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Short Communication

High-performance liquid chromatographic method for the determination of free gossypol in chicken liver

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ABSTRACT

A high-performance liquid chromatographic method for the determination of free gossypol in chicken liver at levels down to 0.5 ppm has been developed. Tissue was deproteinized with acetonitrile in presence of ascorbic acid and the filtrate was subjected to hydrolysis with hydrochloric acid. The liberated pure gossypol was partitioned into chloroform and analysed by gradient elution on a 10-μm C_{18} column. The overall recovery was 83.5 ± 2.6%, with an overall relative standard deviation of 9%.

INTRODUCTION

Cottonseed meal, a by-product of the cottonseed oil industry, is an important protein supplement for livestock feeding. Its utilization, however, is limited by the presence of gossypol (1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-2,2′-binaphthalene-8,8′-dicarboxaldehyde), a compound with well documented toxic effects on animal species [1–3].

Following feeding to animals, gossypol is absorbed from the digestive tract and retained in the tissues where it occurs in both the free and protein-bound form. In liver tissue where the deposition of the free form appears particularly high, concentrations up to 323 and 94 ppm have been found in freeze-dried samples from pigs [4] and cows [5], respectively, and up to 415 ppm have been detected in fresh trout liver [6]. High gossypol concentrations in animal tissues may represent a concern for public health, considering the most harmful effect of gossypol, cardiotoxicity; in China, a low birthrate and high incidence of heart problems have been associated with gossypol in raw cottonseed oil [7], and hypokalaemic paralysis, an infrequent side-effect, was the major drawback of the use of gossypol (20 mg/day for 75 days) as an antifertility agent in men [8].

It seems, however, that the spectrophotometric method [9] used to determine gossypol in animal tissues overestimates the true concentrations. This method, which is based on the reaction of the aldehyde groups of gossypol with aniline to form dianilinogossypol, suffers from interferences from extraneous materials affecting the absorbance values [9]. False-positive readings have been reported for tissues that were known to be free from gossypol; diaphragm muscle and bile from animals fed gossypol-free diets [4] were found to contain as much as 24.5 and 100.3 ppm of gossypol, respectively. There has therefore been increasing interest in the development of more reliable methods.

Considerable progress has been made recently with the use of liquid chromatography (LC). Vari-

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ous LC methods allowing the accurate, precise and sensitive determination of gossypol in human plasma have been presented [10–13]. A survey of the literature, however, shows that such a method is not available for animal tissues.

This paper describes a high-performance liquid chromatographic (HPLC) method that has been developed for the determination of gossypol in chicken liver.

EXPERIMENTAL

Instrumentation

HPLC was carried out on a Perkin-Elmer (Norwalk, CT, USA) system consisting of a Series 3 modular chromatograph equipped with two reciprocating pumps controlled by mirocomputer, a power solvent mixer, an LC-100 column oven, an LC-55-B UV–VIS spectrophotometer and a Model 023 variable-span recorder. A Perkin-Elmer LC-55-S digital scanner permitted the monitoring of corrected spectra of the eluted compounds under stop-flow conditions; trapping of the eluates in the flow cell could be effected by shutting off the pump power and, simultaneously, closing a stop-flow valve located before the Rheodyne Model 7105 injector.

Injections were made on a 25 × 0.46 cm I.D. column laboratory packed with Spherisorb ODS-2, 10 μm (Phase Separations, Norwalk, CT, USA). Packing was accomplished at pressure of 42.8 MPa by the downwards slurry-packing technique on a Magnus Scientific (Aylesbury, UK) P6060 HPLC slurry-packer using as the suspending medium methanol–water (80:20 v/v) containing 0.0002 g/ml of sodium acetate [14].

Homogenization of tissue samples was performed in a domestic blender and hydrolysis of the extracts in a Tamson (Zoetermeer, Netherlands) Model T.X.V. 45 constant-temperature water-bath (accuracy ±0.1°C).

Chemicals and reagents

Analytical-reagent grade ascorbic acid, acetonitrile, chloroform, hydrochloric (37% min.) and phosphoric acid were obtained from Merck (Darmstadt, Germany), HPLC-grade methanol from Prolabo (Paris, France) and gossypol–acetic acid (89.62% pure gossypol) from Makor Chemicals (Jerusalem, Israel). Stock solutions of gossypol were prepared by weighing *ca.* 25 mg of gossypol–acetic acid and diluting to 50 ml with acetonitrile. Aliquots of these solutions were further diluted with acetonitrile to give working solutions that contained pure gossypol in the range 0.8–8 μg/ml. Working solutions were prepared daily and protected from light throughout the analysis.

