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DETERMINATION OF GOSSYPOL ENANTIOMERS IN PLASMA AFTER ADMINISTRATION OF RACEMATE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PRECOLUMN CHEMICAL DERIVATISATION

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SUMMARY

A high-performance liquid chromatographic assay with precolumn chemical derivatisation was developed for the determination of gossypol enantiomers in plasma, after administration of the racemate. Racemic gossypol acetic acid in plasma was extracted into acetonitrile and analysed using a reversed-phase column and a coulometric detector in the redox mode. To separate the enantiomers, 30 μl of the chiral derivatising reagent, (*R*)-(−)-2-amino-1-propanol (50 mg/ml) and 15 μl of 20% (v/v) acetic acid were added to the acetonitrile layer which was then heated at 60°C for 100 min. The mobile phase used to resolve the derivatised enantiomers was 0.2 *M* phosphate buffer (pH 3.5)–acetonitrile (38:62, v/v). At a flow-rate of 1.5 ml/min, the retention times for derivatised (+)-gossypol and (−)-gossypol were 4.0 and 7.8 min, respectively. Two cancer patients received 10 mg racemic gossypol acetic acid three times a day. In one patient, the racemic, (+)- and (−)-gossypol acetic acid plasma concentrations after 65 days of therapy were 317, 213 and 104 ng/ml, respectively. In the other patient, these values were 362, 210 and 152 ng/ml, respectively, after a week of therapy. This represents, to our knowledge, the first determination of the individual enantiomer levels of gossypol after administration of the racemate.

INTRODUCTION

Racemic gossypol acetic acid, a compound with male antifertility activity, is potentially useful in some gynecologic disorders and has antitumor activity. It is

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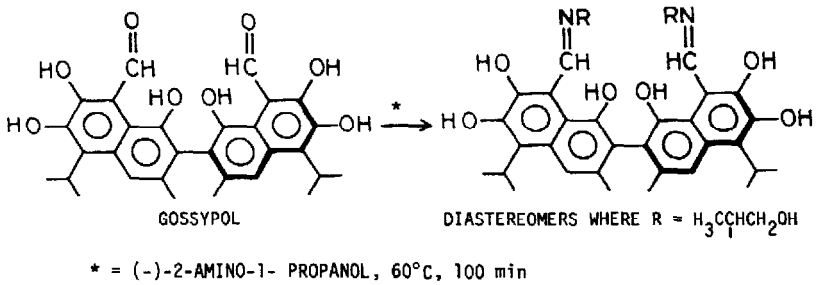

Fig. 1. Chemical structures of racemic gossypol and diastereomeric derivatives.

a polyphenolic, binaphthyl derivative. There is hindered rotation about the internaphthyl bond giving rise to the chiral nature of the molecule (Fig. 1). The antifertility [1–3] and antitumor [4,5] activity reside mainly in the (−)-enantiomer. Many high-performance liquid chromatography (HPLC) methods exist for determining racemic gossypol [6–10], but only one [10,11] is capable of measuring racemic gossypol in human plasma under therapeutic conditions. This method uses a laborious column-switching technique. The HPLC method developed in this paper for measuring racemic gossypol acetic acid in human plasma after therapeutic administration of the racemate uses a reversed-phase column and electrochemical detection in the redox mode (oxidation at detector 1 and reduction at detector 2 with reading of the signal at detector 2). This greatly decreased late-eluting peaks and eliminated the need for column switching, which is a marked improvement. To understand the dispositon of the individual enantiomers of gossypol after administration of the racemate, an HPLC method using precolumn derivatisation with a chiral derivatising reagent was also developed.

EXPERIMENTAL

Standard solutions

Stock solutions of gossypol acetic acid, gossypol dimethyl ether, individual gossypol enantiomers and (*R*)-(−)-2-amino-1-propanol were 1 mg/ml in acetonitrile. Gossypol acetic acid is stable in acetonitrile at 5°C for one year [6]. New stock solutions were made monthly.

