drolytic scission to alloxan itself; electron-withdrawing halogens on the aryl ring would be anticipated to activate the anil to hydrolysis. Compound Va would appear to be an attractive candidate for radiohalogenation and study as an insulinoma-imaging agent with the caveat that *in vivo* hydrolysis may separate the label-bearing aniline and thwart target uptake of the tracer.

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Analysis of Gossypol and Gossypol–Acetic Acid by High-Performance Liquid Chromatography

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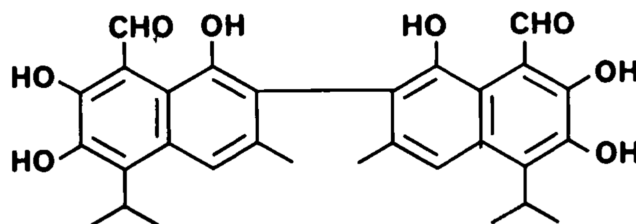
Abstract □ Gossypol, a bis-sesquiterpenoid cotton pigment, is of current interest as a male fertility-regulating agent. For the purposes of analyzing material to be studied biologically, a method is described for the analysis of gossypol by high-performance liquid chromatography. This has been used for examining the purity of gossypol-acetic acid using a UV-absorbance ratio technique.

Keyphrases □ Gossypol—analysis, gossypol-acetic acid, high-performance liquid chromatography □ Contraceptives, male—gossypol-acetic acid, analysis, high-performance liquid chromatography

Gossypol (I), one of several pigments isolated from *Gossypium* (Malvaceae) (1, 2), is found in concentrations of up to 1.7% in cotton seeds (*G. hirsutum* L.). Because of the toxicity of gossypol, cotton seed flour has limited use for humans and domestic animals; the upper limit of gossypol concentration for human consumption has been set at 0.045% (3). Gossypol is reported to be unstable and is, therefore, usually available as the 1:1 complex with acetic acid (1, 2, 4, 5)[1].

Several analyses of gossypol have been described, including complexation with aromatic amines followed by UV analysis (6, 7), GC analysis of the trimethylsilyl ether (8) and *N,O*-bis(trimethylsilyl) acetamide derivatives (9), paper chromatography (10), and TLC (11). A recent (12) communication describing the high-performance liquid chromatographic (HPLC) analysis of gossypol prompts us to report our own efforts in this area[2].

Our interest in gossypol was stimulated by reports that it possessed *in vitro* spermicidal activity (13, 14) and *in*



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[1] For an extensive review of early chemical work on gossypol see Ref. 1; for a broader discussion of gossypol see Ref. 2.

[2] This work was first presented at a meeting of the Core Group of Advisors to the Chemical Synthesis Programme, Task Force on Long-Acting Agents for the Regulation of Fertility, World Health Organization, held in Bethesda, Md., November 1980.

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Table I—Analytical Solvent Systems Used for the HPLC Separation of Gossypol

Solvent	Column			
		μ-Styragel	μ-Bondapak	
	μ-Porasil	500	Cyano	C_{18}
CCl_4		X		
$CHCl_3$				
alone	X	X	X	X
containing 0.01 M HOAc	X			
$CHCl_3$–MeOH (99:1)		X		
CH_2Cl_2		X		
CH_2Cl_2–isopropyl alcohol–H_2O (96:4:0.5)	X			
CH_2Cl_2–MeOH				
10:90			X	X
25:75			X	X
25:75, containing 0.001 M HOAc			X	X
95:5	X			
99:1		X		
CH_3CN				
alone			X	X
containing 0.01 M HOAc			X	X
containing 0.001 M HOAc			X	X
CH_3CN–H_2O (90:10)			X	X
CH_3CN–H_2O–HOAc				
35:65:0.1			X	X
70:20:10			X	X
CH_3CN–H_2O–MeOH (25:30:45)			X	X
MeOH			X	X
MeOH–H_2O (7:3)			X	X
MeOH–0.01 M K_3PO_4 (1:1)			X	X
MeOH–0.01 M KH_2PO_4 (85:15)			X	X
MeOH–0.025 M NaH_2PO_4 (40:60)			X	X
n-Propyl alcohol			X	X
Tetrahydrofuran		X		

vivo antifertility activity in several male mammalian species (rats, hamsters, and rhesus monkeys) (15, 16) including humans (17–19). A preliminary report of our work (20) indicates that gossypol, but not its chemically related impurities, has antifertility activity in male hamsters. A critical basis for these pharmacological experiments was the need to have highly purified gossypol available to be able, categorically, to indicate the nature of the material used for dosing. In this paper we report our efforts on the analysis of gossypol and a determination of the purity of gossypol–acetic acid by a combination of quantitative HPLC and HPLC–UV absorbance ratio techniques (21, 22).

EXPERIMENTAL

Materials—Gossypol [2,2′-bis(1,6,7-trihydroxy-3-methyl-5-isopropylnaphthalene-8-carboxaldehyde), (I)] was supplied as the acetic acid complex[3]. Gossypol, mp 182–184°C, was liberated from the complex by the procedure of Campbell *et al.* (23), followed by crystallization from petroleum ether–ether. Concordance with previously reported physical and spectroscopic data for gossypol (24) was established. A highly purified sample of gossypol–acetic acid[4] was recrystallized three times from methyl ethyl ketone–acetic acid (3:1).

