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Note

High-performance liquid chromatographic determination of gossypol in plasma

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Gossypol, a yellowish phenolic compound occurring in the pigment glands of the seeds of the plants in genus *Gossypium* [1] has been advocated recently by Chinese scientists as a new antifertility agent for males [2, 3]. Oral administration of gossypol—acetic acid to male rats at a dose level of 15—40 mg/kg/day for 2—4 weeks induced infertility. Because of its instability, the existence of gossypol in plasma should be determined. Tang et al. [4] detected plasma gossypol using a radioactive labelling method. However, this method can not really show the existence of gossypol in plasma. This report describes a high-performance liquid chromatographic (HPLC) method that was developed for the quantitative and qualitative analysis of plasma gossypol. The method can show the occurrence of gossypol in plasma after oral administration.

EXPERIMENTAL

Materials

Gossypol (1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-2,2′-

0378-4347/84/$03.00

binaphthalene-8,8′-dicarboxaldehyde) was purchased as gossypol–acetic acid from Sigma (St. Louis, MO, U.S.A.) and standard solutions were freshly made in methanol (HPLC grade) before use. HPLC solvents were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Glacial acetic acid (RPE) was purchased from Carlo Erba (Milan, Italy). Ethylenediaminetetraacetic acid (EDTA) disodium salt (AR) and benzene (AR) were products of Koch-Light Labs. (Colnbrook, U.K.) and Mallinckrodt (Paris, KY, U.S.A.), respectively.

Gossypol extraction from plasma

Rats were fed with a single dose of gossypol–acetic acid at a dose level of 10, 30 and 100 mg per kg body weight. Blood samples were collected from tails at 0, 1.5, 2, 3, 5, 6, 8 and 9 h after the oral administration of gossypol. Heparin was used as anticoagulant. For extraction of gossypol, 0.15 ml of plasma separated from the blood was added with 0.15 ml of absolute ethanol to precipitate proteins and then followed with 1 ml of saturated solution of EDTA disodium salt. Gossypol was extracted from the water–ethanol phase by adding 1 ml of benzene. After vortex-mixing for 4 min the sample was centrifuged for 10 min at 800 *g* at 10°C. The benzene layer was transferred to another tube. The water–ethanol phase was extracted again by the same method. The combined benzene phases were evaporated to dryness in a stream of nitrogen. The residue was redissolved in 100 μl of absolute methanol (HPLC grade). A 5–15-μl volume of the extract was injected into the column. The same procedure was also applied to smaller plasma samples (down to 100 μl).

High-performance liquid chromatography

A Water Assoc. liquid chromatograph consisting of M 6000A solvent delivery system, M 440 absorbance (UV) detector, a U6K universal injection system and a reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm) were used. Solvents were filtered through Millipore membrane filters, type FH, pore size 0.5 μm, and degassed in an ultrasonic bath prior to use. Standard and extracted gossypol were injected in 5–15 μl of methanol and eluted from the column isocratically at room temperature. The mobile phase consisted of methanol–water–glacial acetic acid (77:20:3). The flow-rate was 2 ml/min. Gossypol was detected and quantitated by monitoring the ultraviolet (UV) absorbance of the column eluates at 254 nm. The peaks of substances were drawn by an Omni-Scribe recorder (Houston Instruments).

RESULTS AND DISCUSSION

Chromatogram

One of the prime objectives of this study was to develop a simple analytical HPLC method that could be used for qualitative and quantitative analysis of gossypol in plasma after oral administration. Since gossypol has many ionizable phenolic groups, the mobile phase has to be acidified to obtain a good performance peak. Abou-Donia et al. [5] added phosphoric acid to the mobile system to decrease ionization of gossypol. But the gossypol used in the experiment was in the form of gossypol–acetic acid (a loosely bound complex of one molecule of gossypol and one molecule of acetic acid [1]), therefore

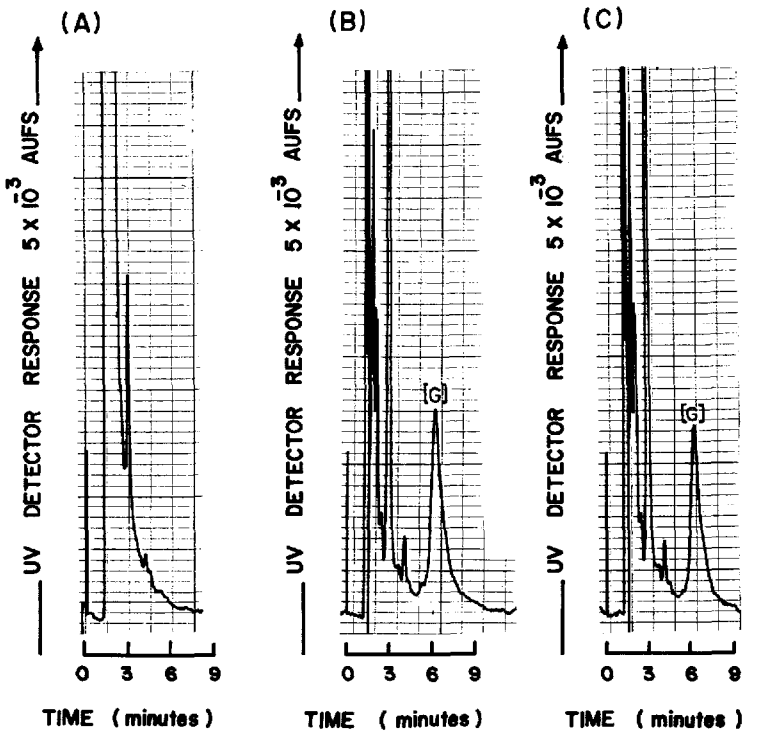

Fig. 1. Chromatograms of HPLC analysis of plasma extracts on a μBondapak C_{18} column of (A) blank plasma extract, (B) plasma extract spiked with 20 ng of gossypol—acetic acid [G], and (C) a 1-h plasma sample obtained from a rat taking a single dose of 10 mg/kg gossypol—acetic acid (corresponding to a plasma level of about 2.44 μg/ml).