Determination of racemic gossypol acetic acid

Heparinized blood (10 ml) was immediately placed on ice and 25 μl of 0.8 *M* reduced glutathione (in water) were added to stabilize gossypol acetic acid. After centrifugation at 1100 *g* (15 min at room temperature), 0.4 ml plasma, 200 ng gossypol dimethyl ether (internal standard) and 0.6 ml acetonitrile were added to a glass test tube. After mixing, 100 mg of sodium chloride were added. After mixing and centrifugation (1100 *g*, 15 min, room temperature), 25 μl of the top acetonitrile layer were injected into the HPLC system.

Chromatographic conditions

A Waters Assoc. (Milford, MA, U.S.A.) reversed-phase Novapak C_{18} column (150 mm × 4.6 mm I.D., 5 μm particle size) in series with a Waters Assoc. guard column containing a μBondapak C_{18} insert was used. The column was connected to a Waters Assoc. 510 pump modified for electrochemical detection. The detector was an ESA Coulochem electrochemical detector (Model 5100A, Bedford, MA, U.S.A.) with conditioning cell at +0.65 V and analytical cell (Model 5011) with detector 1 at +0.55 V and detector 2 at −0.35 V. Measurement of signal was made at detector 2. The mobile phase consisted of 0.1 *M* phosphate buffer (pH 3.5)–acetonitrile (30:70, v/v). At a flow-rate of 1.2 ml/min, the retention times for racemic gossypol acetic acid and internal standard were 6.0 and 11.0 min, respectively.

The detection limit was 25 ng/ml (at a signal-to-noise ratio of 2.0) for racemic gossypol acetic acid in plasma. A standard curve for racemic gossypol acetic acid was always determined concurrently with unknown samples. Peak height of racemic gossypol acetic acid divided by peak height of the internal standard was plotted as a function of racemic gossypol acetic acid concentration. All unknown plasma concentrations of racemic gossypol acetic acid were determined from the standard curve.

Resolution of gossypol enantiomers

Precolumn chemical derivatisation of racemic gossypol acetic acid to form permanent diastereomers was employed to resolve the enantiomers and determine the plasma concentration ratio of (+)- to (−)-gossypol.

Procedure. A 30-μl volume of the chiral derivatising agent (*R*)-(−)-2-amino-1-propanol (50 mg/ml and 15 μl of 20% (v/v) acetic acid were added to a freshly prepared acetonitrile extract of plasma. After heating at 60°C for 100 min, in a Fisher Isotemp dry bath (Model 145, Springfield, NJ, U.S.A.), 25–50 μl were injected into the HPLC system (guard cell, +0.65 V; detector 1, +0.2 V; detector 2, +0.5 V). The mobile phase was 0.2 *M* phosphate buffer (pH 3.5)–acetonitrile (38:62, v/v). At a flow-rate of 1.5 ml/min, the retention times for the derivatised (+)-gossypol and (−)-gossypol were 4.0 and 7.8 min, respectively. Knowing the concentration of racemic gossypol in plasma and the ratio of (+)- to (−)-gossypol enables one to determine the actual concentration of each enantiomer.

In addition, plasma samples containing (+)- and (−)-gossypol enantiomers in different ratios were prepared as follows: 50 ng/ml (+) was added to 500 ng/ml (−) for a (+)/(−) ratio of 0.1; 100 ng/ml (+), 500 ng/ml (−), (+)/(−) ratio of 0.2; 250 ng/ml (+), 250 ng/ml (−), (+)/(−) ratio of 1.0; 500 ng/ml (+), 100 ng/ml (−), (+)/(−) ratio of 5.0; 500 ng/ml (+), 50 ng/ml (−), (+)/(−) ratio of 10.0. These plasma samples were analyzed and the (+)/(−) HPLC peak-area ratios were determined (in duplicate).

Chemicals

Gossypol acetic acid was a gift from Dr. G.M. Waites (World-Health Organization, Geneva, Switzerland). Gossypol dimethyl ether and the pure enantiomers [the (+)-enantiomer was 100% pure while the (−)-enantiomer was 98% pure

as determined by HPLC] were a gift from the Institute of Materia Medica, Chinese Academy of Medical Sciences (Beijing, China). (*R*)-(−)-2-Amino-1-propanol was obtained from Aldrich (Milwaukee, WI, U.S.A.).

