Bioanalytical Report

Determination of R(-)Gossypol (AT-101) Concentrations in Precipitated Human Plasma from "A Phase 1, Open Label Study of AT-101 Plus Adjuvant Temozolomide for Patients with Newly-Diagnosed Glioblastoma Multiforme"

Ascenta Study AT-101-CS-007

Prepared For:

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ANALYST ACKNOWLEDGEMENT

I, the undersigned, acknowledge that the laboratory work performed on this project was executed in accordance with USFDA regulations and MicroConstants' SOPs.

Christy Bethel
Staff Scientist
Bioanalytical Chemistry
MicroConstants, Inc.

8/28/08
Date

QUALITY ASSURANCE STATEMENT

It is the objective of MicroConstants, Inc. that all studies conducted by our facility shall be of the highest quality and meet or exceed the criteria set forth by the USFDA to ensure the quality and integrity of the data generated.

The QA department has audited bioanalytical report MC07B-0032.01 entitled "Determination of R(-)Gossypol (AT-101) Concentrations in Precipitated Human Plasma from 'A Phase 1, Open Label Study of AT-101 Plus Adjuvant Temozolomide for Patients with Newly-Diagnosed Glioblastoma Multiforme" for accuracy and compliance with all applicable SOPs and has documented any deviations from these requirements.

The audit findings were submitted to the analyst and the CSO on the following dates:

Study Phase	Audit Date(s)	Date Reported to Analyst and CSO
Reporting	06/25-26/08	06/26/08

Based on the audit findings, the report, raw data and records were found to be an accurate reflection of the study.

During the conduct of this study, there were no deviations from USFDA regulations, which affected the quality or integrity of the study. All audit findings have been appropriately addressed by the analyst and accepted by the QA department.

Jose V Buenviaje
Quality Assurance Director
MicroConstants, Inc.

08/28/08
Date

COMPLIANCE STATEMENT

We, the undersigned, declare that this study was performed under our guidance and supervision and was conducted and reported in compliance with USFDA regulations and MicroConstants' SOPs. The information contained in this report presents a true, unaltered and accurate record of the results.

David F. Beyerlein
Laboratory Director
Bioanalytical Chemistry
MicroConstants, Inc.

08/28/08
Date

Gilbert N. Lam, Ph.D.
President/CSO
MicroConstants, Inc.

08/28/08
Date

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1.0 INTRODUCTION

The Bioanalytical Chemistry department at the San Diego facility of MicroConstants, Inc. has determined the concentrations of R(-)gossypol in precipitated human plasma samples in support of an Ascenta Therapeutics study (AT-101-CS-007) entitled "A Phase 1, Open label Study of AT-101 Plus Adjuvant Temozolomide for Patients with Newly-Diagnosed Glioblastoma Multiforme." The project was initiated on April 19, 2007 and completed on November 27, 2007.

2.0 EXPERIMENTAL

2.1 Principles of the Method

Human plasma samples containing R(-)gossypol with reduced glutathione and maleic anhydride as stabilizing agents and K_3EDTA as the anticoagulant were precipitated with acetone, vortex mixed and centrifuged. Hypophosphorus acid was added to an aliquot of the supernatant. Gossypol-d_2 was added as the I.S. and the samples were vortex mixed and centrifuged. The samples were diluted with water and analyzed by reversed-phase HPLC using a Thermo Electron BetaBasic-4 HPLC column maintained at 40°C. The mobile phase was nebulized using heated nitrogen in a Z-spray source/interface and the ionized compounds were detected using MSMS. Details of the method can be found in MicroConstants' analytical method MN05086.

2.2 Relevant Method Validation Findings

This method was validated following MicroConstants' SOPs for validation of chromatographic analytical assays. Based on the validation results summarized in MicroConstants' report MC05373, the calibration range of the assay was from 10.4 to 1040.0 ng/mL. R(-)gossypol is stable in precipitated human plasma for 66 days when stored frozen at -70°C (MicroConstants' report MC05373). All samples were received without the precipitation solution. No stability has been generated for non-precipitated samples. Samples were stored between 3-36 days before precipitation.

2.3 Reference Standards

R(-)Gossypol (Ascenta) was used to prepare the stock standard and working standard solutions. An overall correction factor, as shown in the following table, was used to adjust for purity (0.993), water content (0.17% = 0.9983) and salt correction (518.5634/578.616 = 0.89621). Two weighings for R(-)Gossypol were prepared 6 days after the expiration date. Gossypol-d_2 (Ascenta) was used as the I.S.

