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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION OF GOSSYPOL IN HUMAN PLASMA

MU-ZOU WANG*

Department of Analytical Chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing (China)

and

DA-FANG WU and YU-WEN YU

Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing (China)

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SUMMARY

A sensitive and selective high-performance liquid chromatographic method with electrochemical detection for the determination of gossypol in human plasma is described. Glutathione is used as a protective agent and gossypol dimethyl ether as an internal standard. Acetonitrile-treated protein-free plasma sample is first introduced on to a C_{18} pre-column for enrichment and clean-up. By using a column-switching technique, gossypol and the internal standard are subjected to further separation on a C_8 analytical column, while the major interfering components are eliminated before entering the column. Methanol—0.1 *M* citrate buffer (pH 3.2) (80:20) is used as the mobile phase. The detector potential on the glassy carbon electrode is maintained at +0.6 V vs. an Ag—AgCl reference electrode. The linearity with human plasma ranged from 5 to 250 ng/ml. The absolute recoveries of gossypol and gossypol dimethyl ether were 91.3 and 97.5%, respectively, with a within-day precision of 2.5% and a day-to-day precision of 3.8%. The limit of detection is 5 ng/ml (signal-to-noise ratio = 3:1). The method is considered to be suitable for the clinical pharmacokinetic studies of gossypol.

INTRODUCTION

Considerable attention has been paid to gossypol since the male antifertility activity of the compound was confirmed both experimentally and clinically in

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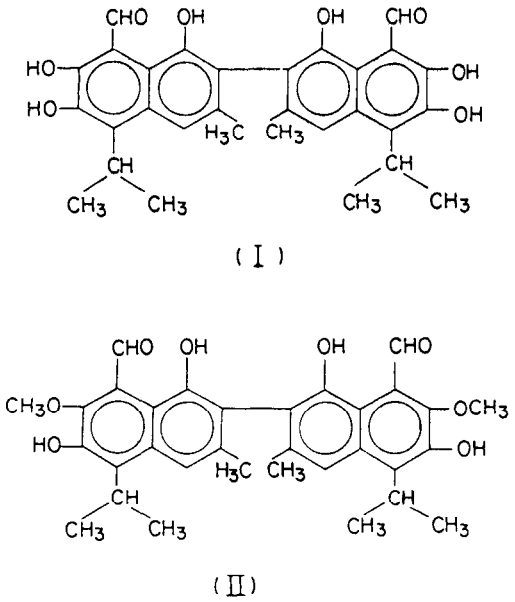

Fig. 1. Structural formulae of gossypol (I) and gossypol dimethyl ether (II).

the early 1970s in China [1]. In spite of the fact that gossypol has proved to be effective in more than 8000 male volunteers in China, the treatment regimen hitherto widely accepted as the "optimum" was completely empirical and requires re-evaluation on a sound pharmacokinetic basis.

Although the metabolism and physiological disposition of gossypol have been studied either colorimetrically or radioisotopically in different species of animals [2—6], no report on the clinical pharmacokinetics of gossypol was found, probably owing to the lack of a sensitive and selective method for the determination of gossypol in human biological fluids.

Gossypol is a binaphthaldehyde compound with polyphenolic hydroxy groups (Fig. 1) which exhibit high electrochemical activity. Thus, Jiang and Zhou [7] were able, although with low sensitivity, to establish a polarographic method for the determination of the compound in plant samples. This paper describes an assay for gossypol in human plasma at clinically achievable concentrations based on reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection. In addition, as gossypol is chemically unstable on handling and the recovery is low after solvent extraction, glutathione is added as a protective agent at the stage of sampling and an acetonitrile-treated protein-free plasma sample is introduced directly into a C_{18} precolumn. A column-switching technique is used, which involves a double pump and double injection valve design and enables gossypol and gossypol dimethyl ether (i.e., the internal standard) to be well separated on a C_8 analytical column, while avoiding entry of the major interfering components into the column. The method was found to be selective, precise and sensitive with high recovery, and was suitable for the clinical pharmacokinetic study of gossypol.