Precision studies

Six replicate extractions of plasma containing 200 ng/ml racemic gossypol acetic acid were analyzed and the intra-assay coefficient of variation was determined. Also 200 ng/ml racemic gossypol acetic acid standards in plasma were analyzed on six different days and the inter-assay coefficient of variation was obtained. Ten replicate extractions of plasma containing 250 ng/ml ($n=5$) or 500 ng/ml ($n=5$) racemic gossypol acetic acid were analyzed after derivatisation for the individual enantiomers and the intra-assay coefficient of variation of this part of the assay was determined.

Recovery studies

The recovery of racemic gossypol acetic acid was determined by comparing peak height obtained by injection of a plasma extract of known initial plasma volume and gossypol acetic acid concentration and final extract (acetonitrile) volume with that obtained by direct injection of pure standard. This procedure was also used to measure recovery of the internal standard.

Pharmacology

Two dogs (13.6 and 15.4 kg) received racemic gossypol acetic acid orally at a dose of 40 mg per day. Blood was drawn in the morning just before the next dose on days 2, 6, 10 and 17 for determination of racemic gossypol acetic acid and the individual gossypol enantiomers. In addition, a 77-year-old woman (weight of 87 kg) (patient 1) received racemic gossypol acetic acid orally for experimental treatment of metastatic endometrial cancer. Similarly, an 80-year-old woman (weight of 50 kg) (patient 2) received racemic gossypol acetic acid orally for experimental treatment of ovarian cancer. Blood was drawn 2 h before the morning dose for determination of racemic gossypol acetic acid and the individual enantiomers on several of the days they received the drug.

RESULTS

A chromatogram obtained from analysis of a plasma extract from patient 1 receiving racemic gossypol acetic acid is presented in Fig. 2. The individual gossypol enantiomers, derivatised with (*R*)-(−)-2-amino-1-propanol, from this patient's plasma sample is presented in Fig. 3. The intra-assay coefficient of variation of the HPLC assay for racemic gossypol acetic acid obtained by analysis of a 200 ng/ml plasma standard ($n=6$) was 6.6%. The coefficient of variation of this method obtained from the daily analyses ($n=6$) of a 200 ng/ml racemic gossypol acetic acid standard was 7.7%. The mean derivatised (−)/(+)-enantiomer peak-area ratio after analysis of 250 ng/ml ($n=5$) and 500 ng/ml ($n=5$) racemic gossypol acetic acid in plasma was 0.99 with a coefficient of variation of this part of the assay of 5.9%. The relationship between different (+)/(−)-gossypol en-

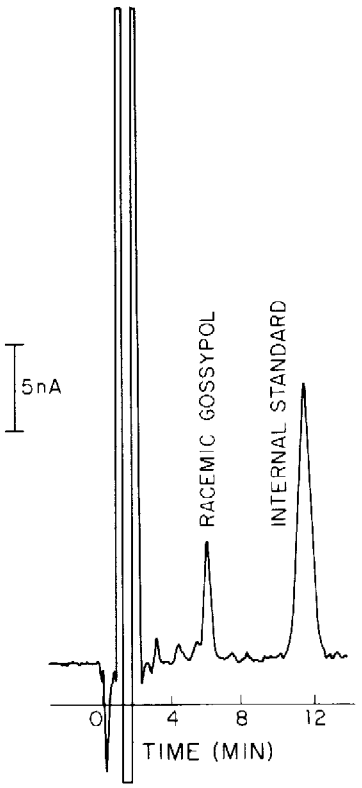

Fig 2. HPLC profile of a plasma extract from patient 1, receiving racemic gossypol. The racemic gossypol level is 138 ng/ml.

antiomer ratios in plasma with the corresponding derivatised (+)/(−)-enantiomer HPLC peak-area ratios is presented in Fig. 4. As can be seen, the slope is close to unity and the line passes very close to the origin ($y=0.92x+0.08$).

A linear relationship between peak-height ratios (peak height racemic gossypol acetic acid/peak height of internal standard) was found from 25 ng/ml to 1.6 μg/ml in plasma ($y=0.96x-0.02$, $r=0.998$).

The recovery of racemic gossypol acetic acid (200–500 ng/ml) and internal standard (500 ng/ml) from plasma was 97 and 103%, respectively).

