HPLC system.

It is not clear whether the GLY and GA levels obtained in this study were equivalent to the levels in the liver before formalin fixation, because they had been preserved in 10% formalin for one year. Consequently, GLY and GA levels obtained in this study may be lower than those of GLY and GA in the liver before formalin fixation. Kuo and Kuo (1988) reported an extraction method for paraquat with a far greater recovery rate from formalin-fixed tissue. Using the superior extraction method described in this study, it may be possible to determine GLY and GA in much smaller samples.

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