Reference Standard	Lot #	Retest Date	Overall Correction
R(-)Gossypol	3146.D.05.601	08/02/06[a]	0.88842

[a] The sponsor approved the use of this lot beyond the expiration date

2.4 Calculations

Peak heights of R(-)gossypol and gossypol-d_2 were acquired using MassLynx v. 4.0 (Waters, Milford, MA). The calibration curves were obtained by fitting the peak height ratios of R(-)gossypol/gossypol-d_2 and the standard concentrations to a power regression equation using MassLynx. The equations of the calibration curves were then used to interpolate the concentration of R(-)gossypol in the samples using their peak height ratios.

3.0 RESULTS AND DISCUSSION

3.1 Study Conduct

3.1.1 Sample Receipt

A total of sixty-three (63) samples were received from Cleveland Clinic Foundation, Henry Ford Hospital and Hospital of the University of Pennsylvania between March 29, 2007 and October 25, 2007. The samples were received frozen, in good condition and were stored in a freezer set to maintain -70°C. Sample discrepancies were reported to the study director and resolved prior to analysis.

3.1.2 SOP Deviations

One deviation from MicroConstants' SOPs occurred during the conduct of this project. The reference standard was used beyond the expiration without client authorization. Based on the known stability data for gossypol, the use of the reference standard beyond the original expiration is not expected to impact the results of this study.

3.1.3 Sample Analysis

Of the sixty-three (63) samples received, forty-two (42) were unique samples and twenty-one (21) were duplicate samples. All the unique samples were analyzed in two (2) analytical runs. The analytical runs met the acceptance criteria set for the standard curve and QC samples. The values for the samples are reported following MicroConstants' SOPs, which contain criteria for determining the reported value.

During the analysis, the standards and QC concentrations were shifted by 4% resulting in standards ranging from 10.0 to 1000.0 ng/mL and QC samples of 30.0, 300.0 and 800.0 ng/mL. The shift in concentration was not expected to affect the results of the analysis. A partial validation will be initiated to demonstrate the validity of the shifted standards and QCs and the results shall be reported in MicroConstants report, MC08B-0018.

3.2 Performance of the Analytical Method

The standards and QC samples for the analytical runs calculated within the acceptance criteria documented in the MicroConstants' SOPs. The results of the analysis are summarized below.

3.2.1 Regression Parameters

The regression parameters of the calibration curve for R(-)gossypol are summarized in Table 1. The correlation coefficients of the calibration curve were not less than 0.9991 (Table 1). The results show that the assay can be described by a power equation in human plasma for the concentration range of 10.0 to 1000.0 ng/mL.

3.2.2 Accuracy

The accuracy of the method was determined by comparing the mean measured concentrations with the theoretical concentrations of the compound in the calibration standards and QC samples. For R(-)gossypol standards, the deviation of the mean from theoretical values did not exceed ±10.5% (Table 2). For the R(-)gossypol QC samples, the deviation of the mean from theoretical values did not exceed ±5.9% (Table 3).

3.2.3 Precision

For the QC samples prepared in precipitated human plasma, the precision of the method was determined from the %CV of the mean of the QC sample replicates at each concentration level. The %CV for the R(-)gossypol QC samples ranged from 4.5% to 9.7% (Table 3).

3.3 Sample Results

The human plasma concentrations of R(-)gossypol for cohorts 1 and 2 are reported in Tables 4 and 5, respectively. All sample concentrations are reported in accordance with applicable MicroConstants' SOPs.

4.0 CONCLUSIONS

A validated method was used for the determination of R(-)gossypol concentrations in precipitated human plasma in support of an Ascenta study (AT-101-CS-007). Based on standard curve and QC sample acceptance criteria outlined by MicroConstants' SOPs, the data presented in this report are an accurate determination of the concentration of R(-)gossypol in all study samples.

5.0 SAMPLE AND DATA RETENTION

Any remaining study samples will be archived at MicroConstants in accordance with MicroConstants' SOPs. The samples will be retained in a freezer pending notification of the Sponsor to determine the final disposition of the samples.

All raw data, documentation, records, protocols and an original signed copy of the final report will be retained in the archives of MicroConstants following the signing of the final report in accordance with MicroConstants' SOPs.