All chemicals and solvents used in this investigation were reagent grade and when used for HPLC were redistilled in glass or were HPLC grade[5]. The melting point was determined using a Kofler hot-stage instrument and is uncorrected.

Equipment—The initial separations were conducted using a liquid chromatograph[6] equipped with a syringe-loading sample injector with 20-μL sample loop[7], a variable-wavelength UV spectrophotometer[8] at 285 nm, and a 25.4-cm strip-chart recorder[9]. Separations were carried out using 30 × 0.39-cm i.d. prepacked columns with a typical flow rate of 1 mL/min[10]. For subsequent analysis of gossypol–acetic acid, the HPLC apparatus used was a combination instrument consisting of a system controller[11], pumps[9] with an added stop–flow valve, a variable-wavelength UV spectrophotometric detector[12] with an automated control[13], an integrator–recorder[14], and an injector[15].

Separation of Gossypol—Table I indicates the analytical solvent systems which were used in a preliminary attempt to separate gossypol from its impurities. Thus, four systems were used with μ-Styragel 500[10], 17 with μ-Bondapak cyano[10], and 17 with μ-Bondapak C_{18} columns[10]. Only one of these systems [μ-Bondapak C_{18} column, CH_3CN:H_2O:HOAc (7:2:1)] was found to be effective for the required separation. Detection was carried out at 254 nm. Gossypol and gossypol–acetic acid had identical retention times in this system.

Purity of Gossypol–Acetic Acid—To examine the purity of gossypol–acetic acid by HPLC, the best wavelength(s) for its detection were first established. Using the analytical solvent system described above, UV spectra of gossypol–acetic acid were obtained on a grating spectrophotometer[16] [complex dissolved in CH_3CN–H_2O–HOAc (7:2:1)] and using a variable-wavelength UV spectrophotometric detector[11] (directly on the solution eluting from the reverse-phase column). Maxima were observed at 254, 263 (sh), 283, 293, and 371 nm. When the absorbance was monitored at 254 nm[17], 260 nm[17], and 290 nm[17], gossypol–acetic acid eluted as a single peak (retention time, 9.18 min), and integration of the area under the peak indicated a minimum purity of 99.90%[18].

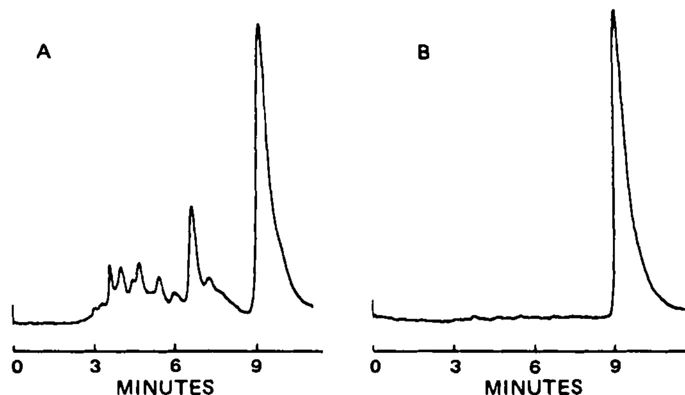

Figure 1—*Typical chromatograms of impure (A) and recrystallized (B) gossypol–acetic acid. For conditions see footnote 17.*

RESULTS AND DISCUSSION

Figure 1 shows typical chromatograms of impure gossypol–acetic acid (A) and the recrystallized gossypol–acetic acid (B) used as a standard. The lower limit of detection was 5 μg at 0.04 AUFS and 5 ng at 0.01 AUFS.

For the chromatographic conditions used, the peak representing gossypol–acetic acid has some tailing. To demonstrate that this peak was intrinsically a single peak and to further substantiate the purity of the gossypol–acetic acid sample, an absorbance ratio technique was utilized (21, 22). In this experiment, the flow of the eluting peak was stopped at the leading edge (point 1), and crest (point 2), and the trailing edge (point 3), as illustrated in Fig. 2. Based on the UV-absorption spectra of gossypol–acetic acid taken in CH_3CN–H_2O–HOAc (7:2:1), absorbancy was