EXPERIMENTAL

Standards, reagents and solvents

Racemic gossypol (in the form of gossypol—acetic acid) and gossypol dimethyl ether were purified/synthesized in the Department of Medicinal Chemistry in this Institute. Stock standard solutions of both compounds in acetone (at concentrations of 100 and 400 μg/ml, respectively) were prepared and stored at −40°C, and were diluted before use to 1 and 4 μg/ml, respectively. These stock solutions were found to be stable for at least one month.

Reduced glutathione was purchased from Sigma (St. Louis, MO, U.S.A.). Sodium thiosulphate, chemical pure grade, was obtained from the Shanghai No. 1 Sulfuric Acid Products Factory and acetonitrile (analytical-reagent grade) from Huang-yan Chemical Products, Zhe-jiang Province (China). The following reagents and solvents were obtained from the Beijing Chemical Engineering: methanol, acetone, and citric acid (all analytical-reagent grade) and trisodium citrate, C.P.; 0.1 *M* citrate buffer solution (pH 3.2) was prepared with redistilled water, kept at 4°C and renewed monthly.

Instrumentation and chromatographic conditions

Apparatus. The high-performance liquid chromatograph included two pumps, two injection valves, a pre-column, an analytical column, an electrochemical detector and a recorder. Pump A was a constant-flow high-pressure pump (Shanghai Chemical Engineering School), pump B was an SY-01 pneumatic pump (Beijing Analytical Instruments), valve A was a Rheodyne 7125 injection valve and valve B was a Shimadzu SIL-1A injector. A Pye Unicam PU 4022 electrochemical detector with a glassy carbon working electrode and an Ag—AgCl reference electrode was used. A Model XWT 264 recorder (Second Shanghai Automatic Meter Factory) was used. These accessories were arranged as illustrated in Fig. 2 so that column switching could easily be performed.

Chromatography. The pre-column was a 30 × 4.6 mm I.D. stainless-steel column packed with Nucleosil C_{18} (10 μm), and the analytical column was a 250 × 4.6 mm I.D. column packed with LiChrosorb RP-8 (10 μm) (Merck). The mobile phase was methanol–0.1 *M* citrate buffer (pH 3.2) (80:20). The flow-rates were set at 1.5 and 1.2 ml/min for pumps A and B, respectively. The detector potential of the glassy carbon electrode was maintained at +0.6 V vs. the Ag—AgCl reference electrode. The sensitivity of the detector was set at 10 nA for full-scale deflection. The chromatography was performed in an air-conditioned room.

Analytical procedure

Sample handling. To each 5-ml glass test-tube were added 0.2 ml of 1% heparin and 0.1 ml of 0.2 *M* reduced glutathione, and the solutions were evaporated to dryness under vacuum. Volumes of 1.0–2.0 ml of human blood were placed in these tubes immediately after sampling and were transported at 0°C. The sample was centrifuged for 15 min and the plasma was transferred into another glass-stoppered test-tube and kept at −40°C.

