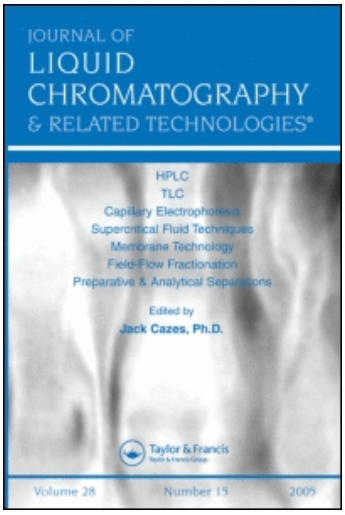

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High Pressure Liquid Chromatography Assay for Determination of 18-β-Glycyrrhetinic Acid in Plasma

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HIGH PRESSURE LIQUID CHROMATOGRAPHY ASSAY FOR DETERMINATION OF 18-β-GLYCYRRHETINIC ACID IN PLASMA

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ABSTRACT

This report presents a useful high pressure chromatography assay for determination of the proposed chemopreventive agent 18-β-glycerrhetinic acid (GA) in murine and human plasma. Drug was released from plasma proteins through precipitation with a mixture of sodium bisulfate and sodium chloride after which it was extracted with acetonitrile. Standard calibration curves of GA covered the concentration range of 2.5 to 120 μg/ml. The lower limit of detection was 0.5 μg/ml. No endogenous plasma constituents from plasma were found to interfere with the determination of GA. Recover of GA from plasma was greater than 95% over a concentration range of 20 to 100 μg/ml. Linearity of the assay was excellent; within-run precision showed a C.V. of 4.2% at 25 μg/ml, 5.6% at 20 μg/ml and 3.4% at 120 μg/ml. Between-run assay precision and accuracy was also considered to be excellent. The specificity, sensitivity and reproducibility of the procedure are adequate for proposed clinical pharmacology studies of this agent.

INTRODUCTION

Glycerrhetininc acid (GA; see Fig. 1), obtained from the water extract of dried licorice root *(Glycyrrhiza glabra)*, is currently being investigated for its potential to prevent the formation of cancer [1-4]. As an investigational

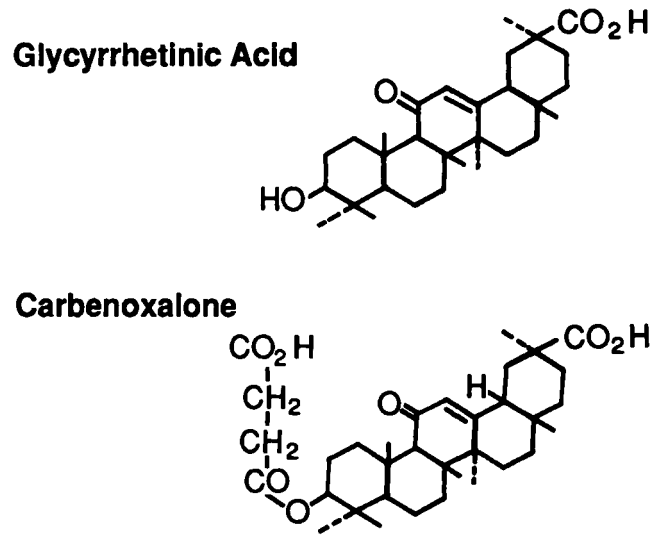

FIG. 1 Structures

Chemical structures of GA and carbenoxolone

"chemopreventive" agent, there is an important need to understand the pharmacology of this compound such that clinically relevant questions pertaining to dose-response, efficacy and toxicity can be answered.

Although analyses of glycyrrhetic glycosides have been made with an enzyme immuno-antibody technique [5], this method is neither specific for glycerrhetininc acid nor generally applicable for the routine analysis of GA in biological fluids. While a previous report of an HPLC procedure for determination of GA in rat plasma has been made published, no information was provided with respect to within-day or between day reproducibility or the applicability of this procedure for determination of GA in human plasma [6]. The present report describes a facile extraction and HPLC analytical approach for

the routine analysis of GA in murine and human plasma. The specificity, sensitivity and reproducibility of the procedure are adequate for proposed clinical pharmacology studies of this chemopreventive agent.

MATERIAL AND METHODS

Instrumentation

The HPLC system consisted of a Waters Assoc. (Milford, MA) Model 6000A pump, Model 6K injector and a Model 990 photodiode array detector. The separations were performed on a Hibar Lichrospher (5 μm particle size) 100 RP-18 column (200 mm x 4.6 mm; Merck, Darmstadt, F.R. Germany). A μBondapak C18 guard column (Waters Associates, Milford, MA) was routinely used to protect the analytical column.

