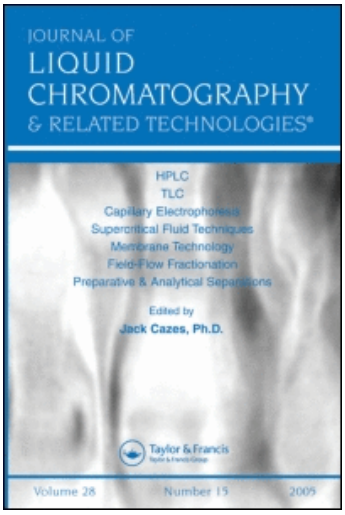

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FUMARIC ACID IN RAT PLASMA, URINE, AND FECAL SAMPLES

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ABSTRACT

Fumaric acid, a Krebs cycle intermediate, is a potential cancer chemoprevention agent. A high performance liquid chromatographic procedure with UV detection for determination of fumaric acid in large numbers of rat plasma, urine and fecal samples was developed. Fumaric acid was extracted from

plasma, urine, and fecal samples utilizing solid phase extraction using Clean Up® Quaternary Amine 1 mL (plasma, fecal samples) and 3 mL (urine) extraction columns followed by reverse phase high performance liquid chromatography with UV detection at 215 nm. Standard curves for plasma (1 μg/mL - 200 μg/mL), urine (5 μg/mL - 200 μg/mL), and fecal material (25 μg/g - 500 μg/g) were analyzed and replicate analysis of controls were used to determine intra-day and inter-day variability. Chlorofumaric acid was used as an internal standard for plasma and fecal samples; trans-glutaconic acid for urine samples. Precision and accuracy were studied using control solutions (low and high) prepared in naive rat plasma, urine, and fecal material. Intra-day variability was determined using 3 - 6 replicates of each control solution on a single day. Coefficient of variation (CV) for 20 μg/mL control (low) in plasma was 6.11% and for 80 μg/mL (high) was 7.07%; relative accuracy (RA) was 0% and 6.01%, respectively. CV for 15 μg/mL low control in urine was 38.95% and for 150 μg/mL high control was 8.84%; RA values were -12.2% and -15.16%, respectively. For the fecal material, CV for low control (100 μg/g) was 0.92% and for the high control (400 μg/g) was 4.67%. RA values were -4.02% and -4.14%, respectively. Inter-day variability was determined over a four day period. For the 20 and 80 μg/mL plasma control solutions, CVs were 12.97% and 9.09%, respectively, and RA values were 1.1% and 4.04%, respectively. For the 15 and 150 μg/mL urine control solutions, CVs were 26.85% and 14.17%, respectively, and RA values were 33.07% and -1.24%, respectively. For the 100 and 400 μg/g fecal material controls, CVs were 2.5% and 4.39%, respectively, and RA values were -4.07% and -3.87%, respectively. The standard curves for plasma, urine, and fecal samples were linear over the range of fumaric acid assayed and the means for the regression coefficient were 0.9939, 0.9972, and 0.9956, respectively. The limits of quantitation for plasma, urine, and fecal material were 1 μg/mL, 5 μg/mL and 25 μg/g, respectively.

INTRODUCTION

Fumaric acid, a naturally occurring metabolic intermediate, is currently undergoing preclinical development by National Cancer Institute as a cancer

chemoprevention agent.[1] It has been shown to inhibit hepatocarcinogenesis in rats induced by 3'-methyl-4-(dimethylamino)azobenzene (0.06% in diet for 50 days) when given by dietary admixture (1%) and in drinking water (0.025%) for 51 weeks.[2,3] Fumaric acid also reduced the hepatocarcinogenicity of mitomycin C and aflatoxin B1.[4] Dietary administration of fumaric acid totally suppressed hepatocarcinogenesis in mice[5] and rats[6] fed thioacetamide for 40 weeks when followed by fumaric acid treatment for 48 weeks. An inhibitory effect of fumaric acid on forestomach and lung carcinogenesis induced by a 5-nitrofuran naphthydrine derivative in mice has also been evident.[7]