6.0 TERMS AND DEFINITIONS

%CV: Percent Coefficient of Variation (Relative Standard Deviation)

%DEV: Percent Deviation of a Value from Theoretical

CSO: Chief Scientific Officer

K_3EDTA: Potassium Ethylene Diamine Tetra-Acetic Acid

HPLC: High-Performance Liquid Chromatography

I.S: Internal Standard

LLOQ: Lower Limit of Quantitation

MS/MS: Tandem Quadrupole Mass Spectrometer

QA: Quality Assurance

QC: Quality Control

SD: Standard Deviation

SOPs: Standard Operating Procedures

USFDA: United States Food and Drug Administration

7.0 REFERENCES

Ascenta Study AT-101-CS-007
A Phase 1, Open Label Study of AT-101 Plus Adjuvant Temozolomide for Patients with Newly-Diagnosed Glioblastoma Multiforme, Ascenta Therapeutics, Inc., San Diego, CA.

MC05373
Validation of a Method for the Determination of R(-)Gossypol in Human Plasma using Racemic High-Performance Liquid Chromatography with Mass Spectrometric (MS/MS) Detection, MicroConstants, Inc., San Diego, CA.

MN05086
Method for the Determination of R(-)Gossypol in Human Plasma using Racemic High-Performance Liquid Chromatography with Mass Spectrometric (MS/MS) Detection, MicroConstants, Inc., San Diego, CA.

Table 1

Calibration Curve Parameters for R(-)Gossypol in Precipitated Human Plasma

Analytical Run	Analysis Date	Coefficient (A)	Exponent (B)	Correlation Coefficient
AR06[a]	05/04/07	1.628E-01	0.9030	0.9991
AR01	11/26/07	7.755E-02	0.9181	0.9995
	Mean	1.202E-01	0.9106	0.9993

Equation: $y = Ax^B$; where y = peak height ratio and x = concentration

[a] Samples were analyzed in PF05504

Table 2

Back-Calculated Calibration Standard Data and Statistics for R(-)Gossypol in Precipitated Human Plasma

Analytical Run	Standard Concentration (ng/mL)										
	10.0		**20.0**	**40.0**	**70.0**	**100.0**	**200.0**	**400.0**	**700.0**	**1000.0**	
AR06[a]	9.00	10.2	21.2	41.3	68.4	b	221.4	377.9	755.6	b	904.9
AR01	9.36	10.1	19.4	38.7	73.8	110.5	212.5	410.9	668.1	940.9	985.6
Mean	9.67		20.3	40.0	71.1	110.5	217.0	394.4	711.9	943.8	
%DEV	-3.3		1.5	0.0	1.6	10.5	8.5	-1.4	1.7	-5.6	

[a] Samples were analyzed in PF05504
[b] Standard excluded due to greater than fifteen percent deviation from theoretical

Table 3

Quality Control Sample Data and Statistics for R(-)Gossypol in Precipitated Human Plasma

Analytical Run	Replicate	Concentration (ng/mL)		
		30.0	**300.0**	**800.0**
AR06[a]	1	31.9	317.9	661.2[b]
	2	33.6	355.9[b]	760.7
	3	32.9	312.8	763.0
	4	28.8	321.1	767.4
	5	29.4	325.0	717.6
	6	37.7[b]	319.1	782.8
AR01	1	28.3	301.6	742.5
	2	28.9	301.9	805.9
	3	27.2	304.0	767.6
	4	28.5	318.3	800.6
	5	30.1	314.7	758.8
	6	29.4	320.6	805.4
	Mean	30.6	317.7	761.1
	%CV	9.7	4.5	5.4
	%DEV	2.0	5.9	-4.9

[a] Samples were analyzed in PF05504
[b] QC sample calculates outside fifteen percent deviation from theoretical

Table 4

Plasma Concentrations (ng/mL) of R(-)Gossypol in Cohort 1 Following Oral 20 mg/kg/day Doses

Time (hr)	Subject I.D.		
	0130[a]	0132[a]	0133[a]
Pre-dose	BQL	BQL	BQL
0.5	BQL	89.9	BQL
1	23.0	371.8	BQL
2	239.2	545.7	14.0
4	556.7	171.2	236.7
6	389.5	419.8	295.8
24	21.6	18.7	16.8

