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Quantitative determination of apogossypol, a pro-apoptotic analog of gossypol, in mouse plasma using LC/MS/MS

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Abstract

A simple and selective liquid chromatography/tandem mass spectrometry (LC/MS/MS) method based on internal standard quantitation using apigenin as the internal standard has been developed and validated for the analysis of the gossypol analog apogossypol, a pro-apoptotic compound, in mouse plasma. The methodology involves protein precipitation of plasma samples followed by LC/MS/MS analysis. Ascorbic acid was added to the spiking solutions and plasma samples to stabilize the easily oxidized compound. Separation of apogossypol and the internal standard from the plasma matrix was achieved using a C18 column with a gradient elution profile consisting of 5 mM ammonium acetate and methanol. The validated range of the method extended from 10 to 2000 ng/mL with accuracies of 85–115% and precision of <15%. The average recovery of apogossypol at three concentrations (50, 200 and 1000 ng/mL) assayed in triplicate using this methodology was determined to be 90.8 ± 12.9%. Recovery for the internal standard (apigenin) at a concentration of 500 ng/mL was found to be 99.9 ± 6.41%. Apogossypol concentrations of 50 ng/mL and above were found to be stable in extracted plasma for 24 h when stored at 25 °C. This method has been applied to the determination of apogossypol concentrations in plasma collected from mice given an IV dose of apogossypol.

Keywords: Apogossypol; Gossypol; LC/MS/MS; Quantitative analysis

1. Introduction

Overexpression of anti-apoptotic Bcl-2-family proteins Bcl-2 or Bcl-X_L occurs during apoptosis dysregulation in many malignancies, resulting in resistance to cytotoxic anticancer drugs. Recent studies have shown that Bcl-2 and Bcl-X_L are targets of (±)gossypol [1], a polyphenolic dialdehyde compound found in cottonseed which has been tested for treatment of metastatic adrenal cancer, glioblastoma and breast cancer with low but measurable responses.

Enantiomeric (−)Gossypol has been purified and shows some improved efficacy and pharmacokinetic properties compared to the racemic compound [2]. The presence of two highly reactive aldehyde groups in gossypol is very likely a limitation to its therapeutic successes. By using a combination of molecular modeling and structural biology techniques, (±)-apogossypol, which lacks the reactive aldehyde groups and retains pro-apoptotic activity, has recently been synthesized and shown to bind to and inhibit the Bcl-2-family proteins, Bcl-2 and Bcl-X_L, inducing apoptosis of the tumor cell lines [3]. Quantitative measurement of gossypol and its analogs in bio-matrixes has been achieved and refined for more than 25 years, including high-performance liquid chromatography [4–6] electrochemical detection [7–9], radiolabeling [10] and fluorescence quenching titrations [11]. However, these methods can be time-consuming and labor-intensive, in that they involve several steps in sample preparation. In addition, UV detection is not always feasible with biological samples due to potential interferences with the analytes. In preparation for preclinical studies, a selective liquid chromatography/tandem mass spectrometry (LC/MS/MS) method has been developed and validated for the analysis of apogossypol in mouse plasma. The methodology involves protein precipita-

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tion of plasma samples followed by LC/MS/MS analysis and has been applied to the determination of apogossypol concentrations in plasma collected from mice dosed IV with apogossypol. The results of the studies are reported herein.

2. Experimental

2.1. Chemicals and materials

(±)-Apogossypol (NSC D732804-K) was provided by the National Cancer Institute (Bethesda, MD) (Fig. 1). Apigenin (internal standard) was purchased from Indofine Chemical Co. (Hillsborough, NJ). HPLC-grade acetonitrile and ammonium acetate were purchased from Fisher Scientific (Atlanta, GA, USA). Reagent grade ascorbic acid was purchased from Acros Organics (Pittsburgh, PA). Deionized organic free water was obtained from an in-house system.