[3] Kindly supplied by Dr. V. Graci, Southern Regional Research Center, ARS, USDA, New Orleans, La.
[4] Kindly supplied by Dr. H. K. Kim, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20205.
[5] Fisher Scientific Co., Pittsburgh, PA 15219.
[6] Model 6000A; Waters Associates, Milford, MA 01757.
[7] Rheodyne model T120; Waters Associates, Milford, MA 01757.
[8] Model 450; Waters Associates, Milford, MA 01757.
[9] Beckman Instruments, Fullerton, CA 92634.
[10] Waters Associates, Milford, MA 01757.
[11] Model 21421; Beckman Instruments, Fullerton, CA 92634.
[12] Model LC85; Perkin-Elmer Corp., Norwalk, CT 06856.
[13] Autocontrol, Model LC75; Perkin-Elmer Corp., Norwalk, CT 06856.
[14] Model C-R1A; Altex Scientific, Inc., Berkeley, CA 94710.
[15] Model 210; Altex Scientific, Inc., Berkeley, CA 94710.
[16] Model DB-G; Beckman Instruments, Fullerton, CA 92634.
[17] Conditions: μBondapak C_{18} (10-μm particle size) column (3.9 mm × 30 cm); eluant CH_3CN:H_2O:HOAc (7:2:1); flow rate, 1.0 mL/min; temperature, 26°C; sample concentration, 0.27 mg/mL; injection volume, 4 μL; sensitivity, 0.04 AUFS; inlet pressure, 1057 psi; chart speed, 10 mm/min.
[18] The chromatographic profile generally indicated a single peak eluting at 9.18 min. Occasionally, a small peak (≤0.3%) eluted at the solvent front (3.42 min) which could be attributed to a system artifact.

Table II—Absorbance Ratios for Gossypol-Acetic Acid

Stopping Point	Absorbance Readings				Absorbance Ratios, λ_1/λ_2					
	254 nm	260 nm	290 nm	370 nm	254/260 nm	254/290 nm	254/370 nm	260/290 nm	260/370 nm	290/370 nm
1. Leading edge	0.063	0.054	0.056	0.031	1.167	1.125	2.032	0.964	1.742	1.806
	0.083	0.071	0.074	0.041	1.169	1.122	2.024	0.959	1.732	1.805
	0.057	0.049	0.051	0.028	1.163	1.117	2.036	0.961	1.750	1.821
	0.064	0.055	0.057	0.031	1.164	1.122	2.064	0.965	1.774	1.838
2. Crest	0.105	0.090	0.094	0.049	1.167	1.117	2.100	0.958	1.837	1.918
	0.106	0.091	0.095	0.051	1.165	1.116	2.078	0.958	1.784	1.863
	0.105	0.090	0.094	0.049	1.167	1.117	2.100	0.958	1.837	1.918
	0.106	0.091	0.095	0.051	1.165	1.116	2.078	0.958	1.784	1.863
3. Trailing edge	0.049	0.042	0.044	0.023	1.167	1.114	2.130	0.955	1.826	1.913
	0.032	0.028	0.029	0.015	1.143	1.103	2.133	0.965	1.866	1.933
	0.043	0.037	0.039	0.021	1.162	1.103	2.048	0.948	1.762	1.857
	0.043	0.037	0.039	0.021	1.162	1.103	2.048	0.948	1.762	1.857
Mean					1.164	1.115	2.073	0.958	1.788	1.866
SD					0.007	0.008	0.037	0.006	0.043	0.045
RSD, %					0.60	0.72	1.78	0.42	1.85	2.41

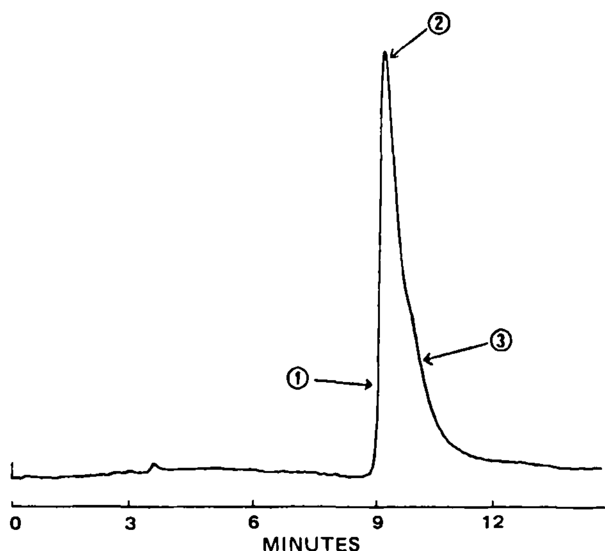

Figure 2—*Points of eluting HPLC peak of gossypol-acetic acid at which absorbance measurements were made at preselected wavelengths. Key: (1) leading edge, (2) crest, (3) trailing edge.*

determined at four preselected wavelengths (254, 260, 290, and 370 nm) each time the flow was stopped. The sensitivity was reduced for higher absorbance readings. Absorbance ratios were then calculated for (λ_1/λ_2) 254/260, 254/290, 254/370, and 290/370 nm. The experiment was done in replicate. The results (Table II) strongly indicate that the chromatographic peak for gossypol-acetic acid consists of a single, spectroscopically (UV, IR, ^{1}H-NMR, ^{13}C-NMR, and MS) pure substance, since the absorbance ratios at the various points of the peak did not differ significantly.

From these results the most consistent and reliable ratios are those involving wavelengths of 254, 260, and 290 nm. From an analysis of variance of the calculated absorbance ratios taking the leading edge, the crest, and the trailing edge as three distinct populations, the results were $F_{2,69} = 0.03$; the reference shows $F_{0.005,2,60} = 5.80$. The three sets of ratios are therefore similar with a 99.5% degree of confidence. This method appears to be a highly effective HPLC technique for the analysis of gossypol-acetic acid, which is superior to those reported previously.

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