The frozen plasma samples were thawed shortly before analysis and 0.2–0.4

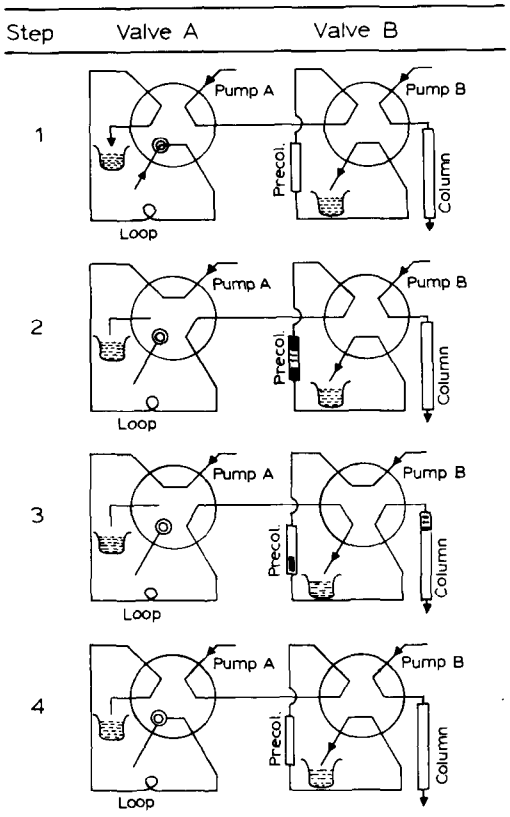

Fig. 2. Schematic diagram of column-switching technique.

ml aliquots were placed in 5-ml glass test-tubes. At the same time, two extra tubes containing drug-free plasma and known amounts of gossypol (10 and 50 ng, respectively) were processed and analysed alongside the unknown samples as simultaneous external standards. To each tube 80 ng of the internal standard and 0.6 ml of acetonitrile were added. The tubes were vortexed and centrifuged and the supernatant was transferred into another conical glass test-tube containing 1 ml of 0.1 *M* citrate buffer solution (pH 3.2). After mixing and then centrifuging for 10 min, the protein-free samples were kept at 0°C and were ready for chromatographic analysis. Normally a 1.0-ml aliquot of each sample was injected into the 1.0-ml loop of valve A.

Instrument operation. The operation cycle for each analysis in which the column switching technique is accomplished can be resolved into four steps as illustrated in Fig. 2.

Step 1.

1. A 1-ml volume of protein-free plasma sample is injected into the loop of valve A, which is in the "loading" mode.
2. Pump A is eluting the pre-column when valve B is in the "loading" mode.
3. Pump B is eluting the analytical column.

Step 2.

1. Pump A is driving the sample into the pre-column as valve A is switched to the "injecting" mode.

2. Pump B continues to elute the analytical column as valve B remains in the "loading" mode.
3. This step lasts for 1 min.

Step 3.

1. Valve A remains in the "injecting" position.
2. Valve B is switched to the "injecting position.
3. This step lasts for 4 min. During this step, components to be analysed are driven into the analytical column, leaving the less polar contaminants behind in the pre-column.

Step 4.

This step lasts for 6 min, and is actually the same as step 1, i.e., chromatography on the analytical column and clean-up of the pre-column (usually this is done by flushing the system with 2 ml of methanol, followed by normal elution), and at the same time another sample is loaded in the loop of valve A.

Calculation. Six plasma standards covering the concentration range from 5 to 250 ng/ml were processed as described above and analysed as calibration samples. A calibration graph was obtained by least-squares regression of the peak height ratios of gossypol to the internal standard against the concentration of gossypol. This calibration graph, corrected by the simultaneous external standards, was used to calculate the concentration of gossypol in unknown samples.

RESULTS AND DISCUSSION

Stability studies

As gossypol is easily oxidized in aqueous solution at ambient temperature, some protective measure is required at the very beginning of sample handling. Thus, the effects of some antioxidants and temperature on the stability of gossypol in plasma samples were studied. The results (Fig. 3) showed that reduced glutathione exhibited the best protective effect. After adding reduced glutathione and keeping at 0°C for 6 h, the loss of gossypol in plasma was found to be less than 5.6%, which was much lower than that of the unprotected control sample (80%). When stored in a −30°C deep-freeze, no appreciable loss was found in such protected samples for at least two months.

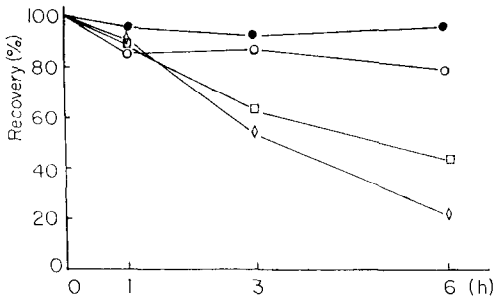

Fig. 3. Effects of some reagents and low temperature on the stability of gossypol (25 ng) in human plasma. (◇) No reagent; (□) sodium thiosulphate; (○) glutathione; (●) glutathione and low temperature (0°C).