[a] Samples were analyzed in PF05504, AR06

BQL - Below the Quantifiable Limit < 10.0 ng/mL

Table 5

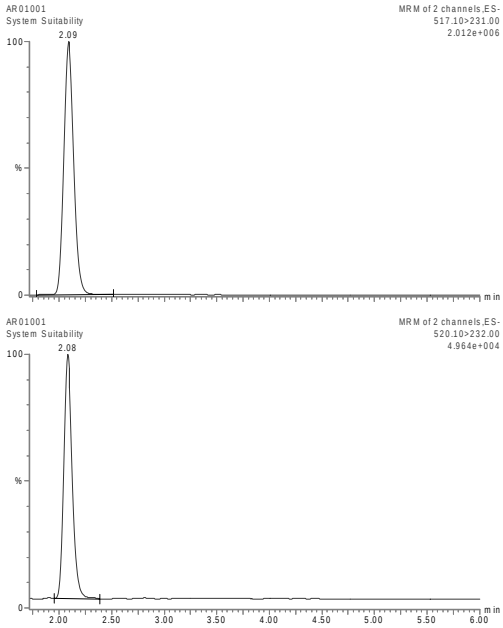
Plasma Concentrations (ng/mL) of R(-)Gossypol in Cohort 2 Following Oral 30 mg/kg/day Doses

Time (hr)	Subject I.D.		
	0447	**0086**	**0449**
Pre-dose	BQL	BQL	BQL
0.5	15.6	BQL	BQL
1	43.2	32.3	107.0
2	176.2	85.1	231.1
4	744.9	489.5	447.5
6	562.7	483.4	507.3
24	24.5	231.5	28.0

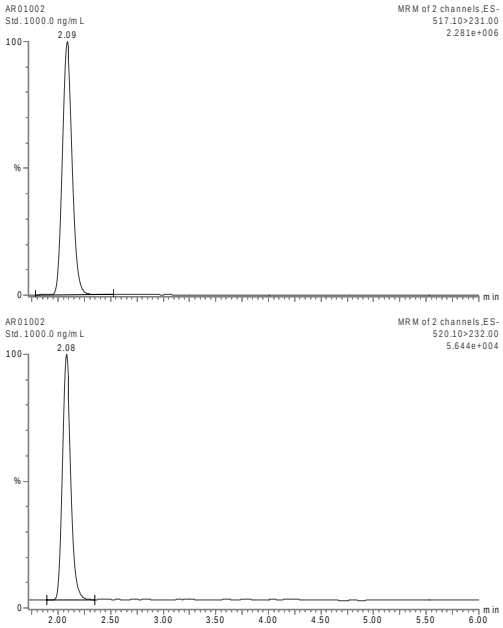
BQL - Below the Quantifiable Limit < 10.0 ng/mL

Appendix A

Chromatograms from AR01



Gossypol on upper panel; I.S on lower panel



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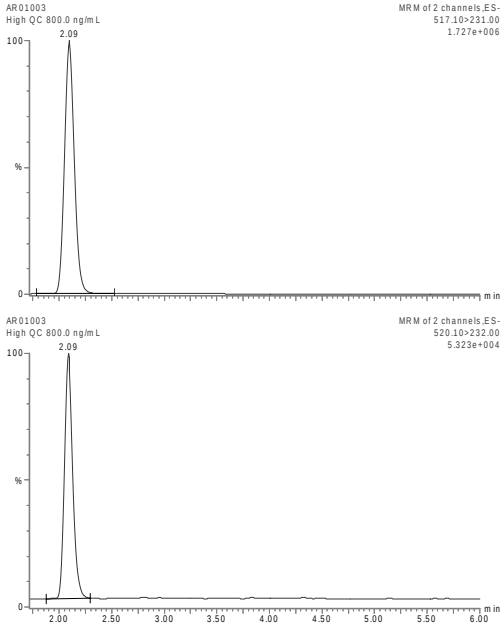

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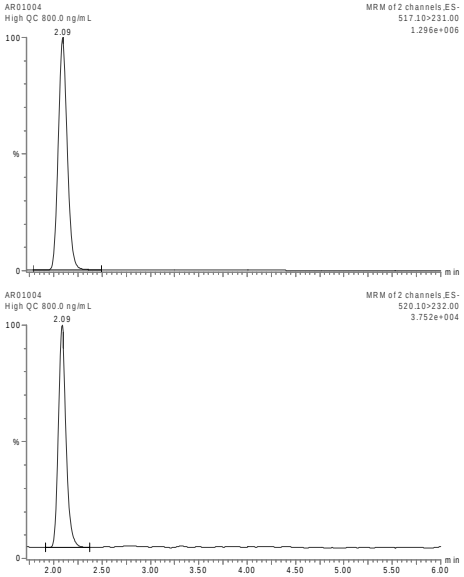