Several plasma samples containing glutathione from patient 1 were pooled and aliquots stored at −20°C. Periodically over a month, samples were removed from the freezer and assayed for racemic gossypol and the derivatised enantiomers. The mean racemic gossypol plasma level was 121 ng/ml ($n=10$) with an inter-assay coefficient of variation of 8.8%. The mean derivatised (+)/(−)-enantiomer ratio was 0.68/0.32 ($n=10$) with an inter-assay coefficient of variation of 2.9%. There was no change in any of the levels with respect to time. Thus, racemic gossypol and the individual enantiomers were stable for at least a month at −20°C.

The mean plasma gossypol enantiomer concentrations at the end of a dosing interval in two dogs taking racemic gossypol acetic acid orally at a dose of 40 mg per day for seventeen days are presented in Table I. The gossypol enantiomer

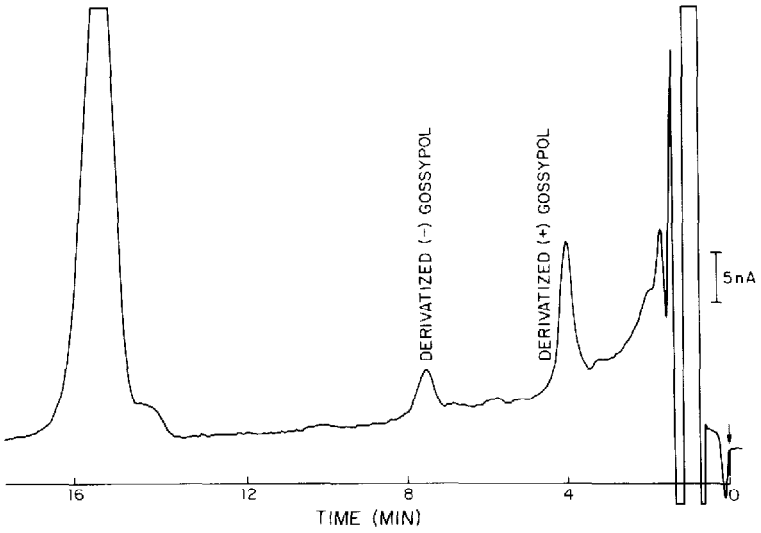

Fig. 3. HPLC profile of derivatised gossypol enantiomers in plasma extract from the patient receiving racemic gossypol in Fig. 2. The (+)/(−)-enantiomer area ratio is 0.71/0.29 The peak at 16 min is from an unknown plasma constituent.

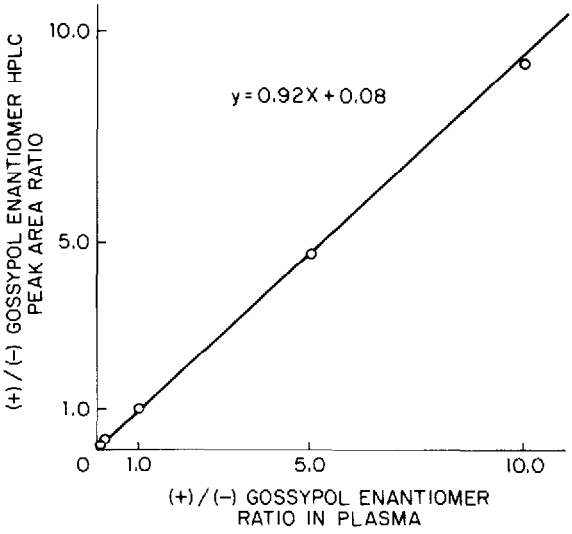

Fig. 4. Relationship between (+)/(−)-gossypol enantiomer ratio in plasma with the corresponding derivatised (+)/(−)-gossypol HPLC peak-area ratio.

plasma levels in the two patients are presented in Table II. In patient 1, the racemic, (+)- and (−)-gossypol plasma concentrations after 65 days of therapy and 2 h before the dose were 317, 213 and 104 ng/ml, respectively. In patient 2, the racemic, (+)- and (−)-gossypol plasma concentrations after seven days of therapy and 2 h before the dose were 362, 210 and 152 ng/ml, respectively.