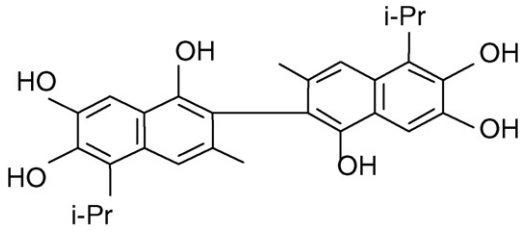
2.2. Standard and sample preparation

2.2.1. Preparation of stock and working solutions

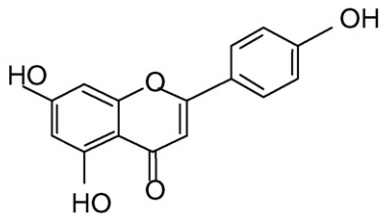
A stock solution of apogossypol (1 mg/mL) was prepared in acetonitrile:10% ascorbic acid (9:1, v/v) and subsequently diluted with acetonitrile:10% ascorbic acid (9:1, v/v) to yield eight working solutions over a concentration range of 100–20,000 ng of apogossypol/mL. A stock solution of apigenin (1 mg/mL) was prepared in acetonitrile and diluted with acetonitrile to make a 5 μg/mL spiking solution.

2.2.2. Spiking and extraction from plasma

For the preparation of the calibration standards, mouse plasma (100 μL), fortified with 1% ascorbic acid (final concentration), was spiked with the appropriate working solution of apogossypol to achieve concentrations in plasma ranging from 10 to 2000 ng/mL. After the addition of 10 μL of apigenin spiking solution to each plasma standard, plasma proteins were precipitated by the addition of acetonitrile (200 μL). After centrifugation for 5 min at 9000 × *g*, the resulting supernatant was analyzed by LC/MS/MS.



Apogossypol, MW =462



Apigenin, MW = 270

Fig. 1. Chemical structures and molecular weights for apogossypol and apigenin (internal standard).

2.3. Instrumentation

The LC/MS/MS system consisted of a Perkin-Elmer (Norwalk, CT) series 200 autosampler, and two Series 200 micro-flow pumps with a Phenomenex Synergi Fusion RP, 100 mm × 2 mm 4 μm particle column (Torrance, CA, USA) maintained at ambient temperature. Detection was accomplished using a PE Sciex API 3000 (Toronto, Canada) triple quadrupole mass spectrometer operated in the positive ion mode. Mass calibration, data acqusition and quantitaion of apogossypol were performed using Applied Biosystem Analyst 1.4 software (Applied Biosystems, Foster City, CA).

2.4. Chromatographic conditions

The mobile phase consisted of 5 mM ammonium acetate (A) and methanol (B) and was delivered at a flow rate of 400 μL/min using a gradient elution profile. The starting mobile phase composition was 80% A/20% B and was increased linearly to 5% A/95% B over a 3 min period after an initial 0.5 min hold, then held at 5% A/95% B for 1.5 min, before returning to 80% A/20% B (step gradient), with subsequent re-equilibration for 2.5 min. The injection volume for the method was set at 10 μL.

2.5. Mass spectrometer conditions

The LC/MS/MS was equipped with a Turboflow electrospray ion source operated at 450 °C and at a potential of 5 kV. The orifice and ring potentials were set at 30 and 200 V, respectively. High purity nitrogen was used as the curtain and collision gas with a CAD gas setting of 5 producing a pressure of 3.5×10^{-5} Torr. The analyte and internal standard were detected using multiple reaction monitoring (MRM) for the following transitions: apogossypol (*m/z* 463.2 → 231.4); apigenin (*m/z* 271.1 → 152.9). A dwell time of 200 ms was used for each ion transition. Collision energies were optimized for each transition and consisted of 33 eV for apogossypol, and 45 eV for apigenin.

2.6. Calculations

Concentration calculations were performed using Analyst (Version 1.4). The amount of analyte in each plasma sample (ng/mL) was back calculated using a calibration curve generated from a set of calibration standards. A quadratic fit with 1/*X* weighting was determined to be the best fit due to the wide concentration range investigated.

2.7. Stability

2.7.1. Stability in solvent

During the course of method development an apparent instability of apogossypol was noted in the stock and spiking solu-

tions. In order to address this instability, the effect of ascorbic acid on the stability of apogossypol prepared in acetonitrile was investigated. Stock solutions containing apogossypol at a concentration of 2.5 μg/mL were prepared in acetonitrile and stored at 4 and 25 °C for 72 and 6 h, respectively, in the presence and absence of 1% (v/v) ascorbic acid. These times were chosen to emulate the amount of time solutions would be needed. The samples were assayed using LC/MS/MS for apogossypol concentration.

