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## Short Communication

# Determination of glycyrrhetinic acid in human plasma by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method has been developed for measuring 18$\beta$-glycyrrhetinic acid (GRA) in human plasma in the range of 0.1–3 $\mu$g/ml. The acetate ester of GRA is added to the plasma as an internal standard, plasma proteins are denatured with urea to release GRA, and the GRA and the internal standard are extracted in an ion-pairing solid-phase extraction process. An isocratic, reversed-phase HPLC separation is used, followed by ultraviolet absorbance detection at 248 nm. The results from the analysis of five GRA-fortified plasma pools show a mean relative standard deviation of 7% and are accurate to within 10%. With evaporative concentration of the extract, the limit of detection for GRA in plasma is approximately 10 ng/ml.

## INTRODUCTION

Interest in investigating the cancer chemopreventive properties of herb-derived substances such as licorice root extracts of 18$\beta$-glycyrrhetinic acid (GRA; see Fig. 1) has created a need for simple, sensitive, and accurate methods for the determination of GRA in human plasma. GRA, the main metabolite of the glucoside glycyrrhizin, is obtained from licorice root, *Glycyrrhiza glabra*, and is believed to act as an anti-tumor agent for humans [1–3].

Only a limited number of methods for quantitative determination of GRA in human plasma have been published [4–9]. Recently, Yasuda *et al.* [5] described the use of ammoniacal ethanol to precipitate the proteins, followed by extraction of GRA with methylene chloride. This extraction procedure gives good recovery of GRA, approximately 90%. However, the procedure requires a solvent exchange on the extract to make it compatible with reversed-phase chromatogra-

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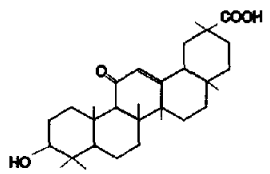
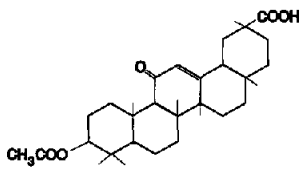



**Glycyrrhetinic acid**




**Glycyrrhetinic acid acetate**

Fig. 1. Structures of 18β-glycyrrhetinic acid and the acetate ester of glycyrrhetinic acid.

phy. De Groot *et al.* [7] have reported results using a multi-dimensional ion-pairing–reversed-phase chromatography scheme which requires a sophisticated valving arrangement for column switching. Most recently, Newman and Welch [8] have reported the development of a chromatographic method for determining plasma levels of GRA that uses a mixture of sodium bisulfate and sodium chloride to precipitate proteins, and acetonitrile to extract GRA. They report accurate and precise measurement capabilities, though the limit of detection is about an order of magnitude greater than the method we report here. Takeda *et al.* [9] have developed a reversed phase high-performance liquid chromatographic (HPLC) method that is useful in the evaluation of the pharmacokinetic profile of GRA in human serum following the oral administration of Kampo medicines containing glycyrrhizin. Their method provides a limit of detection and measurement capabilities that are comparable to those reported in our method. Other reports provided information on only qualitative measurement of GRA [10–13].

In this paper, we describe an HPLC method for the determination of GRA in human plasma. Denaturation of the plasma proteins with urea renders the GRA and the internal standard readily extractable from the matrix by ion-pairing solid-phase extraction (SPE). Analysis of the extract is by isocratic reversed-phase separation with ultraviolet (UV) absorbance detection at 248 nm. Quantitation is accomplished through use of an internal standard, the acetate ester of GRA (see Fig. 1), which is not commercially available, but can be easily prepared. This method allows reliable measurements of GRA in human plasma with a detection limit of 10 ng/ml and with reliable quantitative measurements being possible at greater than or equal to 100 ng/ml.

## EXPERIMENTAL[a]

### *Standards and materials*

Standard solutions with concentrations ranging from 0.1 to 10 μg/ml GRA (Sigma, St. Louis, MO, USA) and from 3 to 7 μg/ml GRA acetate were prepared (synthesis described below) in methanol and in plasma. Working standards were prepared from the methanol solutions for use in calibration and were run prior to sample analysis to determine retention times and detector response factors. The purities of the individual reference compounds were determined by direct injection of the respective reference solutions into the chromatograph, using the HPLC separation described below.

Tetrabutylammonium phosphate (TBAP; Waters Assoc., Milford, MA, USA) was used as the ion-pairing reagent. SPE cartridges ($C_{18}$ Sep-Pak, Waters Assoc.) were used for extraction. The heparin-treated plasma used for this work was a commercial product that had been tested for hepatitis-B and HIV virus.