Fumaric acid is presently used to treat psoriasis vulgaris. This approach was initiated by the German biochemist Schweckendiek and standardized by Schäfer.[8,9] Current fumaric acid therapy consists of the daily oral intake of dimethyl fumaric acid ester or monoethyl fumaric acid ester,[8,9,10,11,12] as fumaric acid itself is poorly absorbed from the gastrointestinal tract.[8] Topical application of fumaric acid and monoethyl fumaric acid in ointment has also been used as a supporting treatment.[8] A variety of serious side effects such as lymphopenia with a decrease of T lymphocytes and nephrotoxicity (acute tubular necrosis), nausea, diarrhea, severe stomach ache, or skin irritation and contact urticaria have been reported during fumaric acid and/or fumaric acid esters therapy.[8,13]

A gas chromatographic method can be used for the determination of fumaric acid and other carboxylic acids in variety of biological matrices. Tsuda et al.[14] developed a gas chromatography method with a flame ionization detector for the determination of fumaric acid and other carboxylic acids in soft drinks and jams. All carboxylic acids were extracted using anion exchange columns. Gas chromatography - mass spectrometry methodology was described for determination of carboxylic acids including fumaric acid in rat tissues as their tert-butyldimethylsilyl derivatives.[15] In another study, concentrations of carboxylic acids as their benzyl esters were measured in human serum, urine, and rat heart ventricle using gas chromatography and capillary column with flame ionization detection.[16] High performance liquid chromatography (HPLC) has been successfully applied for determination of several carboxylic acids in food and beverages as their p-nitrobenzyl esters,[17] in wines and different beverages after derivatization,[18] in human blood,[19] anaerobic bacteria cultures,[20] and routinely in wines and champagne.[21]

The purpose of this study was to develop and evaluate a simpler, precise, accurate and fast HPLC analytical method with isocratic elution for quantitation of fumaric acid in complex biological matrices, ie. plasma, urine, and feces.

MATERIALS AND METHODS

Chemicals

Fumaric acid, rat plasma, urine, and rat fecal material were supplied by the Toxicology Research Laboratory, Department of Pharmacology, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA. Chlorofumaric acid and trans-glutaconic acid were purchased from Aldrich Chemical Company, Milwaukee, WI, USA. Acetonitrile, methanol, potassium phosphate dibasic, sodium fluoride, potassium phosphate monobasic, and o-phosphoric acid (85%) were purchased from Fisher Scientific, Itasca, IL, USA, and were HPLC grade. Physiological saline solution was acquired from Baxter Diagnostics Inc., McGaw Park, IL, USA. Clean Up® Quaternary Amine 1 mL and 3 mL extraction columns were acquired from Worldwide Monitoring, Horsham, PA, USA.

HPLC Instrumentation

Fumaric acid concentrations in plasma, fecal material, and urine were determined isocraticaly using an HPLC system. Chlorofumaric acid was used as an internal standard for plasma and fecal samples; trans-glutaconic acid was used as an internal standard for urine samples. A Waters 600E Solvent System, Waters 484 Tunable UV/VIS Detector, and a Waters 746 Data Module (Waters Chromatography Division, Milford, MA, USA) were used. The samples were introduced to the analytical column via a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA). For plasma and fecal samples, separation was achieved using a C_{18} Beckman Ultrasphere 5 μm, 250 x 4.6 mm analytical column purchased from Alltech Associates, Inc., Deerfield, IL, USA; for urine samples, a C_{18} Waters μBondapak 10 μm, 300 x 3.9 mm (Waters Chromatography Division, Milford, MA, USA) was used. The Rheodyne 7125 injector was equipped with a 20 μL sample loop and a C_{18} precolumn (Guard-Pak, Waters Chromatography Division, Milford, MA, USA) in a precolumn holder.

Quality Control

Quantitation of fumaric acid in rat plasma, urine, and fecal samples was performed using an internal standard method. The standard curves were determined by linear least squares regression analysis of the ratio of peak area of fumaric acid to peak area of the internal standard as a function of

concentration. Control solutions were prepared in naive rat urine. Concentrations of fumaric acid in naive urine were measured against standard curves prepared in 0.9% saline. Standards and controls for plasma and fecal material were prepared in spiked naive rat plasma and fecal material, respectively. Standard solutions and controls were prepared with each set of plasma, urine, and fecal samples. The concentration range of the standard curve for plasma was 1 μg/mL to 200 μg/mL; for urine it was 5 μg/mL to 200 μg/mL; and for fecal material it was 25 μg/mL to 500 μg/mL. A 1,000 μg/mL fumaric acid standard stock solution, 800, 1,000, and 2,000 μg/mL control stock solutions, and 1 mg/mL and 2 mg/mL solutions of internal standards (chlorofumaric acid and trans-glutaconic acid, respectively) were prepared in 0.9% physiological saline solution.