acetic acid should be more suitable than phosphoric acid. Fig. 1A and B show typical chromatograms of extracts from plasma samples without and with gossypol—acetic acid as internal standard. The chromatogram of the extract from the plasma of the rat at the first hour after oral administration (force-feed) of 10 mg gossypol—acetic acid per kg body weight is shown in Fig. 1C. Standard gossypol and gossypol extracted from rat plasma have the same retention time. It is 6 min and varied by at most 2% from day to day. The relationship between the amount of gossypol injected and the peak areas was linear and detection by UV at 254 nm was very sensitive.

Percentage recovery and precision

Gossypol can form complexes with many kinds of metal ions [1]. This is why gossypol can not be easily extracted from plasma. However, some chelating agents such as EDTA can break the complexes into free gossypol that can be easily extracted. Benzene was used for the extraction because it can dissolve gossypol rapidly. The intra-assay linearity and precision of the method was evaluated over a concentration range of 1.5—9 μg gossypol—acetic acid per ml of plasma (225—1350 ng gossypol—acetic acid per 0.15 ml) are shown in Table I. Triplicate samples at each concentration of the compound were added to 0.15 ml of plasma and taken through the analytical procedure. The data

TABLE I

LINEARITY AND INTRA-ASSAY PRECISION OF THE HPLC ASSAY FOR GOSSYPOL–ACETIC ACID IN PLASMA

Correlation coefficient (r) = 0.999.

Concentration range	N	Conc. added (μg/ml)	Mean conc. found ± S.D. (μg/ml)	Coefficient of variation (%)	Mean recovery (%)
1.5–9 μg/ml	3	1.50	1.31 ± 0.10	7.63	87.50
	3	3.00	2.48 ± 0.15	6.05	82.72
	3	4.50	3.61 ± 0.18	4.99	80.15
	3	6.00	5.10 ± 0.17	3.33	84.95
	3	9.00	7.35 ± 0.45	6.12	81.62
Mean				5.62	83.39 ± 4.90

TABLE II

LINEARITY AND INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR GOSSYPOL–ACETIC ACID IN PLASMA

Correlation coefficient (r) = 0.999. Each determination was combined from four different dates of assay.

Concentration range	N	Conc. added (μg/ml)	Mean conc. found ± S.D. (μg/ml)	Coefficient of variation (%)	Mean recovery (%)
1.5–16.5 μg/ml	4	1.50	1.33 ± 0.09	6.77	88.50
	5	3.00	2.52 ± 0.12	4.76	84.12
	5	4.50	3.72 ± 0.20	5.38	82.61
	4	6.00	5.04 ± 0.18	3.57	84.00
	5	9.00	7.31 ± 0.44	6.02	81.17
	3	16.5	14.28 ± 0.41	2.87	85.71
Mean				4.90	84.20 ± 4.58

indicated the high degree of linearity of the method with a correlation coefficient (r) of 0.999. The method showed an average coefficient of variation of 5.62%. The recovery of gossypol from plasma was 83.39 ± 4.90%.

Table II shows the inter-assay linearity and precision for the method with a correlation coefficient (r) of 0.999 and average coefficient of variation of 4.90%. The overall recovery was 84.20 ± 4.58%.

Another chromatographic system was also tested in order to ensure that the peaks in the system described above are of gossypol itself and not its degradation products. The mobile phase was changed to a more polar system by increasing the percentage of water. With this system, the retention times of standard gossypol and the extracted gossypol were extended but it is still the same time. However, the peak of gossypol in this system was broader than in the first one.

Gossypol in plasma

Concentrations of gossypol in plasma at different times that were

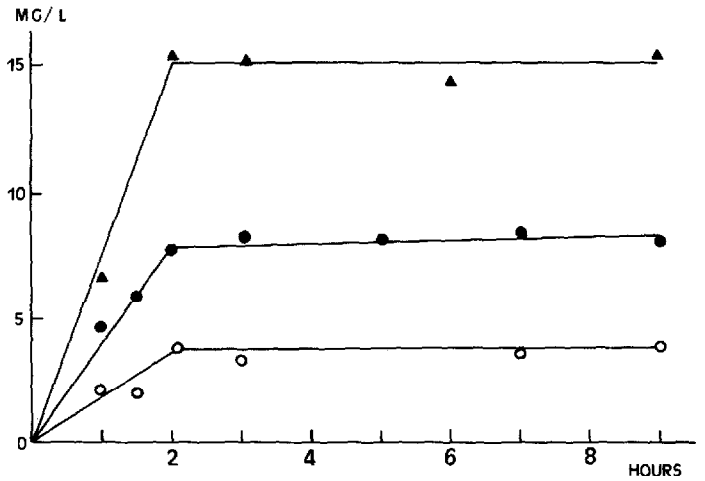

Fig. 2. Gossypol—acetic acid concentrations in plasma from three rats after single oral administration of gossypol—acetic acid: (○), 10 mg/kg body weight; (●), 30 mg/kg body weight; (▲), 100 mg/kg body weight.

quantitated by peak height are presented in Fig. 2. The maximum levels of plasma gossypol appear between the second and the third hour after oral administrations and the levels are maintained for many hours. The maintenance of the level may result from complex formation between gossypol and some metal ions in plasma [1].

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