Published in final edited form as:

J Pharm Biomed Anal. 2012 July ; 66: 371–375. doi:10.1016/j.jpba.2012.03.029.

A validated HPLC assay for the determination of R-(-)-gossypol in human plasma and its application in clinical pharmacokinetic studies

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Abstract

R-(-)-gossypol acetic acid (AT-101), a natural BH3 mimetic, is investigated in a Phase I/II clinical trial for the treatment of advanced solid tumor malignancies. Gossypol undergoes rapid degradation in solution phase, which causes major technical difficulty for its quantitation in plasma. We developed and validated a sensitive HPLC assay for pharmacokinetic evaluation of gossypol. Acetonitrile deproteinization method was chosen for sample preparation and Schiff's base derivative, R-(-)-gossypol-diamino-propanol (GDP), was used as internal standard. Chromatographic separation of gossypol in plasma was performed using a Zorbax Eclipse XDB column C₁₈ at 30°C. The mobile phase consists of 10 mmol/L KH₂PO₄ (pH=3.0) and acetonitrile (20:80) at 1.0 mL/min flow rate. Linearity ranged over 56-3585 ng/mL ($R^2=0.9997\pm0.0003$, $n=4$), and the limit of detection was 28 ng/mL. The intra- and inter-assay precision was less than 13.7% and the bias ranged from -7.4 to 7.0%. The method was successfully applied to characterize the pharmacokinetics of AT-101 in a Phase I clinical trial. The validated assay is accurate, and sensitive with minimum loss and rapid analysis time and suitable for quantification of gossypol for pharmacokinetics evaluation.

Keywords

R-(-)-gossypol; HPLC-UV; pharmacokinetics

1. Introduction

Gossypol [1,1',6,6',7,7',-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2'-binaphthelene 8,8'-dicarboxaldehyde], a natural compound isolated from cottonseed and roots, has been investigated extensively as a male contraceptive agent [1, 2]. Recently gossypol and its analogs apogossypolone and apogossypol are shown to have anti-proliferative and cytotoxic effects on a variety of human cancer cell lines, e.g. prostate: VCap, PC-3, and DU145 [3, 4],

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breast: MCF-7 [5], colon: HT-29 [6], leukemia: HL-60 [7], and diffuse large cell lymphoma WSU-DLCL2 [8], and tumor growth inhibition in preclinical xenograft models [9, 10]. R-(-)-gossypol, the biologically more potent gossypol enantiomer (AT-101, Ascenta Pharmaceuticals Inc.) has received development support from National Cancer Institute (USA), and a phase I/II clinical trial was initiated for determining the safety and the maximum tolerated dose of AT-101 in combination with paclitaxel and carboplatin in patients with advanced solid tumor malignancies. Previous phase I and II studies on safety evaluation of AT-101 as a single agent, and in combination with docetaxel or topotecan [11-15] showed some possible survival benefit. As a promising anti-tumor agent, a better understanding of the pharmacokinetics is necessary for the correlation of its therapeutic effect. Previously high performance liquid chromatography (HPLC) methods were reported for the quantitative analyses of gossypol in human, and dog plasma using electrochemical (EC) [16, 17] or ultraviolet (UV) detector [18], and its analog apogossypol in mouse plasma by liquid chromatography-mass spectrometry (LC/MS-MS) [19]. However, HPLC-UV method was not fully validated for the quantitative analysis of gossypol in human plasma. In this present study, we report a fully validated HPLC-UV method for quantitative analysis of gossypol in human plasma using R-(-)-gossypol-diamino-propanol (GDP), as internal standard (a stable Schiff's base gossypol derivative), for the evaluation of clinical pharmacokinetics of AT-101 in patients with advanced solid tumor malignancies.

2. Experimental

2.1. Chemicals and reagents

R-(-)-gossypol was a gift from Ascenta Therapeutics Inc (Malern, PA). (R)-(-)-1-amino-2-propanol (D-alaninol), and dimethylformamide, glutathione (reduced) were purchased from Sigma-Aldrich Co. (St. Louis, MO). HPLC grade solvents methanol and acetonitrile were purchased from Fisher Scientific Inc. (Pittsburg, PA). The control plasma used for the preparation of calibration standards was obtained from the New Brunswick affiliated hospital blood bank (New Brunswick, NJ).