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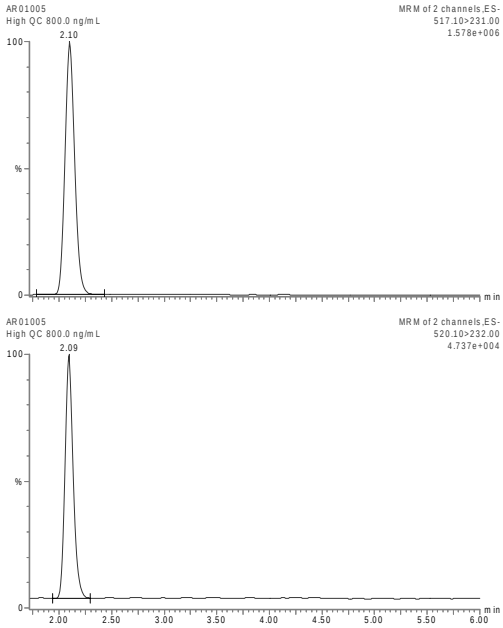

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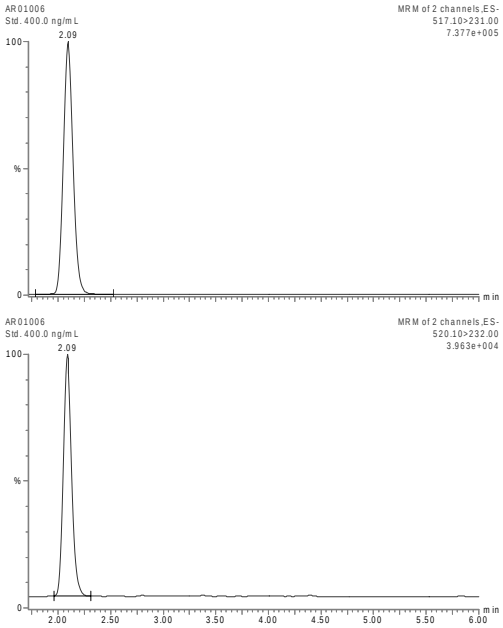

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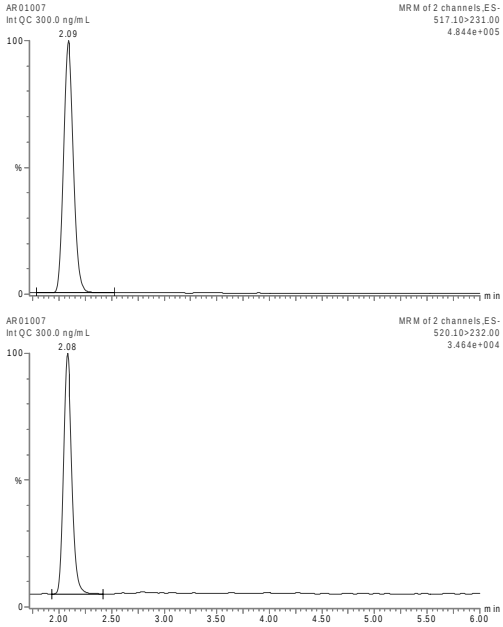

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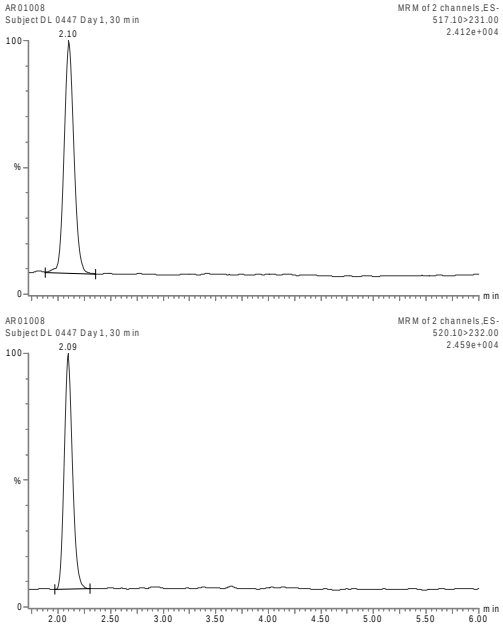