Two levels of control solutions were prepared for each specimen (20 and 80 μg/mL for plasma, 15 and 150 μg/mL for urine and 100 and 400 μg/g for fecal samples) and were analyzed. Precision and accuracy were determined by analyzing controls prepared in naive rat plasma, urine, and fecal material. Intra-day variability was determined using 3-6 replicates of each control solution analyzed on a single day. Inter-day variability was determined over a four day period analyzing replicates of each control solution. Relative accuracy (%) was determined employing the following equation:

RA = [(Mean Measured Conc. - Theoretical Conc.)/Theoretical Conc.] x 100%

Extraction Procedures

All plasma samples (0.2 - 0.8 mL) were diluted to 2 mL with 0.9% saline. All control solutions, standards and samples were vortex mixed, and 1 mL of 0.1 M phosphate buffer, pH 5, and 100 μL of internal standard (chlorofumaric acid) were added to each solution. All urine samples were mixed and 1 mL of each urine sample was centrifuged for 5 minutes at 13,605 *g*. A 0.25 mL volume of urine was transferred to a test tube and a 0.75 mL volume of 0.1 M potassium phosphate dibasic buffer, pH 5, was added. A 0.5 mL volume of 0.1 M potassium phosphate dibasic, pH 5, was then added to all controls and standards. A 25 μL volume of internal standard (trans-glutaconic acid) was added. All controls, standards and samples were vortex mixed.

Fecal samples were prepared as follows: each sample was weighed, placed into a mortar, and macerated with a pestle. Three mL of 0.1 M potassium phosphate monobasic buffer, pH 5, was added to each gram of fecal material. A homogenous paste was prepared and transferred to a 50 mL plastic tissue

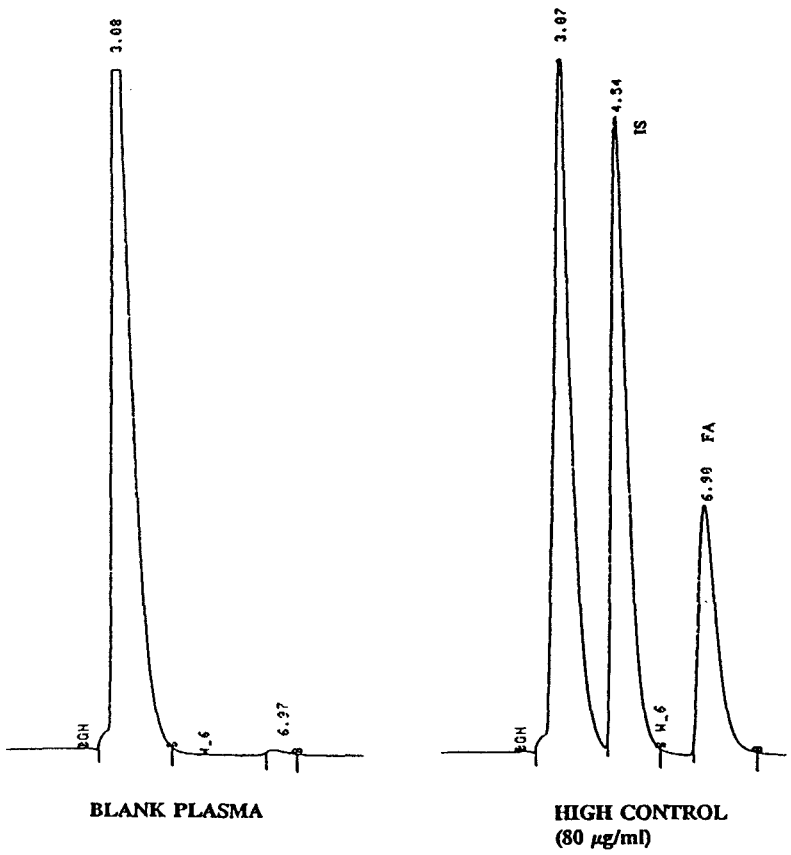

Figure 1. Chromatogram of blank rat plasma extract and extract of plasma spiked with fumaric acid (FA) and the internal standard (IS), chlorofumaric acid.

collection tube. Two hundred mg of fecal material paste was then taken from each sample for analysis. The blank rat fecal material, from untreated animals used for standards and controls, was prepared in the same way before it was spiked with fumaric acid. One mL of 0.9% saline was added to 200 mg of each sample. All controls, standards and samples were vortexed, and 2 mL of the phosphate buffer, pH 5, was added to each test tube. A 100 μL volume of internal standard (chlorofumaric acid) and 100 μL of sodium fluoride, 10 mg/mL, were added. All samples were centrifuged at 1,500 *g* for 20 minutes. The supernate was removed, placed in another glass test tube and stored at -20°C until analyzed.