2.7.2. Stability in plasma

As a result of the observed instability of apogossypol in acetonitrile, stability in mouse plasma was also investigated. Mouse plasma was spiked with apogossypol (10 μg/mL) in the presence and absence of ascorbic acid (1% final concentration). Individual plasma mixtures were then incubated at 37 °C for 1, 2, 4, 8 and 24 h. After incubation, the samples were extracted and analyzed by LC/MS/MS. The stability of apogossypol was determined from the amount of apogossypol measured in each stability study sample as compared with the amount of apogossypol measured in a freshly prepared standard of apogossypol in the same biological matrix.

2.8. Method validation

Method validation consisted of analyzing two composite curves comprised of three individual standard curves, each prepared and analyzed on separate days. The selectivity of the method was determined by measuring the level of interfering components in blank pooled mouse plasma. Accuracy and precision were determined from triplicate determinations per day of calibration standards at eight different concentrations of apogossypol. Recovery of apogossypol from plasma was determined by comparing the response of apogossypol in plasma after extraction to the response of the same concentration of apogossypol spiked into extracted plasma water.

The short term stability of apogossypol in mouse plasma under sample preparation conditions was determined. Plasma was fortified with ascorbic acid, spiked with apogossypol (200 and 2000 ng/mL), and maintained at 25 °C for 1 h. The samples were then extracted as described above and compared to freshly prepared standards.

To determine the integrity of apogossypol in extracted plasma samples during the time course of an analytical run, the stability was determined for a 24-h time period. Calibration standards from a curve prepared on the first day of validation were stored in the autosampler and were re-injected on the second day of validation. The composite curve from the freshly prepared day 2 standards were used to quantitate the calibration standards from day 1.

2.9. Pharmacokinetic study of apogossypol in mice

Male CDF1 mice were procured from Charles River Laboratories (Raleigh, NC). The mice were approximately 12 weeks old and weighed between 24 and 27 g on the day of dosing. The mice were group housed in polycarbonate cages lined with hardwood chip bedding (P.J. Murphy Forest Products Corp., Montville, NJ). Animals were fed Certified Rodent Diet #5002 (PMI Feeds, Inc., St. Louis, MO) and provided city (Birmingham, AL) tap water, *ad libitum*. Cage size, animal care, and environmental conditions conformed to the guidelines of the U.S. Department of Agriculture (Animal Welfare Act; Public Law 99-198) and to those of the *Guide for the Care and Use of Laboratory Animals*.

The dose formulation of apogossypol was prepared in ethanol:cremophor EL:saline (10/10/80, v/v/v) to contain 2.8 mg/mL of apogossypol. Individual mice were dosed IV, via a tail vein, with 28 mg/kg of apogossypol, in a dose volume of 10 mL/kg. At each of the following times after dosing, four mice were anesthetized with CO_2/O_2 and then bled from the retro-orbital sinus: 2, 5, 10, and 30 min and 1, 2, 4, 8, 12, and 24 h. Each blood sample was collected into a 0.6 mL tube containing EDTA and 0.06 mL of 10% ascorbate (yielding an approximate ascorbate concentration of 1% in each blood sample) and immediately placed on ice. Individual blood samples were centrifuged to separate plasma. Each plasma sample was stored ≤ -70 °C until analyzed by LC/MS/MS for levels of apogossypol.

Pharmacokinetic parameters were calculated from the plasma concentration versus time data using WinNonlin® (Professional Version 4.1; Pharsight Corp., Mountain View, CA).

3. Results and discussion

3.1. Stability

3.1.1. Solvent stability

In that apogossypol is easily oxidized in acetonitrile, it has previously been shown that the addition of the anti-oxidant ascorbic acid (1%, v/v) increases apogossypol stability [12]. Briefly, apogossypol (2.5 μg/mL) was incubated in acetonitrile at two temperatures (4 and 25 °C) at two different time intervals (Table 1). At 4 °C, apogossypol appeared to be stable after 72 h with or without the addition of ascorbic acid, while at 25 °C the apogossypol concentration decreased to 76.6% after 6 h in the absence of ascorbic acid. In the presence of 1% ascorbic acid, apogossypol was determined to be stable at 25 °C for 6 h in acetonitrile.