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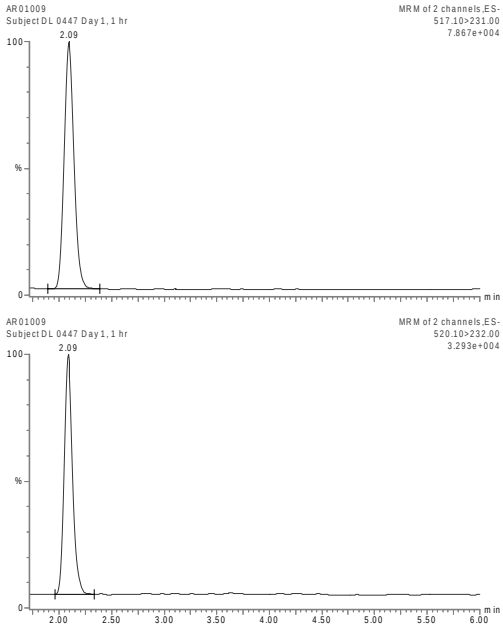

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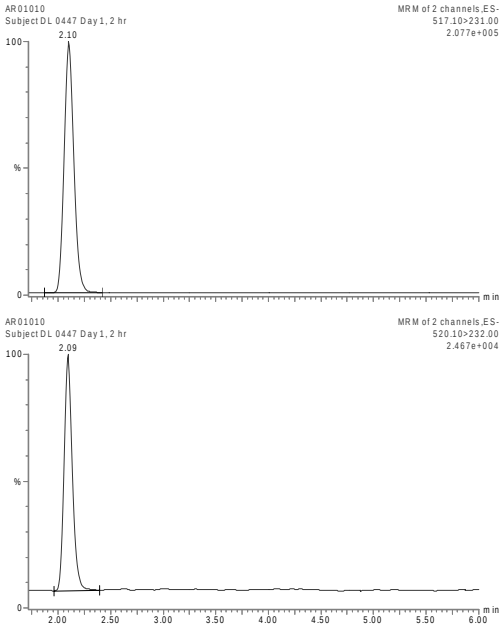

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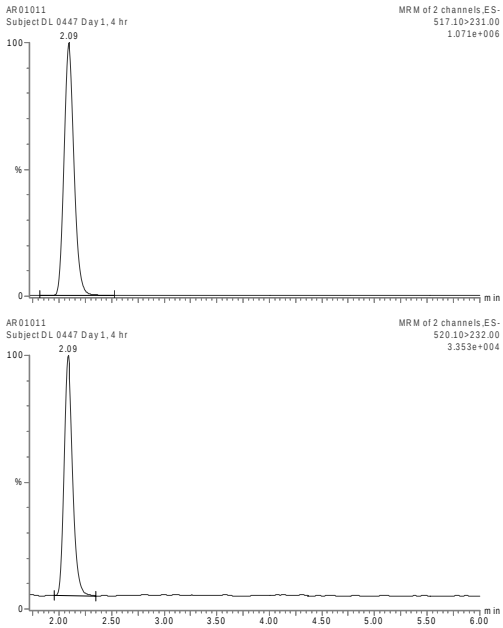

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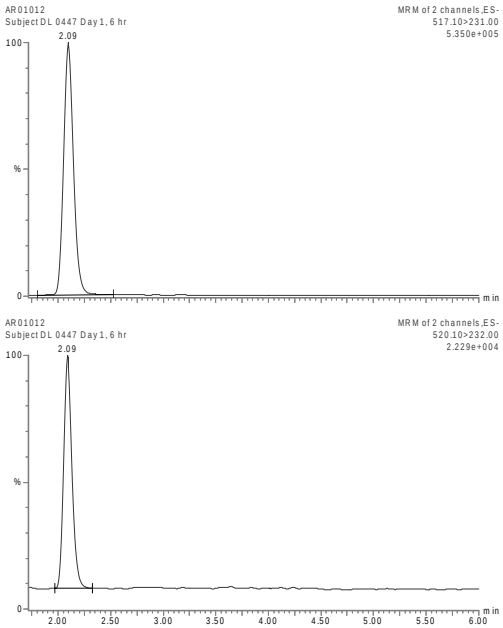