Sample preparation

A 2-g sample of chicken liver was blended for 2 min with 50 ml of acetonitrile–water (40:10, v/v) containing 2% of ascorbic acid. After the precipitated proteins has settled, the supernatant liquid was filtered through Whatman No. 40 paper, discarding the first 5 ml of the filtrate. A 25-ml aliquot of the clear extract was pipetted into a 50-ml volumetric flask and 0.05 ml of hydrochloric acid was added. The flask was placed in a 65°C water-bath, stoppered after equilibration for 5 min and then heated for 100 min. After cooling to room temperature, the flask contents were transferred into a 250-ml separating funnel and 50 ml of 0.3% aqueous ascorbic acid followed by 0.5 ml of hydrochloric acid were added. The suspension formed was extracted with 25 ml of chloroform and the separated bottom layer was filtered through anhydrous sodium sulphate on Whatman No. 40 paper into a 100-ml flask to be further evaporated under vacuum at 35°C. Traces of solvents were expelled with a stream of nitrogen and the remaining residue was dissolved in $\geqslant 1$ ml of acetonitrile, the volume depending on the expected gossypol content of the processed sample.

Chromatography and quantification

Aliquots (25 μl) of sample extracts were injected into the chromatograph and analysed at a mobile phase flow-rate of 1.5 ml/min, a detection wavelength of 254 nm, a chart speed of 15 cm/h and a recorder sensitivity of 0.050 a.u.f.s. Chromatography was performed at 30°C to isolate the column from fluctuations in ambient temperature.

The mobile phase consisted of two solvents, methanol and water, both containing 0.1% of phosphoric acid. The water used in the mobile phase was glass-distilled water that had been further purified by passing it through a C_{18} column. Elution of gossypol was carried out by programming the metha-

nol–water mobile phase composition (v/v) as follows: 2 min isocratic at 82:18; 2 min linear gradient to 92:8; 5 min isocratic at 92:8; 3-min purge at 99:1, and 10-min equilibration at 82:18. After each day's work, the column was flushed with water until free from acidity and maintained filled with methanol.

Calibration graphs were prepared daily by running 25-μl aliquots from the series of the working solutions and plotting the recorded peak heights versus the amount of gossypol injected. The concentration of gossypol in the samples was calculated by reference to this calibration graph and multiplication by appropriate dilution factor as follows:

$$\text{Gossypol in samples (ppm)} = (QV \cdot 2)/(0.025\ W)$$

where Q = amount of gossypol found (ng), V = volume of final sample dilution (ml) and W = weight of sample (g).

RESULTS AND DISCUSSION

As gossypol is easily oxidized in aqueous solution, attempts were made to protect the compound against oxidative degradation from the very beginning of sample handling. Wang *et al.* [11] reported that a considerable loss of gossypol in human plasma kept at 0°C for 6 h occurs unless reduced glutathione is added. A series of pertinent experiments with liver samples showed that addition of 2% of ascorbic acid to the extraction solvent could efficiently protect gossypol, increasing its recovery from 10% to more than 80%.

The extraction of gossypol from liver samples was carried out with aqueous acetonitrile, a solvent which effectively precipitates proteins. With this solvent, further clarification of the homogenates such as that proposed by Smith [9], for extracting gossypol from animal tissues with an ethanol–water–diethyl ether–acetic acid solvent mixture, was not needed as clear filtrates were taken after a 1-min settling time. These observations concur with those of Wang *et al.* [11] and Wu *et al.* [12], who also used acetonitrile for deproteinization purposes in the determination of gossypol in human plasma, while Sattayasai *et al.* [10] deproteinized human plasma by using ethanol.

Purification of the protein-free filtrates was performed by partitioning them between chloroform and water, a procedure that has been effectively applied in the determination of total gossypol in cottonseed meals [15]. With this procedure, however, gossypol could not be partitioned into chloroform although this solvent quantitatively extracts the compound from its aqueous solutions provided that acid has been added to suppress gossypol ionization. As this might be due to the well known tendency of gossypol to form complexes with various metal ions [16], amino acids, phospholipids [17], etc., a debinding process was evaluated. Sattayasai *et al.* [10] reported that some chelating agents such as EDTA disodium salt can break gossypol complexes into pure gossypol, which can be easily extracted, and in previous work [18] it was found that an acid hydrolysis procedure at 65°C for 60 min can debind gossypol from cottonseed meal extracts. Application of these procedures in liver analysis showed that both worked equally well as far as the recovery was concerned, provided that the hydrolysis time was prolonged to 100 min. The EDTA procedure, however, increased the level of endogenous compounds that were coextracted with gossypol.

HPLC of the injected samples was initially performed under isocratic conditions using a mobile phase of methanol–water (88:12, v/v) containing 0.1% of phosphoric acid. The addition of the acid eliminated peak tailing by suppressing the ionization of the phenolic hydroxyl groups of gossypol, but the separation of the compound from other constituents could not be effected. To improve the resolution, the mobile phase composition was altered to contain less methanol but, surprisingly, even at 87% methanol the gossypol peak almost disappeared. Therefore, a short isocratic step at 82% methanol was finally used to elute the less retained compounds, and a rapid linear gradient to 92% methanol followed by an isocratic run was then applied to elute gossypol at 8.1 min without affecting its peak shape (Fig. 1a). With this solvent programme, however, further purification of the distilled water used in the mobile phase was essential, as otherwise a baseline rise appeared midway through each run even when no sample had been injected. This rise was found to be due to UV-absorbing impurities in the distilled water that concentrated on the top of the column during the equilibration period and eluted once the gradient was started.