All three specimens analyzed (samples, standards, and controls) were then put through a solid phase extraction procedure using quaternary amine solid phase extraction columns placed in a 12-station vacuum manifold (Varian, Harbor City, CA, USA). The procedure for extraction column preparation was as follows: the column was washed with 1 mL of 0.02 M phosphate buffer, pH 2.4, and with 1 mL of deionized water. For column conditioning, 1 mL of methanol and 1 mL of deionized water were used. A 1 mL volume of sample (plasma, fecal material, or whole urine sample) was then put through the column.

The column was next washed with 1 mL of acetonitrile and dried under maximum vacuum for 5 minutes. Samples were eluted using 1 mL of 0.02 M phosphate buffer, pH 2.4, collected into test tubes. A 20 μL of the eluent was injected onto the column. The mobile phase consisted of 0.02 M phosphate buffer, pH 2.4 adjusted with o-phosphoric acid (85%), while the flow rate was maintained at 1 mL/minute. Fumaric acid was monitored at 215 nm.

RESULTS

Typical chromatograms of naive plasma and of plasma spiked with fumaric acid and chlorofumaric acid as an internal standard are presented in Figure 1. The calibration was performed by using 5-7 concentrations for all specimens analyzed. A blank fecal material chromatogram and fecal material with fumaric and chlorofumaric acids added are presented in Figure 2. All chromatograms were recorded over a 12 minute time period. There were no contaminants eluting after that time. Under the assay conditions, reproducible retention times for fumaric acid in plasma and fecal material (Beckman C_{18} Ultrasphere 5μ column) were approximately 7 and 8 minutes, respectively, and for chlorofumaric acid, they were 5 and 7 minutes, respectively. The reproducible retention time for fumaric acid in urine using a Waters C_{18} μBondapak 10 μ analytical column was approximately 6 minutes and for trans-glutaconic acid it was approximately 9 minutes. A standard curve for urine was prepared in 0.9% saline. Calibration curves for plasma, urine (saline) and fecal material containing fumaric acid were linear over the range analyzed and had mean correlation coefficients of 0.9939, 0.9972 and 0.9956, respectively.

A representative calibration curve prepared in naive rat plasma is presented in Figure 3. Results of intra- and inter-day variability including relative accuracy and coefficients of variation for plasma, urine and fecal material are presented in Table 1.

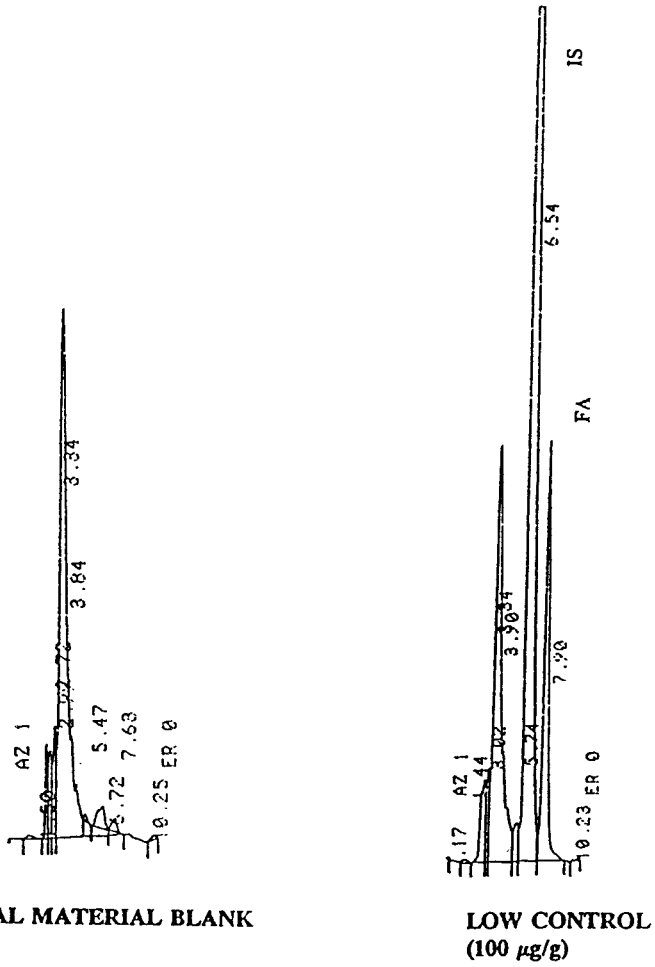

Figure 2. Chromatogram of blank rat fecal material extract spiked with fumaric acid (FA) and the internal standard (IS), chlorofumaric acid.