3.1.2. Plasma stability

The stability of apogossypol in mouse plasma (10 μg/mL) was explored at 37 °C in the presence and absence of ascorbic acid (1%, v/v) at various times over a 24-h time course. In the absence of ascorbic acid, apogossypol was found to be rapidly degraded at 37 °C such that after 1 h only 48.9 ± 2.21% remained. After 8 h less than 2% of the starting concentration

Table 1
Stability of 2.5 μg/mL apogossypol dissolved in acetonitrile with and without ascorbic acid (1%, v/v) and stored at 4 or 25 °C

Storage temperature	Percent of remaining − ascorbic acid	Percent of remaining + ascorbic acid
72 h at 4 °C	95.2	115
6 h at 25 °C	76.6	104

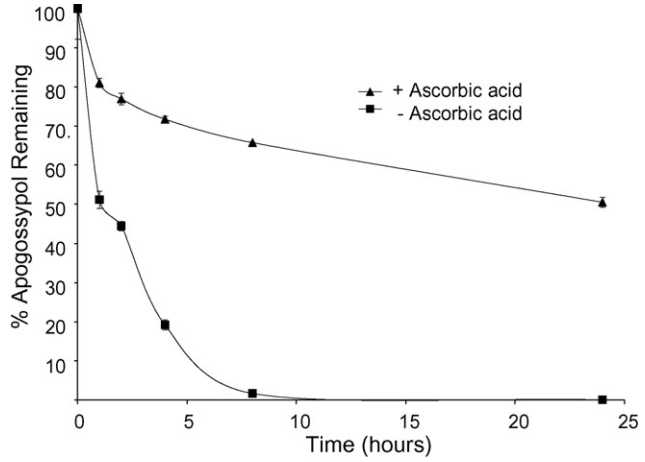


Fig. 2. Mouse plasma was spiked with apogossypol for a final concentration of 10 μg/mL and incubated at 37 °C with (■) and with out (▲) the ascorbic acid.

of apogossypol remained, while essentially none remained after 24 h. In the presence of ascorbic acid, 81.0 ± 1.19% of the original concentration remained after 1 h and 50.5 ± 1.25% after 24 h. The comparison of these results is shown in Fig. 2. The addition of ascorbic acid appeared to somewhat increase the stability of apogossypol in mouse plasma at 37 °C as well as in acetonitrile; therefore, during the course of the in vivo experiments, ascorbic acid (1%) was added to the apogossypol stock solutions and standards, as well as the plasma samples, upon blood collection.

3.2. Method validation

3.2.1. Selectivity and range of the curve

The selectivity of the method was determined by extracting and analyzing pooled drug-free mouse plasma. Representative ion chromatograms of drug-free and spiked mouse plasma containing 100 ng/mL of apogossypol and 500 ng/mL of apigenin are shown in Fig. 3A and B. The absence of peaks at the measured mass transitions and retention times of apogossypol (*m/z* 463.4 → 231.4, 4.5 min) and internal standard (*m/z* 271.1 → 152.9, 4.2 min) in the drug-free trace provide evidence of no endogenous interferences in drug-free plasma (Fig. 3).

Linearity for the method was observed between apogossypol concentrations of 10–2000 ng/mL as detected from a 100 μL sample size of mouse plasma. The resulting regression coefficients of the composite calibration curves were greater than 0.995 on each of the 2 days of validation.

3.2.2. Intra- and Inter-assay accuracy and precision

The intra-assay accuracy and precision of the method was determined from the results of the analysis of three individual standard curves that were prepared and analyzed on two separate days. The intra-assay accuracy of the method ($n = 3$) ranged from 95.5 to 106% on the first day of validation and 97.2 to 102% on the second day of validation. With one exception (19.7% for the LLOQ), the range of CVs was 3.30 to 13.0% on day 1 and 2.12 to 12.1% on day 2 of the validation (Table 2).