Reagents and Chemicals

Sodium chloride, (Sigma, St. Louis, MO) and sodium bisulfate monohydrate (Aldrich Chemical Co., Milwaukee, WI.) were of analytical grade. Acetonitrile (Curtin Matheson, Scientific Inc., Houston, TX) PIC-A, (Waters, Milford, MA) and water were of HPLC grade. Glycerrhetinic acid was kindly provided by Dr. P. Vara (MacAndrews and Forbes Co., Camden, NJ).

Chromatography

The mobile phase consisted of acetonitrile: 0.005M tetrabutylammonium phosphate [PIC-A solution] (55:45, v/v) which was pumped through the column at a flow rate of 1 ml/min. The column was maintained at room temperature. Glycerrhetinic acid was detected at 255nm. A 25 μl volume was used for all injections onto the column.

Sample Preparation

Plasma samples were obtained at timed intervals from rats which had received an intravenous injection of glycerrhetininc

acid at a dose of 60 mg/kg. Outdated human plasma was obtained from the M. D. Anderson Cancer Center Blood Bank (Houston, TX). Samples were prepared by placing 600 µl into a microfuge tube (No. 500, Sarstedt Co., Numbrecht, W. Germany) containing a finely powdered mixture of 0.3 g of sodium bisulfate and sodium chloride (1:4, w/w; as described by Mathies and Austin [7]). The tube was capped and the solution was vortexed for one minute, after which 600 µl of acetonitrile was added. The tube was vortexed again for one minute then centrifuged in a Beckman Microfuge (12,000 x g) for five minutes. An aliquot (25 µl) of the clear organic layer was then injected onto the HPLC.

Standard Curves

A stock solution of glycerrhetinic acid was prepared daily by dissolving the drug in acetonitrile:PIC-A solution (70:30, v/v) to a final concentration of 2 mg/ml. Standard calibration curves were prepared in both human and rat plasma covering the concentration range of 2.5 to 120 µg/ml. At least five standards and one quality control sample were used for each standard curve.

Quality Controls

Quality controls were analyzed in triplicate over three days to determine accuracy and between-run precision. Drug- free human plasma was prepared to contain glycerrhetinic acid at five concentrations: 10, 20, 40, 60 and 120 µg/ml. The solutions were mixed, separated into 600 µl aliquots and stored at -20 •C. Prior to analysis, three QC's from each concentration were brought to room temperature and prepared along with the calibration curve samples. Derived concentrations of the quality control standards were calculated from the least squares regression of the standard curve run on each day.

Statistics

Linearity was calculated by linear regression analysis and reported as r^2. The intercept and slope parameters include standard deviation (S.D.) estimates. The relative standard deviation (%RSD) was determined by dividing the sample S.D. by the mean and expressing the quotient as a percentage.

RESULTS AND DISCUSSION

Selectivity

The method as described provides for the separation and quantitation of 18-beta glycerrhetinic acid from plasma. Typical chromatograms for control and spiked rat plasma are shown in Fig. 2 and those for huamn plasma in Fig. 3. Using this chromatographic system, no endogenous components, extracted from either human or rat plasma were found to interfere with the determination of GA in the sample assay. In a brief examination of structurally related chemoprevenbtive agents, the methodology used in the present study was also found to be suitable for carbenoxolone which had a retention time of 4.0 to 4.25 min. The methopdology was unable to distinguish the 18-alpha isomer of GA from that of 18-beta GA (data not shown). In addition, the methodology as described was not found suitable for monoammonium glycyrrhizinate which eluted at the solvent front.

Recovery

Extraction efficiency was determined by comparing the peak areas from spiked samples with those obtained from injection in the mobile phase (Table 1). Recovery of GA from human plasma was excellent and averaged greater than 95% over a concentration range of 20 to 100 μg/ml. Recovery of GA from rat plasma was similar to that observed using human plasma (data not shown).

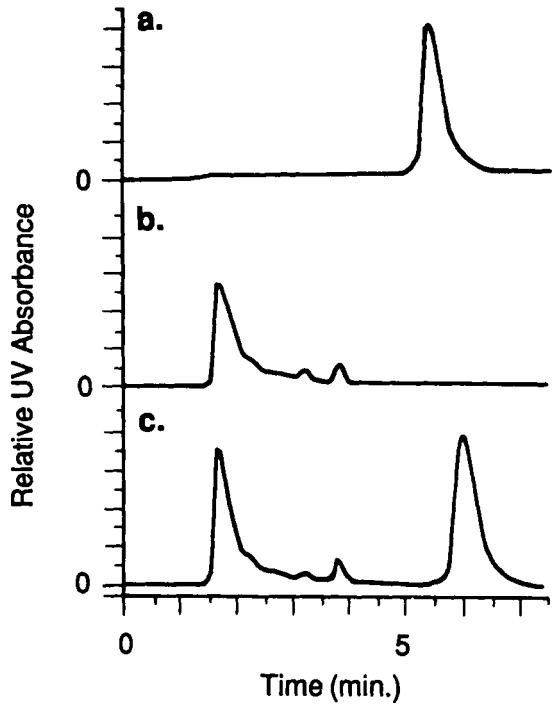

FIG. 2 Representative HPLC chromatograms of a) glycerrhetinic acid (20 μg/ml) extracted from saline; b) extracted rat plasma; c) extracted rat plasma which had been spiked with glycerrhetinic acid to contain 20 μg/ml. Absorbance scale: 0.02 AUFS.