2.2. Instrument and chromatographic conditions

A Finnigan Surveyor LC system equipped with Surveyor quaternary pump, Surveyor auto-sampler and Surveyor PDA and Xcaliber software (Thermo Electron Inc, CA) was used for the analysis and data acquisition. Chromatographic separation of R-(-)-gossypol in plasma was performed using a Zorbax Eclipse XDB C₁₈ column (4.6×150mm, Agilent, Santa Clara, CA) at 30°C. The auto-sampler was maintained at 4°C. The UV detector was set at 254 nm and the mobile phase consisted of 10mM KH₂PO₄ (pH=3.0) and acetonitrile (20:80) at the flow rate of 1.0mL/min. The retention times of R-(-)-gossypol and the internal standard (IS) were 9.7 min and 6.1min.

2.3. Preparation of stock and working solutions

The stock solution of R-(-)-gossypol acetic acid (1mg/mL) was prepared in acetonitrile and was stable for 3 months at -70°C. Since gossypol is unstable in water, methanol, ethanol and alcoholic solvents [20], antioxidant glutathione-reduced (20mM) was added to control blank plasma and patient blood samples to stabilize the drug. The working calibration standards were prepared in plasma by serial dilution of stock standard solution at the concentrations ranging from 0.028 to 3.58µg/mL.

2.4. Preparation of internal standard solution

The internal standard GDP was synthesized as described by Dowd and Pelitire's method [21]. Briefly, 5mg R-(-)-gossypol was added to the complexing reagent consisting of 0.2mL (R)-(-)-1-amino-2-propanol, 1.0mL glacial acetic acid and 8.8mL dimethylformamide and

was heated at 95°C for 30min. After cooling down, the mixture was diluted to 100 times with acetonitrile and used as IS. Under the chromatographic conditions GDP gave a single peak with a retention time ~6 min. The identity of Schiff's base R-(-)-gossypol-diamino-propanol was further confirmed by mass spectrometry m/z 633.1 (Thermo-Finnigan triple Quad TSQ quantum). The plasma standards and the IS solutions were stored in polypropylene tubes at -70°C and thawed on the day of analysis.

2.5. Sample preparation

The calibration standards and patient samples were thawed on ice, and aliquots of 300 μ L samples were pipetted into 1.5 mL polypropylene microfuge tubes and spiked with 10 μ L (8.6nM) of IS. The samples were deproteinized with 600 μ L acetonitrile and vortex mixed for 30s. After centrifuged at 13000rpm (17900 $\times$ g) for 10 min at 4°C, an aliquot of 100 μ L supernatant was injected into the HPLC system for analysis. Extraction of gossypol from plasma in different solvents such as chloroform, dichloromethane, and acetone was attempted; however, on drying even under nitrogen atmosphere, we found gossypol degraded and ended up with poor recovery. Therefore, we choose to deproteinize standards and samples with acetonitrile and direct inject the acetonitrile extracts without further reduction in sample volume.

2.6. Method validation

The selectivity of the method was tested by comparing the chromatograms from four different batches of blank human plasma with the corresponding gossypol spiked plasma. Peak areas of endogenous compounds co-eluting with the analyte were less than 20% of the peak area of the LLOQ standard (lower limit of quantitation). The calibration standards were prepared and assayed on three different days to demonstrate the linearity of this method. Linearity was assessed by the least square linear regression. The lower limit of detection (LOD) was defined as signal/noise ratio of 3. The LLOQ was defined as the lowest analyte concentration that can be determined with precision less than 20% relative standard deviation (RSD). The precision and accuracy of the assay were evaluated at three concentrations (quality control 0.112, 0.896 and 3.58 μ g/mL). Three replicate samples were prepared and analyzed on the same day for intraday and continuous days (n=4) for inter-day accuracy and precision following FDA guide lines [22]. The assay precision was expressed as relative standard deviation and accuracy was calculated as bias. The intra- and inter-day precision was required to be below 15%, and the accuracy to be within $\pm$ 15%. The extraction recovery of R-(-)-gossypol and IS in plasma was measured at three QC concentrations and expressed as the ratio of the peak responses.

The stability of gossypol in plasma was performed under the different storage conditions. The stability test conditions included analysis of QC samples after three cycles of freeze and thaw, storage at room temperature for 4 h and at -70°C for 1 month. Post preparation stability was accessed by re-analyzing the samples after 24hr in auto-sampler at 4°C. All the stability test samples were analyzed in triplicate and determined with freshly prepared calibration standard. The analyte was considered to be stable in plasma when 85% -115% of the initial concentration was found.