DISCUSSION

This paper presents an HPLC procedure for the quantitation of fumaric acid in biological matrices (rat plasma, urine, and fecal samples). Eluent monitoring at 215 nm provides adequate sensitivity, precision, and accuracy for determination of fumaric acid concentrations in all specimens studied. Standard

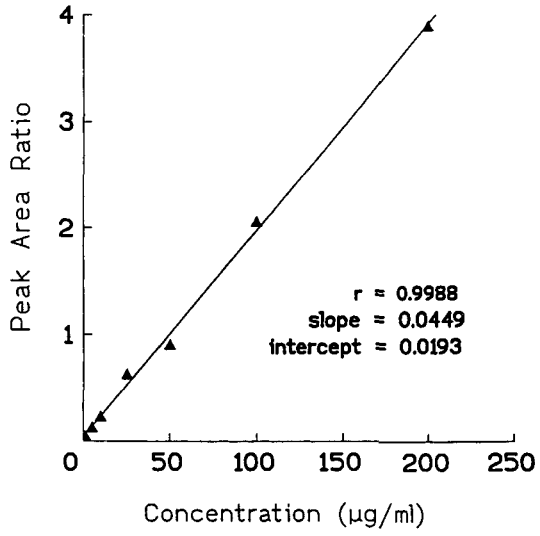

Figure 3. Fumaric acid calibration curve prepared in rat blank plasma.

curves were linear over the range of fumaric acid assayed; 1 µg/mL to 200 µg/mL for plasma, 5 µg/mL to 200 µg/mL for urine, and 25 µg/g to 500 µg/g for fecal material. The quantitation limits were achieved without time consuming derivatization. The results obtained using Ultrasphere 5 µm C_{18} (plasma and fecal samples) and µBondapak 10 µm C_{18} columns were reproducible and similar to previously described studies that utilized two Aminex HPX-87H columns for determination of several citric acid cycle intermediates including fumaric acid.[19] In that experiment, fumaric acid retention time was 26.81 ± 0.14 minutes, which is not practical when large numbers of samples and a variety of different specimens are assayed.

Extractions from rat plasma, urine, and fecal samples were performed using solid phase extraction on Clean Up^R Quaternary Amine columns which provided sufficiently clean samples to achieve good reproducibility, precision and accuracy of the analytical method described. In addition, the extraction procedure presented remains less time consuming in comparison with other published extraction procedures.[15,16,17] In summary, the method described in this paper provides several analytical advantages, e.g., simplicity; clean extracts (even for fecal material); sufficient sensitivity; reproducible retention times, precision and accuracy; low cost; and reliability for analysis of large numbers of plasma, urine, and fecal samples containing fumaric acid.

Table 1

Accuracy and Precision of Fumaric Acid Control Concentrations (μg/mL)

Intra-Day Variability

Parameter	Plasma		Urine		Feces	
	LC[1]	HC[2]	LC	HC	LC	HC
MMC[3]	20.00	84.81	13.17	127.26	95.98	383.45
(± S.D.)	(± 1.22)	(± 5.99)	(± 5.13)	(±11.25)	(±0.88)	(± 17.92)
% CV[4]	6.11	7.07	38.95	8.84	0.92	4.67
% RA[5]	0.00	6.01	-12.20	15.67	-4.02	-4.14

Inter-Day Variability

MMC	20.22	83.23	19.96	148.14	95.93	384.51
(± S.D.)	(± 2.62)	(± 7.57)	(± 5.36)	(20.99)	(± 2.40)	(±16.89)
% CV	12.97	9.09	26.85	14.17	2.50	4.39
% RA	1.10	4.04	33.07	-1.24	-4.07	-3.87

[1] Low control
[2] High control
[3] Mean measured concentration
[4] Coefficient of variation
[5] Relative accuracy

ACKNOWLEDGMENT

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