TABLE 1

ASSAY RECOVERY OF GA FROM HUMAN PLASMA

Concentration (μg/ml)	Recovery (Mean ± S.D., n = 3; %)
10	92 ± 5
20	96 ± 3
40	96 ± 4
60	98 ± 3
80	91 ± 6
100	96 ± 3

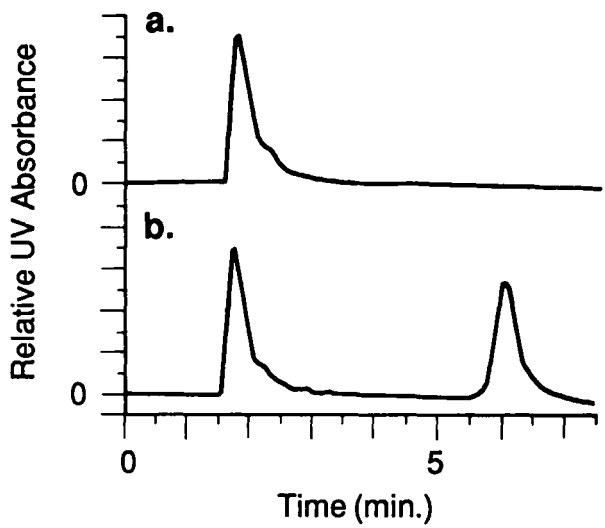

FIG. 3 Representative HPLC chromtatograms of a) extracted human plasma; b) extracted human plasma which had been spiked with glycerrhetinic acid to contain 20 μg/ml. Absorbance scale: 0.02 AUFS.

Plasma calibration curves were described by the regression equation $y = 0.000459 + 0.000589x$, where y is the observed peak area and x is the GA concentration.

Linearity, accuracy and within-run precision

Data describing the linearity, within-run precision and accuracy of a typical calibration curve are shown in Table 2. The linearity was excellent over the concentration range studied (2.5 to 120 μg/ml) with an r^2 value of 0.9995, a slope of 1.0877 ± 0.0379 and an intercept of -1.4676 ± 0.3640. The within-run precision calculated from the QC samples analyzed with the calibration curve showed a C.V. of 4.2% at 0.25 μg/ml, 5.6% at 20 μg/ml and 3.4% at 120 μg/ml.

TABLE 2

Linearity and within-run precision of GA $r^2 = 0.995$; slope = 1.0877 ± 0.0379 (Mean ± S.D.); intercept = 1.4676 ± 0.3640 (Mean ± S.D.)

Concentration added (μg/ml)	Concentration found (Mean ± S.D., n = 3) (μg/ml)	C.V. (%)
2.5	2.55 ± 0.07	2.7
5.0	5.06 ± 0.18	3.6
10	9.84 ± 0.16	1.6
20	19.75 ± 0.22	1.1
40	40.14 ± 0.14	0.3
60	61.81 ± 0.53	0.9
120	120.87 ± 4.00	3.3

TABLE 3

BETWEEN-RUN PRECISION AND ACCURACY OF GA QUALITY CONTROLS

Day	Concentration added (μg/ml)	Concentration found (Mean ± S.D., n = 3/day) (μg/ml) (%)	C.V.
1	2.5	2.45 ± 0.01	0.2
	20	20.20 ± 0.26	1.3
	120	119.08 ± 8.86	7.4
2	2.5	2.55 ± 0.07	2.8
	20	19.75 ± 0.22	1.1
	120	120.26 ± 5.30	4.4
3	2.5	2.56 ± 0.06	2.4
	20	20.18 ± 0.22	1.1
	120	121.36 ± 1.96	1.6

Between-run precision and accuracy

The QC data representing the between-run precision and accuracy of determination of SG in human plasma over a three- day period is shown in Table 3. The 3-day Mean ± S.D. values determined for GA concentrations of 2.5, 20 and 120 μg ml were 2.52 ±0.07 μg/ml (C.V.= 2.8%), 20.04 ±0.28 μg/ml (C.V.= 1.4%), and 120.27 ±4.72 μg/ml (C.V.= 3.9%).

ACKNOWLEDGMENTS

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