2.7. Pharmacokinetics application in patients

The method was applied to evaluate the pharmacokinetics of gossypol in an Institutional Review Board approved phase I clinical trial in patients with advanced solid tumors. AT-101 was administered orally at 40 mg b.i.d. on Days 1, 2 and 3 of a 21 days cycle. On day 1 of each cycle 1 hour after AT-101 treatment, patients were treated with i.v. infusion of paclitaxel at 150 mg/m² (3 hr) followed by i.v. infusion of carboplatin (0.5 hr, AUC 5). Following gossypol administration, blood samples (4 mL) were collected in EDTA tubes at

predose and at 1, 2, 3, 4, 4.5, 5, 6, 8, 10, and 24 hr post administration. Blood samples were placed on ice and transported immediately to the laboratory, and 3 mL whole blood decanted into appropriately labeled polypropylene tubes containing 300 μ L of 200 mM of freshly prepared reduced glutathione in PBS. The contents were mixed and centrifuged at 2300 rpm for 5 min at 4°C and the plasma was transferred to vials and stored at -70°C for future analysis. The pharmacokinetic parameters $C_{\max}$, half-life ($t_{1/2}$), area under concentration-time curve (AUC), apparent clearance (CL) were estimated using WinNonlin 2.1 software (Pharsight Corp., Palo Alto, CA). The data was analyzed using non-compartmental model for PK modeling.

3. Results and Discussion

3.1. Method development

Chromatographic conditions, especially the composition of mobile phase was optimized to achieve resolution of gossypol, IS and other components in plasma. Since gossypol is reported to decompose in methanol even at 5°C, and is stable in acetonitrile [20], we tested mobile phase with different ratio of acetonitrile and water, and acetonitrile:water (80:20) was selected for mobile phase. For the selection of IS, we evaluated two of the previously reported compounds gossypol dimethyl ether [17], and apogonein [19]. Gossypol dimethyl ether is not commercially available and has to be synthesized in multiple steps from gossypol to gossypol hexamethyl ether, which is then demethylated to yield gossypol dimethyl ether, a cumbersome and time consuming procedure [23]. In addition the purity and yield have to be determined. When we used apigenein as an IS, the retention time was close to the void volume and found not suitable for HPLC-UV analysis. Since gossypol and Schiff's base derivative of gossypol have different chromatographic retention time, but have similar UV absorption characteristics, we derivatized R-(-)-gossypol to Schiff's base R-(-)-gossypol-diamino-propanol by following the simple one step procedure and used as IS. Under the chromatographic conditions, GDP separated as a single chromatographic peak with no interference from gossypol at 254 nm. Figure 1 shows the typical chromatograms of blank plasma (A), blank plasma spiked with gossypol and IS (B), a patient plasma sample at predose (C) and 4.5 hr after oral treatment of AT-101 (D).

3.2. Sample preparation method

Sample preparation is important for the acceptable recovery and reproducible results. Since gossypol is unstable in aqueous and alcoholic solutions at room temperature, the stability of gossypol in plasma with different antioxidants was investigated in detail. The percentage of gossypol remaining in plasma as a function of storage time was plotted at 4°C and room temperature (not shown). R-(-)-gossypol showed good stability in mobile phase acetonitrile/water (80:20), which was retained without oxidation after 24hr at 4°C. At room temperature in plasma more than 60% of R-(-)-gossypol was degraded over 24hr, when compared to freshly prepared samples, whereas, similarly prepared samples stored at 4°C retained about 74.5% in plasma over 24hr. The stability of R-(-)-gossypol (5 μ g/mL) in plasma with 20 mM reduced glutathione and 1% ascorbic acid at 4°C can improve the stability of R-(-)-gossypol. However, at room temperature, none of these antioxidants increased the stability of gossypol in plasma. Based on instability of gossypol in water phase, we choose the single step ice cold acetonitrile plasma-deproteinization extraction procedure and performed all operations on ice. It is necessary to add 20 mM glutathione for preparing patient plasma samples during storage. As shown in Table 1, the stability of quality control samples with 20mM glutathione was investigated under the various conditions throughout the validation process. The results show quality control plasma samples with 20 mM glutathione can keep gossypol stable at -70°C for 1 month or on ice for 4 hr. The acetonitrile extracts of samples were stable at 4 °C autosampler in 24 hr. However, the freeze-thaw stability after 3 cycles

showed significant degradation of gossypol at the lower concentration, therefore the patient samples should not be thawed repeatedly.

