Gossypol: High-Performance Liquid Chromatographic Analysis and Stability in Various Solvents

A.A. NOMEIR and M.B. ABOU-DONIA, Department of Pharmacology, Duke University Medical Center, Durham, NC 27710

ABSTRACT

An improved high performance liquid chromatography (HPLC) method of gossypol analysis was developed and compared with the current spectrophotometric method. Gossypol was determined in four samples of cottonseeds of *Gossypium hirsutum* with varying contents of gossypol, as well as in the stems and roots of *G. arboreum* and *G. thurber*. The roots of both species each contained equally high amounts of gossypol, while the stems of each species varied in content. Those of *G. thurber* contained slightly higher amounts of gossypol than those of *G. arboreum*. Among the seeds studied, the glanded cottonseeds contained the highest concentration of gossypol, followed by low gossypol seeds, then glandless cottonseeds, and finally the roasted glandless cottonseeds. The stability of gossypol was studied in five solvents at six different storage temperatures. Both the type of solvent and the temperature were found to affect the rate of decomposition of gossypol. In all the solvents studied, gossypol was found to be highly unstable at 37 C and at room temperature, while its stability increased as the storage temperature decreased. At all temperatures, depending on the solvent used, the rate of decomposition increased in the following order: acetone < acetonitrile < chloroform < ethanol < methanol. Although the decomposition products of gossypol were not identified, their formation was found to be dependent on the solvent used.

INTRODUCTION

Gossypol is a yellow phenolic substance present in various parts of cotton plants (1). The chemical structure of gossypol was determined to be 1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl[2,2′-binaphthalene]-8,8′-dicarboxaldehyde (2,3). Gossypol causes many deleterious effects to nonruminant animals (4-6), although ruminant animals appear to be more tolerant to gossypol toxicity (7). Various aspects of physiological, toxicological and biochemical effects of gossypol have been discussed in the literature (5,8,9).

Recently, gossypol has been tested as an antifertility agent for males in several animal species including human (10-14). Other studies showed that gossypol is a powerful inhibitor of human spermatozoal metabolism (15) and acts as an uncoupler of the mitochondrial respiratory chain and to oxidative phosphorylation (16). Gossypol also was found to be a nonmutagen in the Ames *Salmonella* mammalian microsomal test system (17).

Gossypol reacts with aromatic amines such as aniline to form dianilinogossypol. This reaction has been the basis of the gravimetric and colorimetric analysis of gossypol (18-23). Analysis of gossypol utilizing chromatographic methods including paper (24), thin layer (25) and gas liquid (26) have been developed. However, these methods either lack sensitivity, are time consuming, or require prior derivatization. Recently, high performance liquid chromatography (HPLC) has been utilized for the analysis of gossypol (27, 28).

In this study an improved HPLC method was developed for qualitative and quantitative analysis of gossypol. The developed method was used to determine gossypol levels in roots, stems, and seeds of cotton plants and also was compared with the colorimetric procedure currently being used. In addition, the stability of gossypol in five different solvents at six different storage temperatures was studied.

EXPERIMENTAL

Reagents and Equipment

Gossypol was purchased as gossypol-acetic acid from Sigma (St. Louis, MO), its purity was determined by HPLC to be 99.5%. Stock and standard solutions were freshly made in methanol before use. All HPLC solvents and reagents used were obtained from Fisher Scientific (Raleigh, NC). Glanded, low gossypol, glandless, and roasted glandless cotton plant seeds of *Gossypium hirsutum*, roots and stems of *G. arboreum* and *G. thurber* were supplied by Cotton, Inc. (Raleigh, NC). For stability studies, gossypol solutions were prepared in methanol, 95% ethanol, chloroform, acetonitrile and acetone at a concentration of 1 mg/mL. Each set of five solutions was stored in a water bath at 37 C, room temperature at 22 C ± 2 and refrigeration at 5 C, -10 C, -25 C and -80 C. Solutions were analyzed frequently by HPLC to determine the amount of gossypol remaining under different storage conditions. The experiment was continued for a period of 90 days.

Gossypol Extraction

Stems, roots and seeds of cotton plants were pulverized individually in an electric grinder, type SHG (Markson Scientific, Inc., Del Mar, CA). Using a Polytron Ultrasonic Homogenizer (Brinkman, Westbury, NY) a one-gram sample of each ground plant part was homogenized for 4 min in 50 mL of a solvent mixture which consisted of 715 mL of 95% ethanol, 285 mL of deionized water, 200 mL of diethyl ether and 0.2 mL of glacial acetic acid (23). Each extract was filtered under reduced pressure through an even layer of Celite (2 g) over a filter paper disk placed in a 30-mL sintered glass funnel. The flask and the sample in the funnel were washed with two successive 15-mL portions of the solvent mixture and filtrates were combined and completed with the solvent mixture to attain a volume of 100 mL. The resulting solutions were used for both spectrophotometric and HPLC analysis. Recovery was determined by adding 4 mg of pure gossypol to 1 g of each ground plant part, and then homogenizing, extracting and analyzing in the same manner as described for the regular sample. At least three replicates for each sample analysis and recovery determination were undertaken.