TABLE I

RECOVERIES OF GOSSYPOL AND THE INTERNAL STANDARD FROM PLASMA

Drug	Concentration added (ng/ml)	Recovery (%)
Gossypol	25	88.2
	25	90.3
	25	94.5
	75	93.0
	75	96.7
	75	85.7
	250	92.1
	250	88.5
	250	92.7
Average		91.3
S.D.		3.4
C.V.		3.7
Gossypol dimethyl ether	200	103.6
		99.3
		98.3
		99.3
		93.5
		97.8
		92.8
		97.8
		95.0
Average		97.5
S.D.		3.3
C.V.		3.4

Recovery

The results of our preliminary study showed that the recovery of gossypol after conventional solvent extraction with various organic solvents including peroxide-free diethyl ether, chloroform, ethyl acetate and ethyl acetate—acetone (70:30) were unsatisfactory, as none of the absolute extraction recoveries was higher than 60%.

Therefore, solid-phase extraction procedures were studied and various absorbents, including polyamide, diatomaceous earth, XAD-2 and ODS were tested. Among these, we found a C_{18} pre-column enrichment procedure as described above to be the most satisfactory, with absolute recoveries of gossypol and the internal standard added to plasma averaging 91.3 ± 3.4% (S.D.) and 97.5 ± 3.3%, respectively (Table I).

Selectivity

The overall selectivity of an HPLC method with electrochemical detection is the result of integrating the selectivity of the detector and the resolution power of HPLC. The lower the detector potential chosen, the fewer would be the interfering peaks. The electrochemical behaviour of gossypol and gossypol dimethyl ether on a glassy carbon electrode was first studied so that the lowest potential that gave rise to steady current signals could be selected. The resulting

hydrodynamic voltammograms of gossypol and gossypol dimethyl ether are shown in Fig. 4. When + 0.6 V was applied to the electrode, the signal current of gossypol approached a plateau, while the plateau current of the internal standard would not be approached unless the applied potential was higher than +0.8 V. However, we found +0.8 V impractical as it resulted in many interfering peaks and the method suffered from a loss of selectivity. As a

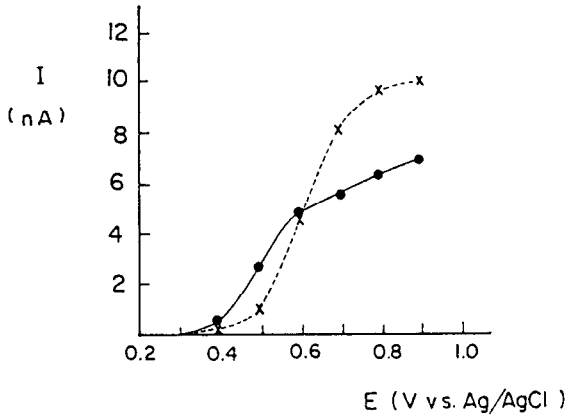

Fig. 4. Hydrodynamic voltammograms of gossypol (20 ng) (•) and gossypol dimethyl ether (40 ng) (×).