The inter-assay accuracy and precision of the method was determined from back calculated values ($n = 6$) for calibration

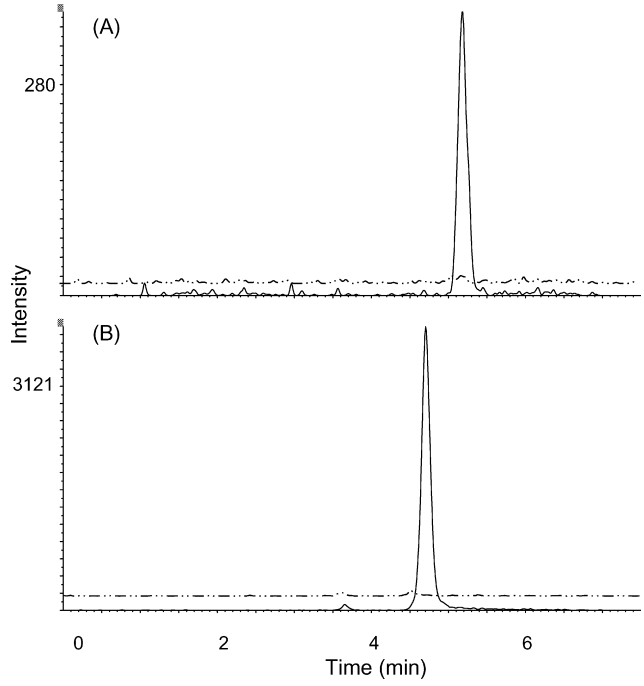


Fig. 3. MRM chromatogram of the transition for (A) apogossypol, *m/z* 463.4–231.4 from mouse plasma spiked with apogossypol for a final concentration 100 ng/mL (lower solid line) and blank plasma (upper dashed line), and (B) apigenin (internal standard) *m/z* 271.1–152.9 spiked mouse plasma (lower solid line) and blank plasma (upper dashed line).

standards at all eight concentrations assayed in triplicate over 2 days. Inter-day accuracy ranged from 98.0 to 104% and inter-day precision ranged from 4.21 to 13.0% (Table 3).

3.2.3. Recovery from plasma

The recovery of apogossypol and the internal standard, apigenin, were determined from mouse plasma. Drug-free plasma was spiked at three separate concentrations of apogossypol (50, 200, and 1000 ng/mL) along with one concentration of apigenin (500 ng/mL) and extracted as described above. An identical volume (100 μL) of plasma-water produced from drug-free plasma was spiked with apogossypol and apigenin at the same concentrations as for plasma. The percent recovery was determined by comparing the peak areas of the spiked plasma to those in

Table 2
Intra-day accuracy and precision of calibration standards spiked with apogossypol and extracted according to the method ($n = 3$ unless noted otherwise)

Nominal concentration (ng/mL)	Day 1		Day 2	
	Accuracy (%)	Precision (%CV)	Accuracy (%)	Precision (%CV)
10	97.2	19.7[a]	97.2	12.1
20	105.9	9.2[a]	102	6.12
50	100.6	12.5	100	7.96
100	95.5	3.3	102	2.12
200	103.4	5.4	98.9	3.49
500	97.0	12.8	99.0	2.52
1000	102.7	13.0[a]	101	5.38
2000	99.8	12.7	99.9	3.47

[a] $n = 2$.

Table 3
Inter-day accuracy and precision of calibration standards spiked with apogossypol and extracted according to the method

Nominal concentration (ng/mL)	Accuracy (%)	Precision (%CV)	*n*
10	97.2	13.0	5
20	104	6.64	5
50	100	9.40	6
100	98.6	4.21	6
200	101	4.76	6
500	98.0	8.26	6
1000	102	7.64	5
2000	99.8	8.32	6

Table 4
Extraction recovery of apogossypol in mouse plasma ($n = 3$)

Nominal concentration (ng/mL)	% Recovery
50	108
200	84.9
1000	80

the spiked plasma water. The average recovery of apogossypol from plasma ranged from 80 to 108% with an overall average of 90.8 ± 12.9%. The recovery appeared to be concentration dependent, with higher recovery at lower concentrations. The recovery of apigenin at a single concentration spiked into plasma was 99.9 ± 6.41% (Table 4).

3.2.4. Short term stability in mouse plasma

The measured values of plasma standards maintained at 25 °C for 1 h were 109 ± 13.6% for 200 ng/mL and 101 ± 0.89% for 2000 ng/mL when compared to freshly prepared standards. This data suggests that apogossypol is stable under sample preparation conditions.

3.2.5. Stability in mouse plasma extracts

The stability of apogossypol in extracted mouse plasma was determined by re-injecting a standard curve from the first day of validation approximately 24 h later after storage at room temperature. The results suggested that after storage for approximately 24 h, apogossypol was stable in the extracts only at concentrations greater than 50 ng/mL. The average response for concentrations ranging from 50 to 2000 ng/mL was 108% of day 1 (Table 5).