3.3. Method validation

The HPLC assay showed acceptable linearity over the range of 0.056–3.585 $\mu\text{g/mL}$ ($R^2=0.9997\pm0.0003$, $n=4$) with the LOD of 28 ng/mL and LLOQ of 56 ng/mL. Table 2 summarizes the intra and inter-day precision and accuracy. Intra-day and inter-day precisions were less than 13.2%. Accuracy (bias) ranged from -7.4 to 7.0%. The direct deproteinization method showed satisfactory intra and inter-assay precision and accuracy for quantitation of gossypol in plasma. In the PK studies, the measured concentrations of R-(-)-gossypol at 12 hours post dose were within calibration range and the limits of detection.

The recovery of R-(-)-gossypol and IS in control plasma at the different concentrations was compared to the same concentration in acetonitrile:water (80:20). The recovery of R-(-)-gossypol and IS in plasma standards was consistent. The extraction recoveries of gossypol and IS in plasma were in the range of 87.8% ~101.1%.

3.4. Application in pharmacokinetic studies

This HPLC assay was applied to determine the concentration of R-(-)-gossypol in plasma of solid tumor patients treated with AT-101, paclitaxel and carboplatin. The large inter-individual variation following oral administration could be due to food and other factors (transporters) in the gastrointestinal tract which might affect the absorption of gossypol. The calculated pharmacokinetic parameters are listed in Table 3. Average of R-(-)-gossypol $\text{AUC}_{0-10\text{hrs}}$ was $3.53 \pm 1.33 \mu\text{g/mL}\times\text{h}$. The pharmacokinetic parameters of gossypol ($t_{1/2}$, C_{max}) were comparable with a previous study using HPLC coupled with electrochemical detector [16].

4. Conclusions

A simple and sensitive HPLC assay was developed and fully validated for quantitation of R-(-)-gossypol in plasma. The method was developed using 300 μL of human plasma for determination of gossypol with a lower limit of quantitation (56ng/mL) using GDP as the internal standard. The HPLC assay is applicable for R-(-)-gossypol quantitation in clinical pharmacokinetics studies.

Acknowledgments

The authors gratefully acknowledge NCI and the Cancer Institute of New Jersey for support. The authors acknowledge the assistance of the Tissue Analytic Service of CINJ for patient sample collection.

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Highlights

- The studies describe a validated and sensitive HPLC assay for R-(-)-gossypol pharmacokinetic evaluation of gossypol.
- A simple acetonitrile deproteinization method was applied for plasma sample preparation with satisfying recovery.
- A Schiff's base derivative, R-(-)-gossypol-diamino-propanol was synthesized and used as internal standard.

Figure 1.

Representative HPLC chromatogram from extractions of a blank plasma (A); 224.0 ng/mL gossypol with IS (B); and a patient plasma sample at predose (C) and 4.5hr (D).

Table 1
Stability of R-(-)-gossypol in human plasma by HPLC assay (n=3, Mean± S.D., * n=2)

Added concentration (µg/mL)	Remained concentration (%)			
	On ice, 4hr	Three freeze-thaw cycles	-80°C, 1 month	Autosampler, 4°C, 24h*
0.112	100.1±2.4	71.9±4.5	99.3±4.0	95.6
0.896	101.5±2.0	81.6±1.7	109.3±5.3	97.3
3.585	103.8±6.1	86.9±1.1	111.8±5.6	100.6

Table 2

Precision and accuracy of R-(-)-gossypol

Conc. nominal ($\mu\text{g/mL}$)	Intraday (n=3)		Interday (n=4)	
	Measured ($\mu\text{g/mL}$) Mean $\pm$ S.D.	Precision (%)	Accuracy (Bias %)	Precision (%)
0.056(LLLOQ)	0.058 $\pm$ 0.002	2.9	-7.3	13.2
0.112	0.105 $\pm$ 0.002	1.6	-6.4	7.4
0.896	0.892 $\pm$ 0.014	1.5	0.2	2.4
3.585	3.575 $\pm$ 0.120	3.5	0.3	0.5

Table 3
Plasma pharmacokinetic parameters of R-(-)-gossypol (Cycle 1 Day 1)

Patients [*]	AUC _(0-10hrs) (µg/mL*h)	Clearance (L/h)	t _{1/2} (hr)	C _{max} (µg/mL)
#1	1.74	17.86	3.19	0.32
#2	5.12	6.35	3.14	0.92
#3	3.69	8.64	3.11	0.68
#4	4.34	7.90	2.91	0.87
#5	2.74	9.21	4.30	0.49
Average	3.53	9.99	3.33	0.66
SD	1.33	4.53	0.55	0.25

^{*}Treated with combination of AT-101 40mg bid,(p.o.), paclitaxel (150mg/m², i.v. 3 hours), followed by carboplatin (AUC 5, i.v. 30min).