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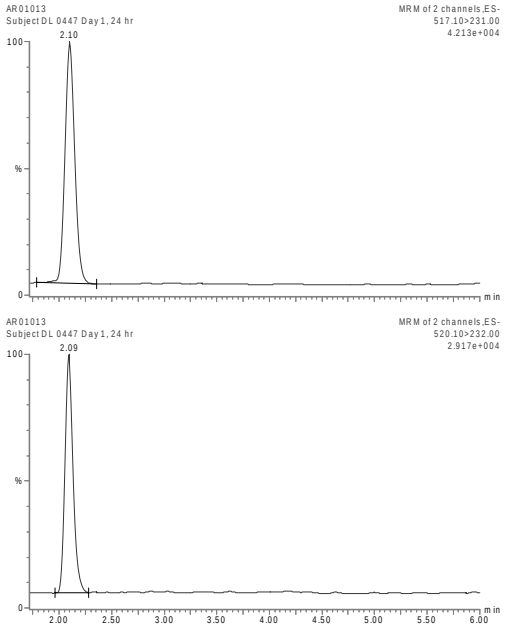

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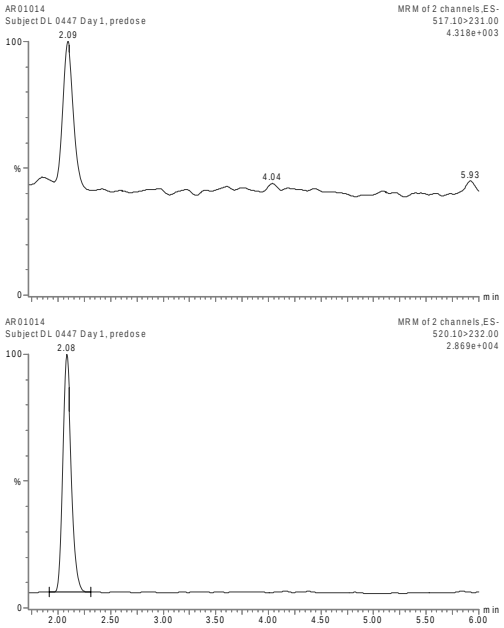

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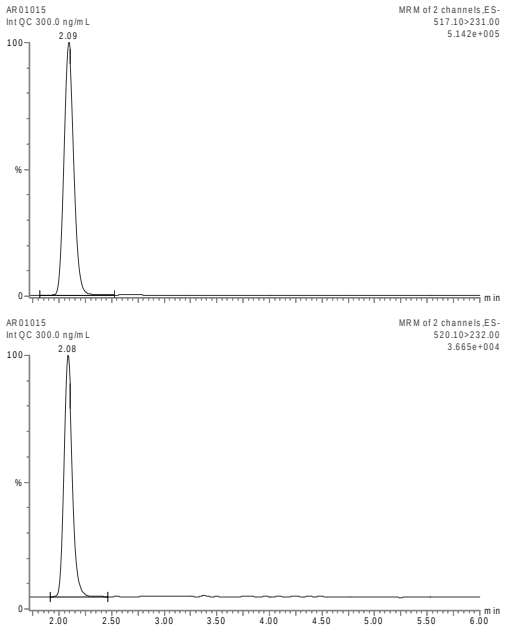

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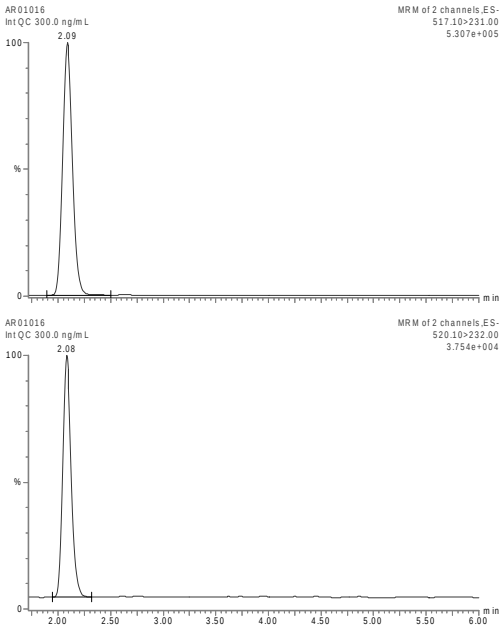