Table 5
Stability of apogossypol in processed calibration standard extracts after 24 h at 24 °C

Nominal concentration (ng/mL)	Day 1	Day 2	Percent of day 1
20	22.6	16.4	72.6
50	57.3	53.8	93.9
100	94.8	94.3	99.5
200	194	236	122
500	482	537	111
1000	933	1080	116
2000	1990	2150	108

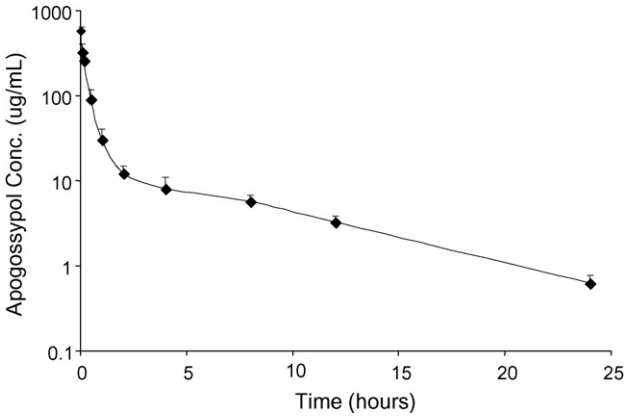


Fig. 4. Plasma concentration vs. time profile of apogossypol for mice given an intravenous dose of 28 mg/kg.

3.3. Plasma concentrations of apogossypol in mice

The plasma concentration–time profile of apogossypol in mice given a single IV bolus dose of 28 mg/kg is shown in Fig. 4. At 2 min after dosing, plasma concentrations of apogossypol ranged from 492 to 662 μg/mL (mean ± S.D.: 574 ± 70.6 μg/mL). Plasma concentrations decreased rapidly during the first hour after dosing and ranged from 21.3 to 43.4 μg/mL (mean ± S.D.: 30.6 ± 9.46 μg/mL) at 1 h. Thereafter, plasma concentrations decreased at a much slower rate. At 24 h after dosing, apogossypol was measured at concentrations ranging from 0.473 to 0.787 μg/mL (mean ± S.D.: 0.622 ± 0.148 μg/mL).

Pharmacokinetic parameters were calculated from the plasma concentration versus time data using compartmental analysis. Data were fit to a three compartment model (Model 18). The estimated half-lives of apogossypol were <0.05, 0.22, and 5.2 h. The extended half-life of the third compartment, relative to the *in vitro* stability of apogossypol in plasma, may have been reflective of protein binding of apogossypol and/or redistribution from tissues to plasma. The estimated total body clearance (CL) and volume of distribution at steady state (V_{ss}) of apogossypol were 82 mL/h kg and 219 mL/kg, respectively (Table 6).

Table 6
Pharmacokinetic parameters calculated from plasma concentrations of apogossypol for mice given a single intravenous dose of 28 mg/kg

Parameter	Value
C_0[a]	12799 μg/mL
Alpha-HL[b]	<0.05 h
Beta-HL[c]	0.22 h
Gamma-HL[d]	5.2 h
AUC[e]	341 μg h/L
CL[f]	82 mL/h/kg
V_{ss}[g]	219 mL/kg

[a] C_0 was the extrapolated 0 h concentration.
[b] Half-life of the alpha-phase.
[c] Half-life of the beta-phase.
[d] Half-life of the gamma-phase.
[e] Area under the plasma vs. time concentration curve from 0 to ∞ calculated as: $\mathrm{AUC} = \int_b^a c(t)\,dt$.
[f] Total body clearance.
[g] Volume of distribution at steady state.

4. Conclusion

A sensitive LC/MS/MS method was developed and validated for the quantitation of apogossypol in mouse plasma. Application of the extraction procedure to mouse plasma samples yielded a recovery of greater than 90% of added apogossypol. The assay showed good intra- and inter-assay accuracy and precision. The lower limit of quantitation of apogossypol in mouse plasma was determined to be 10 ng/mL. The method was applied to the pharmacokinetic analysis of plasma samples collected from mice given an IV dose of apogossypol.

Although apogossypol is readily oxidized in solvent and plasma, it was found that addition of 1% ascorbic acid stabilized apogossypol in both solvent and plasma samples, allowing for the determination of apogossypol concentrations in plasma from *in vivo* samples.

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