Under the mentioned conditions, gossypol could

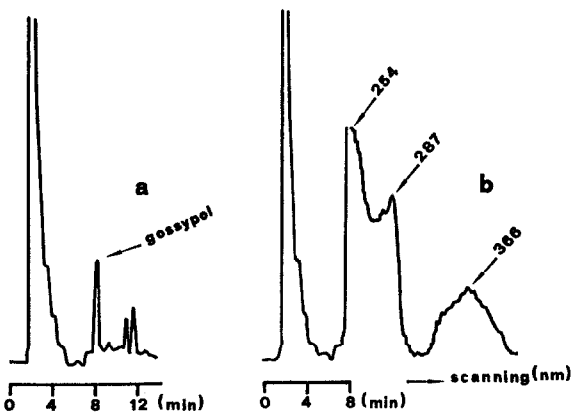

Fig. 1. (a) Typical chromatogram of chicken liver spiked with 2.4 ppm of gossypol and (b) absorbance scan of gossypol peak apex for sample spiked with 5.8 ppm of gossypol. HPLC conditions: solvent programme as in text using methanol and water, each containing 0.1% of phosphoric acid; column, 25 × 0.46 cm I.D., C_{18}, 10 μm; temperature, 30°C; flow-rate, 1.5 ml/min; detection wavelength, 254 nm; recorder sensitivity, 0.050 a.u.f.s.; chart speed, 15 cm/h; injection volume, 25 μl.

be determined in chicken liver at levels down to 0.5 ppm. Characterization of the recorded peak was based on the retention behaviour of the compound, but further characterization could be made possible by on-line scanning of the peak apex (Fig. 1b) and comparison of the spectral scan with the published absorption curve [19], provided that the sample contained more than 5 ppm of gossypol.

Regression analysis of the data obtained by running a series of working solutions showed the response to be linear within the range studied [0.05 a.u.f.s., $y = -0.24 + 0.421x$, correlation coefficien (r) = 0.9999, where y represents the peak height in mm and x the amount of gossypol injected in ng] Therefore, the recovery of gossypol could be evaluated by adding various amounts of working solutions to liver samples and analyzing three replicates The concentrations examined ranged from 1.4 to 44.8 ppm. Least-squares and regression analysis o the data (Table I) showed that the relationship between the added and found amounts was adequately described by a linear regression (r = 0.9987) Hence the slope (0.835 ± 0.026) of the regression line ($y = -0.08 + 0.835x$) could be used as an estimate of the overall recovery (83.5 ± 2.6%) in the determination of gossypol in chicken liver.

TABLE I

RECOVERY DATA FOR THE DETERMINATION OF GOSSYPOL IN CHICKEN LIVER

Concentration added (ppm)	Mean concentration found[a] (ppm)	Mean recovery (%)
1.4	1.2 ± 0.06	85.7
2.8	2.2 ± 0.15	78.6
5.6	4.3 ± 0.36	76.8
11.2	9.7 ± 0.40	86.6
22.4	18.2 ± 0.71	81.2
44.8	37.4 ± 1.43	83.5

[a] Mean of three (2-g) replicates ± S.D.

TABLE II

PRECISION DATA FOR THE DETERMINATION OF GOSSYPOL IN CHICKEN LIVER

Day	Concentration of gossypol found (ppm)	Mean concentration (ppm)	S.D.	R.S.D.[a] (%)
1	2.6, 2.6, 3.1, 2.9, 2.9, 2.9	2.8	0.20	7.1
2	3.2, 3.3, 3.2, 2.6, 3.0, 2.7	3.0	0.29	9.7
3	3.0, 3.4, 3.1, 3.3, 3.3, 3.0	3.2	0.17	5.3

Variance estimates

Source	R.S.D.[a] (%)
Within-day	7.5
Between-days	5.0
Overall	9.0

[a] Relative standard deviation.

The precision of the method was also studied by assaying, on each of three different days, six liver samples spiked with gossypol at the 3.7 ppm level and submitting the data (Table II) to analysis of variance and expected mean squares for the one-way classification-balanced design [20]. The within-day precision was found to be 7.5%, the between-days precision 5.0% and the overall precision 9.0% (relative standard deviation).

In conclusion, the results show that the proposed HPLC method is an efficient means of determining free gossypol in chicken liver. As the method can easily be applied to the analysis of other tissues, it offers the opportunity to study the deposition of the compound in chickens fed cottonseed meal diets.

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