Analysis

HPLC. The system consisted of a Model 600 Solvent Programmer equipped with two Model 6000 A pumps, a Model 440 ultraviolet (UV) detector, a U-6K injector, a RCM-100 radial compression separation system with a C_{18} Cartridge (Waters Associates, Inc.,), a guard column filled with C_{18} bondapak (E. Merck Darmstadt, Germany), a recorder (Fisher Recordall series 5000) and a Chromatopac E1A data processor (Shimadzu, Seisakusho, Ltd., Kyoto, Japan). The data processor was used to measure the retention time, peak areas, and the percentage of each peak in the chromatogram. Solvents were filtered through Millipore membrane filters, type HA or fH, pore size 0.45 μm (Millipore, Bedford, MA). Gossypol solutions and plant extracts were injected directly in 1-50 μL volume and eluted from the col-

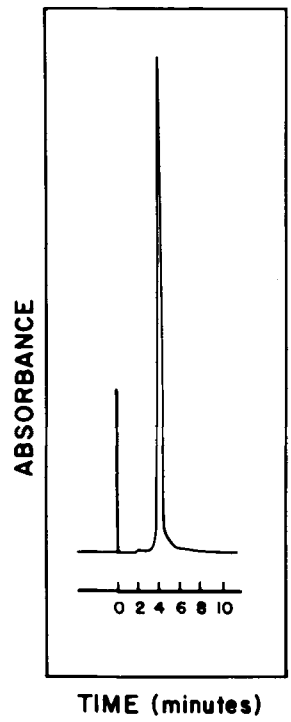

FIG. 1. HPLC chromatogram of standard gossypol. Gossypol was analyzed on a C_{18} cartridge using the RCM-100 radial compression system. The mobile phase was an isocratic solvent consisting of 0.1% phosphoric acid in methanol/water (9:1). Quantification was performed by measuring UV absorbance at 254 nm.

umn isocratically by 0.1% phosphoric acid in methanol/water (9:1) at a solvent flow rate of 2 mL/min. Detection and quantification were carried out by monitoring the UV absorbance of the column eluates at 254 nm. A standard curve was obtained by injecting known amounts of the standard gossypol and measuring the peak areas.

Spectrophotometry. Gossypol was determined spectrophotometrically according to the method described earlier by Smith (21-23). In a 25-mL volumetric flask, standard amounts of gossypol in methanol were added, methanol was evaporated to dryness by a gentle stream of nitrogen and the residue was dissolved in 5 mL of the solvent mixture used in the extraction procedure described above. Aliquots of either cotton seeds, stem, root extracts or the fortified samples were added, then the solvent mixture was added to bring the volumes up to 5 mL. Gossypol was converted to its dianilino derivative by adding 1 mL of colorless aniline, which had been distilled over granular zinc and stored in the freezer, and then the mixture was heated in a water bath at 75 C for 40 min. After cooling, the volume was brought up to 25 mL by adding the solvent mixture and the absorbance was measured at 445 nm using Stasar II Spectrophotometer (Gilford Instrument Labs, Columbia, MD). Reference samples without added aniline were prepared simultaneously.

RESULTS AND DISCUSSION

HPLC Method

One of the main objectives of this study was to improve the HPLC analytical method for gossypol which had been developed earlier in our laboratory (28). Because of its broad peaks, the previous method could allow closely related compounds to interfere with gossypol. In the present method, sharper peaks were achieved by increasing the percentage of the organic solvent in the mobile phase from 80 to 90%, since gossypol has a very low water solubility (Fig. 1). However, increasing methanol higher than 90% resulted in a loss of resolution in the presence of some of the gossypol degradation products (data not shown). In the mobile phase, the presence of phosphoric acid was necessary in order to suppress the ionization of the phenolic hydroxyl groups and subsequently prevent peak tailing. Such a change in the mobile phase made the analysis faster, more specific, and more sensitive. The standard curve for gossypol, using the peak area method, was linear over an entire range of 5 ng to 2 μg. At an absorbance range of 0.2 AUFS, the standard curve gave an average area of 103 ± 5(SD) μV sec/ng.

TABLE 1

Analysis of Gossypol Solutions After Storing at 37 C for 29 Days Using HPLC and Spectrophotometry[a]

Solvent	Percent of gossypol remaining as determined by:	
	Spectrophotometry	HPLC
Methanol	12	0.0
Ethanol	60	29
Chloroform	90	40
Acetonitrile	30	15
Acetone	20	10

[a]The original solutions were 1 mg/mL.