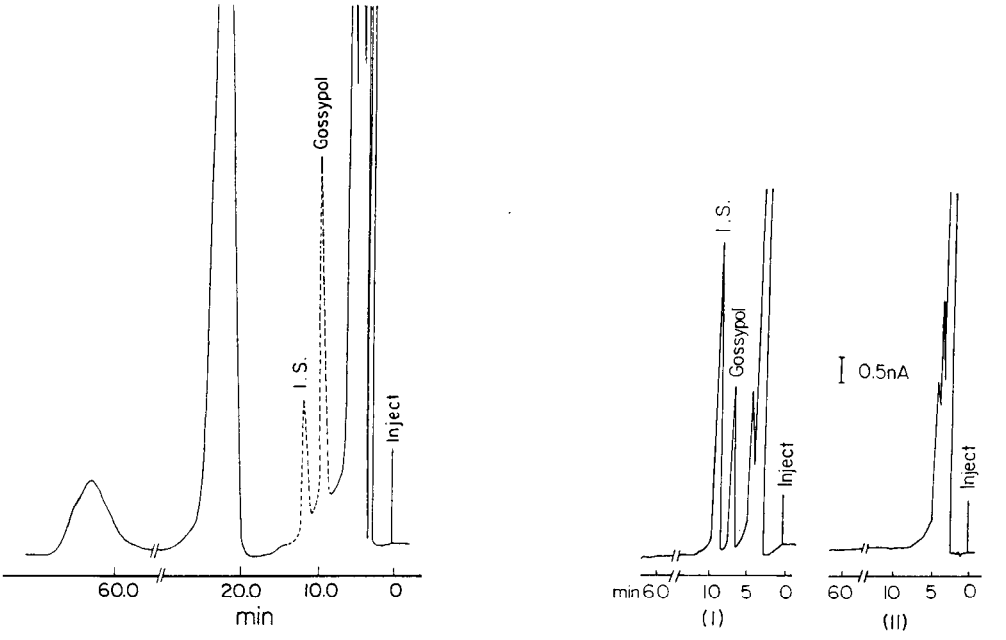

Fig. 5. Chromatogram of normal human plasma without using the column-switching technique, showing many contaminant peaks. The dotted lines represent locations of the peaks of gossypol (30 ng) and gossypol dimethyl ether (25 ng).

Fig. 6. Chromatogram of (I) human plasma containing gossypol (20 ng) and internal standard (100 ng) and (II) drug-free plasma using the column-switching technique.

compromise, we chose +0.6 V as the detector potential in order to gain better selectivity and a plateau signal current of gossypol at the cost of losing some stability of the signal current of the internal standard. Usually the latter decreased gradually by 5.9% during a 2-h period of sample analysis, and this was corrected by regular injections (e.g., one injection after every ten samples) of a fixed amount of the internal standard solution covering the whole period.

As mentioned earlier, we decided to use a C_{18} pre-column enrichment procedure in place of a solvent extraction method for recovery reasons. However, this led to the appearance of large contaminant peaks, both polar and non-polar, in the chromatogram (Fig. 5), which decreased the selectivity of the method, rendered the analysis difficult to carry out and, most important, caused bluntness of the electrode. The column-switching technique as described above was principally designed to solve these problems. A typical chromatogram obtained after column switching is shown in Fig. 6, in which the pre-gossypol polar contaminant peaks decreased markedly and the post-gossypol non-polar interfering peaks disappeared completely.

Interference test

Several drugs commonly used in combination with gossypol in gynaecology clinics were included in the interference study. None of these was found to interfere with the analysis (Table II).

Linearity and sensitivity

A well fitted straight line was obtained by plotting the concentration of gossypol in plasma (ng/ml) against the peak height ratio of gossypol to the internal standard. The relationship was linear over the range 5—250 ng/ml with the following linear regression equation: $y = 0.0198x - 0.0015$ ($r = 0.9999$).

The minimum detectable concentration of gossypol in plasma is 5 ng/ml based on three times the baseline noise.

Precision

Two bulk control standards were prepared by adding known amounts of gossypol to blank plasma at levels of 30.0 and 100.0 ng/ml, respectively. The number of within-day analyses was six for each standard. The estimated con-

TABLE II

RELATIVE RETENTION TIMES (RRT) OF SOME DRUGS

Drug	RRT
Flufenamic acid	0.40
Vitamin K	No peak
Vitamin C	0.36
Glucurone	No peak
Co-phosphatase tablet	No peak
Dicynonum	0.32
Aspirin	No peak
Gossypol	0.76
Gossypol dimethyl ether (internal standard)	1.00

centrations averaged 28.5 ± 0.8 ng/ml (S.D.) and 100.5 ± 2.4 ng/ml, with coefficients of variation of 2.62 and 2.41%, respectively.