### *Synthesis of GRA acetate*

GRA acetate (internal standard) was prepared by mixing 2 g of GRA, 15 ml of acetic anhydride, and 30 ml of pyridine. The mixture was allowed to react overnight and was then poured into cold water. The GRA acetate precipitated, was removed by filtration, and was then further purified by recrystallization from *n*-propanol.

### *Sample extraction*

To 1 ml of plasma, 0.5 g of urea, 3 μg of the internal standard in methanol, and 200 μl of 0.5 *M* TBAP in water were added. The solution was vortex-mixed for 1 min prior to extraction to ensure proper mixing. The pH of the resulting solution was about 10.

A $C_{18}$ SPE cartridge, conditioned sequentially with 3 ml each of methanol, water, and 0.5 m*M* TBAP in water, was used to extract the GRA from the plasma matrix. The 1-ml plasma sample was passed through the cartridge at a rate of about 1 ml/min. After washing the cartridge with 3 ml of 5 m*M* TBAP in water and 3 ml of 45% methanol in water to remove unwanted constituents, the GRA and internal standard were eluted with 2 ml of methanol. This eluate was concentrated under a stream of nitrogen to 1 ml or less, depending on the sensitivity required.

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[a] Certain commercial products are identified to specify adequately the experimental procedure. Such identification does not imply endorsement or recommendation by the National Institute of Standards and Technology, nor does it imply that the materials identified are necessarily the best available for the purpose.

*HPLC apparatus and conditions*

The chromatograph used in this work consisted of a single-piston solvent delivery system, a variable wavelength UV–VIS detector, a data system, and an autosampler. The 5-$\mu$m (30-nm-pore) polymerically bonded octadecylsilane column (25 cm × 0.45 cm I.D., Vydac TP) was protected with a direct-connect pre-column that was packed with 10-$\mu$m $C_{18}$ particles of similar type. Both columns were obtained from The Separations Group (Hesperia, CA, USA). Column temperature was 28°C. Injection volume was 30 $\mu$l. The mobile phase was methanol–water–acetic acid (75:24.7:0.3, v/v), at a flow-rate of 2 ml/min. UV absorbance detection at 248 nm, the absorption maximum of GRA, was used for monitoring the effluent. Analyte concentrations were calculated using peak-area ratios of GRA and internal standard.

## RESULTS AND DISCUSSION

In the development of this method, preliminary studies were made to identify an appropriate internal standard, to investigate the stability of GRA in various solvents under feasible storage conditions, to optimize the extraction of GRA from the plasma matrix, and to develop a chromatographic system for separating GRA and GRA acetate and matric interferences.

*Internal standard*

The required properties of an internal standard for this analysis are that it be a sufficiently close homologue of the analyte so that it would mimic the extractability of the analyte from the matrix and that it would be readily separable from the analyte by HPLC. The acetate ester of GRA meets these requirements, and, although not commercially available, is easily prepared with common reagents, as described earlier.

*Stability of GRA standard solutions*

To verify the stability of GRA in various solvents, solutions at approximate concentrations of 10 $\mu$g/ml were prepared in methanol, acetonitrile, and ethanol, both with and without the addition of 2,6-di-*tert.*-butyl-4- methylphenol (BHT) as an antioxidant. These were stored at room temperature in glass-stoppered flasks wrapped in foil to exclude light. The UV absorbance at 248 nm was monitored for three weeks and no significant changes were observed in solutions with or without BHT. No extraneous peaks appeared upon HPLC analysis of aliquots of these solutions, further confirming that no degradation occurred. Repeated analyses of plasma-based calibrants kept frozen at −70°C showed these materials to be stable over a four-month period.

*Extraction of GRA from plasma*

It is difficult to quantitatively extract GRA from the plasma matrix because of

its polar nature and its affinity for plasma constituents. In evaluating extraction alternatives, acidic (pH 3) and basic (pH 10) aqueous solutions of GRA were extracted with butanol, methylene chloride, and hexane. Butanol and methylene chloride were effective extractants, but hexane was not, suggesting that SPE using *n*-alkane bonded phases would require ion-pairing agents to be effective.