Comparison Between the Spectrophotometric and the HPLC Methods

In order to study the specificity of our HPLC method in comparison with the current spectrophotometric method developed by Smith (21-23), gossypol solutions in five different solvents were analyzed by both methods after being stored for 29 days at 37 C. By the end of the storage period, these solutions contained mostly degradation products of gossypol with gossypol accounting for only 0-40% of the initial concentrations measured by the HPLC method (Table I). The results of this study indicate that the spectrophotometric method exaggerated the amount of gossypol when compared to the HPLC method. This finding is explained by the fact that the spectrophotometric method depends upon the reaction of aniline with gossypol to form dianilinogossypol. This reaction takes place through the two carbonyl groups of gossypol which may be present in some other degradation products and thus interfere with the analysis.

Determination of Gossypol in Cotton Plants

Gossypol concentration was determined by HPLC and spectrophotometry in four different cotton seeds, stems and roots of the two different cotton species (Table II). Among the seeds, the highest amount of gossypol was found in the glanded cotton seeds, while less than half of that amount was measured in the low gossypol cottonseeds. Glandless cotton seeds contained approximately 3.6% of the amount present in the glanded cotton seeds while the roasted glandless cotton seeds contained the smallest amount of gossypol. With respect to glanded and low gossypol cotton seeds, the two analytical methods were comparable, while with glandless and roasted glandless seeds, in which the concentration of gossypol was low, the spectrophotometric method failed to detect gossypol (Table II).

The stem of *G. arboreum* contained less gossypol than the stem of *G. thurber*, while the roots of the two cotton species had almost equal amounts of gossypol. Overall, the roots had the highest concentration of gossypol when compared to seeds or stems. In all gossypol determinations, the HPLC method gave higher recovery than the spectrophoto-

TABLE II

Gossypol Concentration[a] in Cotton Seeds, Stems and Roots as Determined by HPLC and Spectrophotometry

Plant part	HPLC		Spectrophotometry	
	Concentration (mg/g dry wt)	% Recovery	Concentration (mg/g dry wt)	% Recovery
Cotton seeds of:				
G. hirsutum				
Glanded	5.8 ± 0.5	92 ± 4	5.3 ± 0.2	80 ± 1
Low gossypol	2.4 ± 0.2	96 ± 8	1.9 ± 0.02	79 ± 7
Glandless	0.21 ± 0.03	105 ± 5	0.0[b]	92 ± 4
Roasted glandless	0.13 ± 0.01	94 ± 4	0.0[b]	84 ± 4
Stem of:				
G. arboreum	0.4 ± 0.05	88 ± 6	0.35 ± 0.15	83 ± 5
G. thurber	0.66 ± 0.03	105 ± 4	1.03 ± 0.45	92 ± 10
Roots of:				
G. arboreum	9.4 ± 0.2	110 ± 9	7.1 ± 0.2	82 ± 5
G. thurber	10.2 ± 1.5	87 ± 7	7.0 ± 1.2	72 ± 6

[a]Values represent a mean ± standard deviation of at least three replicates and each determination was run in triplicate.

[b]The absorbance was below the recommended minimum detectable limit.

TABLE III

Half-Lives of Gossypol in Different Solvents[a,b]

Temperature	Half-life (days)				
	Methanol	Ethanol	Chloroform	Acetonitrile	Acetone
37 C, Water bath	0.7	1.7 28.5[c]	6.8 26.7[c]	19.5 9[c]	33
22 C, Ambient	2.5 5[c]	4.7 86[c]	24 118[c]	150 20[c]	90
5 C, Refrigeration	16.5	27.5 68[c]	203	338	520
-10 C, Freezer	72	105	255	600	980
-25 C, Freezer	>1,000	>1,000	>1,000	>1,000	>1,000
-80 C, Freezer	>1,000	>1,000	>1,000	>1,000	>1,000

[a]Gossypol concentration was 1 mg/mL in all solvents.

[b]Gossypol solutions were anlyzed by HPLC (see text).

[c]In all cases where there are two half-lives listed, the degradation was biphasic and the left column always describes the initial phase while the right column describes the final phase.

metric method (Table II).

Stability of Gossypol in Different Solvents and at Various Temperatures

To study the stability of gossypol, the percentage of gossypol remaining (logarithmic scale) as a function of storage time was plotted for each solvent at each temperature studied on semilogarithmic papers.

Methanol solutions. At 37 C, gossypol solutions in methanol were highly unstable and its decomposition followed exponential decay. Storing at room temperature (approximately 22 C) resulted in a slower rate of decomposition, and a biphasic degradation curve was observed (data not shown). A smaller but still significant amount of gossypol was decomposed when its methanol solutuions were stored under refrigeration at 5 C. At -10 C, an appreciable amount of gossypol was also degraded, while at -25 C and -80 C, gossypol was highly stable.