TABLE I

MEAN PLASMA GOSSYPOL ENANTIOMER CONCENTRATIONS AT THE END OF A DOSING INTERVAL IN TWO DOGS TAKING RACEMIC GOSSYPOL ACETIC ACID ORALLY AT THE DOSE OF 40 mg PER DAY

The values in parentheses represent the individual concentrations from two dogs.

Day	Mean gossypol concentration (ng/ml)		
	Racemic	(+)	(−)
2	71 (71, 70)	28 (27, 29)	43 (44, 41)
6	224 (199, 249)	114 (105, 122)	110 (93, 127)
10	304 (324, 284)	178 (197, 159)	126 (126, 125)
17	303 (379, 226)	168 (223, 113)	134 (155, 113)

TABLE II

GOSSYPOL ENANTIOMER PLASMA LEVELS IN TWO CANCER PATIENTS RECEIVING RACEMIC GOSSYPOL ACETIC ACID ORALLY

Dose was given at 10 a.m., 4 p.m. and 10 p.m. with blood being drawn at 8 a.m., but for the first three days dose was given at 10 a.m. and 10 p.m.

Day	Dose (mg)	Gossypol concentration (ng/ml)		
		Racemic	(+)	(−)
Patient 1				
7	10	113	75	38
9	10	90	62	28
11	10	138	98	40
18	10	133	98	35
23	10	138	99	39
65	10	317	213	104
Patient 2				
3	10	119	76	43
5	10	315	183	132
7	10	362	210	152

DISCUSSION

A sensitive and selective HPLC method for measuring racemic gossypol acetic acid and the individual gossypol enantiomers has been developed. Racemic gossypol acetic acid was resolved by conversion to diastereomeric Schiff base derivatives using the chiral amine (*R*)-(−)-2-amino-1-propanol as the derivatising agent (Fig. 1). The α value (k'_2/k'_1) for this separation is 2.2. Other chiral amines have been used, but the resolution was nowhere near as good [12,13]. The (+)/(−)-gossypol enantiomer ratio in plasma in patient 1 was about 7:3 and in patient 2 was about 6:4 (Table II), 2 h before the dose (10 mg given three times a

day). This represents, to our knowledge, the first determination of the individual enantiomer levels of gossypol acetic acid after administration of the racemate to man.

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REFERENCES

1 M.C. Lindberg, R.H. Naqvi, S.A. Matlin, R.H. Zhou, G. Bialy and R.P. Blye, Int. J Androl., 10 (1987) 619.
2 Z.D. Kai, S.Y. Kang, M.J Ke, Z. Jin and H. Liang, J. Chem. Soc. Chem. Commun., (1985) 168.
3 N.G. Wang, M.Z. Guan and H.P. Lei, Acta Pharm. Sinica, 19 (1984) 932.
4 A.E. Joseph, S.A. Matlin and P. Knox, Br. J. Cancer, 54 (1986) 511.
5 V. Band, A.P. Hoffer, H. Band, A.E. Rhinehardt, R.C. Knapp, S.A. Matlin and D.J. Anderson, Gynecol. Oncol., 23 (1986) 261.
6 A.A. Nomeir and M.B. Abou-Donia, J. Am. Oil Chem. Soc., 59 (1982) 546.
7 N. Sattayasai, J. Sattayasai and V. Hahnvajanawong, J. Chromatogr., 307 (1984) 235.
8 S.A. Abou-Donia, J.M. Lasker and M.B. Abou-Donia, J. Chromatogr., 206 (1981) 606.
9 C.W. Jefford and H.G. Grant, Anal. Chim. Acta, 166 (1984) 311.
10 M.-Z. Wang, D.-F. Wu and Y.-W Yu, J. Chromatogr., 343 (1985) 387.
11 D.-F. Wu, Y.-W. Yu, Z.-M. Tang and M.-Z. Wang, Clin. Pharmacol. Ther., 39 (1986) 613.
12 S.A. Matlin and R. Zhou, J. High Resolut. Chromatogr. Chromatogr. Commun., 7 (1984) 629.
13 D.S Sampath and P. Balaram, Biochim. Biophys. Acta, 882 (1986) 183.