We found that ion-pairing with $C_{18}$ SPE quantitatively extracted GRA from aqueous solutions, while recoveries from plasma were only 60–70%. This indicated that the loss was not due to solubility considerations, but due to the binding of GRA to plasma constituents. When urea was used to denature the proteins, recoveries of both GRA and GRA acetate were >90%. Both acid and base hydrolyses were also investigated as means for denaturing plasma proteins. GRA appeared to be destroyed by acid at the required temperature (120°C) and only approximately 10% of the GRA was recovered after treatment with base.

To obtain quantitative recovery in the SPE extraction, it was necessary to pass the pre-treated plasma slowly through the cartridge and to prevent intrusion of air bubbles into the cartridge. A flow-rate of 1–2 ml/min was suitable. An intermediate wash with 45% methanol in water was employed to elute interfering components from the plasma while retaining GRA and GRA acetate in the cartridge. The use of higher methanol concentrations, *e.g.* 50%, results in significant loss of GRA.

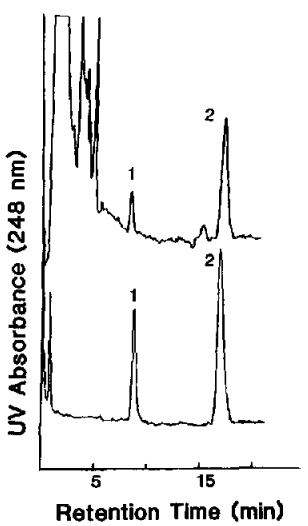


Fig. 2. Lower trace: HPLC from the analysis of a standard solution of (1) 1 μg/ml GRA and (2) 3.4 μg/ml GRA acetate using methanol–water–acetic acid (75:24.7:0.3, v/v) at a flow-rate of 2 ml/min on a $C_{18}$ column with UV detection at 248 nm. Responses shown represent 30 ng GRA and 90 ng GRA acetate. Upper trace: HPLC from the analysis of plasma extract using solid-phase extraction: (1) 0.38 μg/ml GRA and (2) 2.9 μg/ml GRA acetate.

*Chromatographic separation*

A chromatographic separation that resolves GRA and the internal standard from one another, and from potentially interfering plasma components, is easily achieved through use of a mobile phase consisting of methanol–water–acetic acid (75:24.7:0.3, v/v). Acetic acid is added only to effect ionization suppression. Though we used a polymerically bonded $C_{18}$ column [14,15], monomerically bonded $C_{18}$ columns [15] would probably serve as well with suitable adjustments in the mobile phase. Chromatograms of a plasma extract and a GRA solution in methanol using the SPE–HPLC protocol is illustrated in Fig. 2. The elution time of GRA is 8.5 min and of the internal standard is 16.7 min.

*Quantification of GRA*

Calibration curves were established from both plasma and methanol solutions over the range 0.1–3 μg/ml. Linear relationships between concentration and the ratio of peak areas for GRA and the internal standard were obtained for both plasma- and solution-based standards. The co-linearity of these calibration curves demonstrates that the method is free of matrix effects. Thus, for convenience, we used solution-based standards for routine calibration.

Plasma samples that contained GRA at concentrations ranging from 0.1 to 3 μg/ml were analyzed using the SPE–HPLC procedure. At least two aliquots from each plasma sample were extracted, and duplicate HPLC injections were made from each extract.

The results of this study are shown in Table I. Within-day precision is good, with relative standard deviations ranging from 4 to 7%. No significant between-day variability was observed. Agreement with the known values is within 10%. The limit of detection of GRA in plasma using our method is about 10 ng/ml, which corresponds to a limit of quantification of approximately 100 ng/ml.

The procedure we have described has been successfully implemented for our

TABLE I

HPLC MEASUREMENTS OF 18β-GLYCYRRHETINIC ACID IN PLASMA AFTER ION-PAIRING SOLID-PHASE EXTRACTION

Two aliquots of each plasma sample were extracted and analyzed in duplicate by HPLC. Solution-based calibrants were used for these analyses.

| Known concentration (μg/ml) | Mean found concentration (μg/ml) | Relative standard deviation (%) |
|---|---|---|
| 0.104 | 0.104 | 6 |
| 0.520 | 0.571 | 10 |
| 0.966 | 1.03 | 7 |
| 1.90 | 1.74 | 4 |
| 2.74 | 3.04 | 7 |

value assignment of quality control materials distributed to investigators involved in National Cancer Institute-supported pre-clinical trials. It provides a facile, sensitive, precise, and sufficiently accurate means for laboratory use in investigative studies to determine subject compliance in licorice root-dosing studies and for evaluating the efficacy of GRA as a chemopreventive agent.

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