Ethanol solutions. The degradation of gossypol in ethanol was slower than in methanol under all storage temperatures studied. Gossypol solutions in ethanol showed biphasic exponential decay curves at 37 C, room temperature and refrigeration (5 C), while the degradation followed single exponential decay curves at -10 C, -25 C and -80 C (data not shown). The explanation for the high rate of degradation of gossypol in methanol and in ethanol is unknown. However, both solvents are alcohols which behave as a base in some organic reactions (29) and gossypol is known to be unstable in basic solutions (2).

Chloroform solutions. Gossypol degradation in chloroform was investigated and the results showed that its degradation was slower than that observed in ethanol. The biphasic degradation behavior of gossypol was also observed in chloroform, but only at 37 C and at room temperature (data not shown).

Acetone solutions. When acetone was used as a solvent, HPLC analysis of gossypol was difficult because of the high absorbance of acetone at 254 nm. Therefore, in most cases acetone was evaporated and the residue dissolved in the same volume of methanol prior to analysis. Acetone was found to be the solvent in which gossypol showed the highest stability under all storage temperatures.

Acetonitrile solutions. At 37 C and at room temperature, gossypol solutions in acetonitrile showed biphasic degradation curves in which the initial phase of degradation was slower than the final phase contrary to all previous solvents (data not shown). The explanation for this behavior is unknown; however, our speculation is that acetonitrile may be hydrolyzed stepwise to form acetamide followed by the

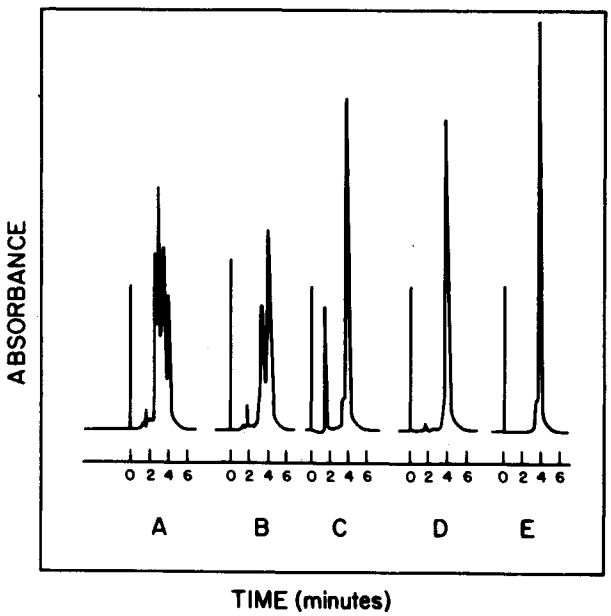

FIG. 2. HPLC chromatograms of gossypol solutions after being stored at room temperature for 6 days in (A) methanol, (B) ethanol, (C) chloroform, (D) acetonitrile, and (E) acetone.

formation of acetic acid and ammonia which may have reacted with gossypol. The sequence of such hydrolysis was dependent on time and temperature; after the hydrolysis occurred in appreciable amounts, the decomposition rate of gossypol increased rapidly. At lower temperatures, gossypol was more stable in acetonitrile than most other tested solvents.

To summarize these stability studies, the half-life of gossypol in each solvent at each storage temperature was calculated and the values were listed in Table III. In cases where there were biphasic degradation curves of gossypol, two half-lives were calculated and listed with the left column describing the initial phase of decomposition. The results show that gossypol in all solvents was highly unstable at 37 C and at room temperature and its stability increased as the storage temperature decreased. It is clear that gossypol solutions in any of the solvents studied should not be stored at 5 C or even at -10 C for long periods of time. However, under very low temperatures, -25 C and -80 C, all gossypol solutions showed greater stability with only 2-5% decomposition observed after three months of storage. At such low temperatures, the solvent used did not have any major effect on the decomposition rate of gossypol. At higher temperatures, the solvent had a pronounced effect on the rate of decomposition of gossypol, and judging from the initial phase of degradation, the stability decreased in the following order: acetone > acetonitrile > chloroform > ethanol > methanol.

Gossypol degradation products formed in various solvents as a result of storage were not identified. However, the profiles of decomposition products were solvent dependent as indicated by HPLC chromatograms (Fig. 2).

The improved HPLC method described in this report was shown to be more reliable, specific and more sensitive than the spectrophotometric method when used to analyze gossypol in various parts of cotton plants. This method also was used successfully in studying the stability of gossypol in various solvents at different temperatures. Thus, this method should prove extremely useful in future studies of gossypol.

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