Day-to-day variation was studied with a bulk sample from a gossypol-taking patient. The sample was protected by adding glutathione and kept in a −30° C deep-freeze and was analysed repeatedly over fifteen days. The estimated concentrations averaged 69.3 ± 2.6 ng/ml (S.D.) with a coefficient of variation of 3.81%.

Study in man

Five healthy male adult volunteers were given a single oral dose (20 mg) of racemic gossypol—acetic acid. Multiple blood samples were taken and plasma gossypol concentrations were determined. The average peak level was found to be 0.996 mg/l 4—6 h after oral administration, and the mean terminal half-life was as long as 9.0 ± 1.7 days. Fig. 7 shows the plasma concentration curve for one subject.

The fact that a chemically unstable compound such as gossypol remains in the body for such a long time is surprising. It has been reported [8] that gossypol was widely bound to tissue macromolecules, especially proteins and peptides, via aldehyde—amino group linkage. We assume that such binding may afford protection against metabolic degradation and the binding, although covalent in nature, is slowly reversible. As the present method merely determines "free" rather than covalently bound gossypol, it is not sufficient to test the hypothesis by using this method only. Therefore, a sensitive and selective method for the determination of covalently bound gossypol is being developed.

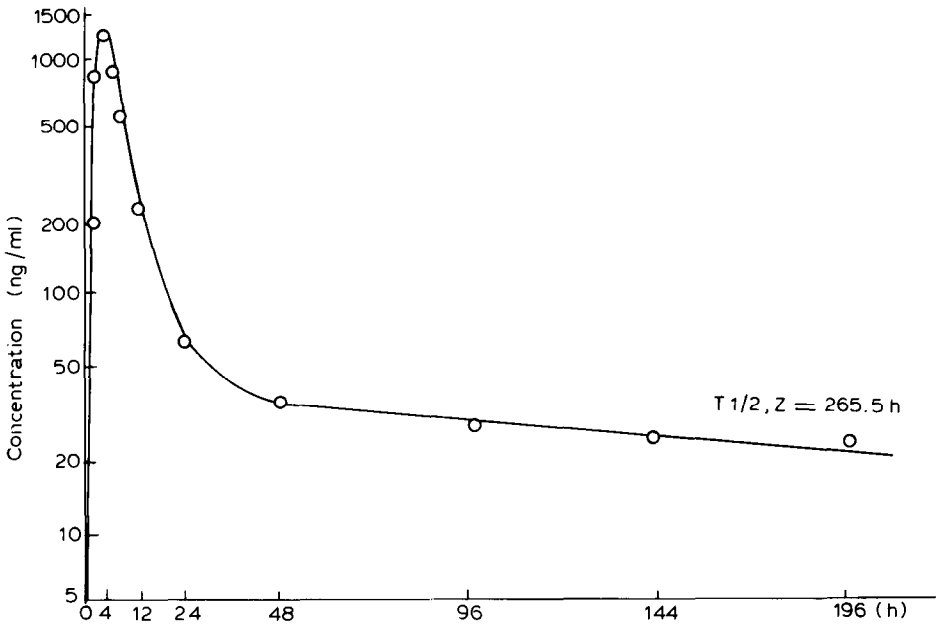

Fig. 7. Plasma concentration—time curve for a male volunteer taking a single dose (20 mg) of racemic gossypol.

CONCLUSION

A sensitive and selective HPLC method with electrochemical detection for the determination of "free" gossypol in human plasma has been developed. The method, although slightly tedious to perform, has been found to be suitable for the clinical pharmacokinetic study of gossypol.

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