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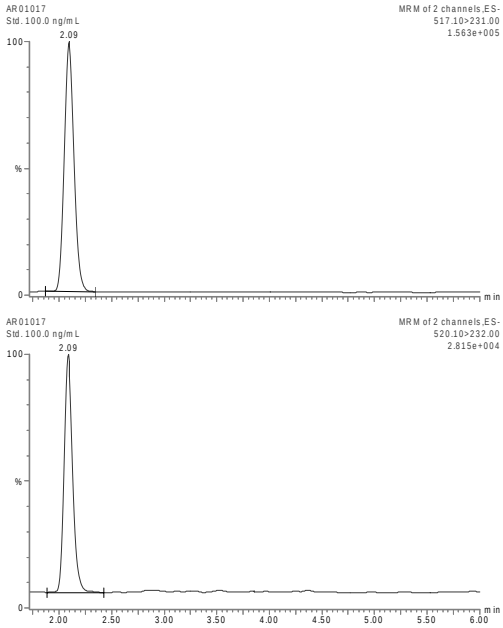

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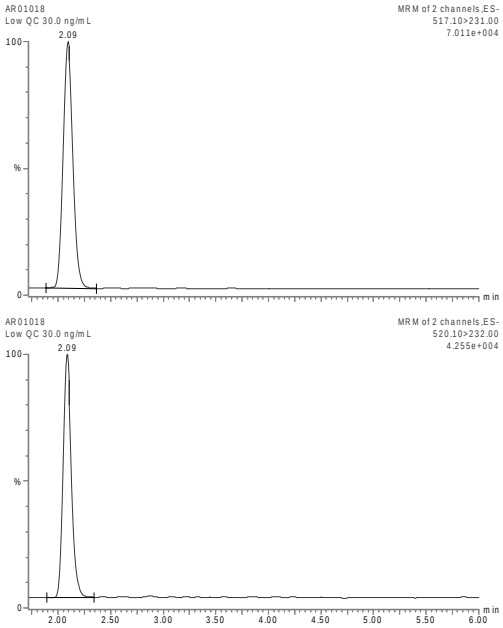

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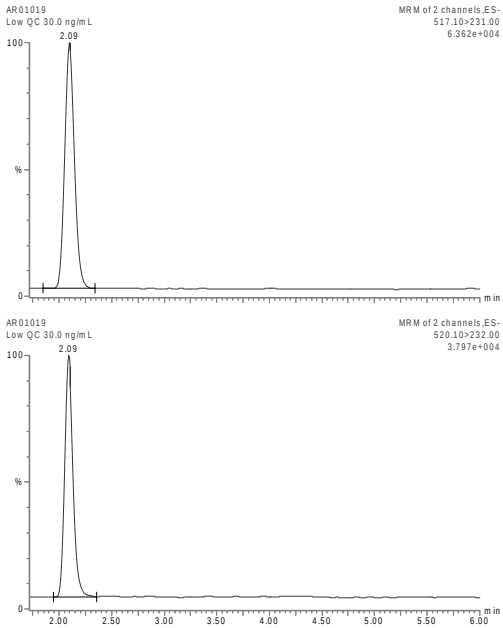

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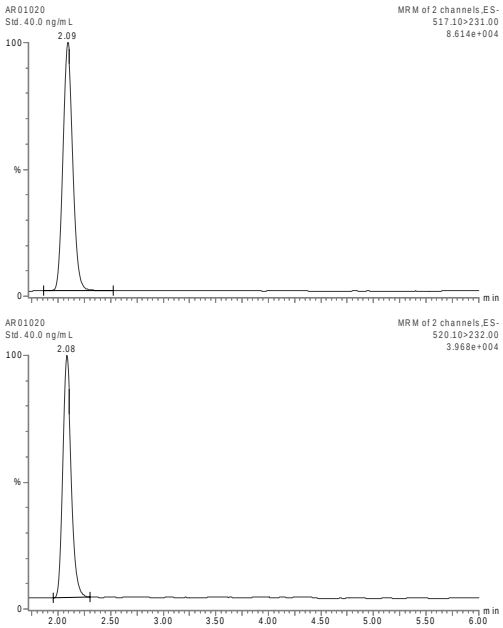

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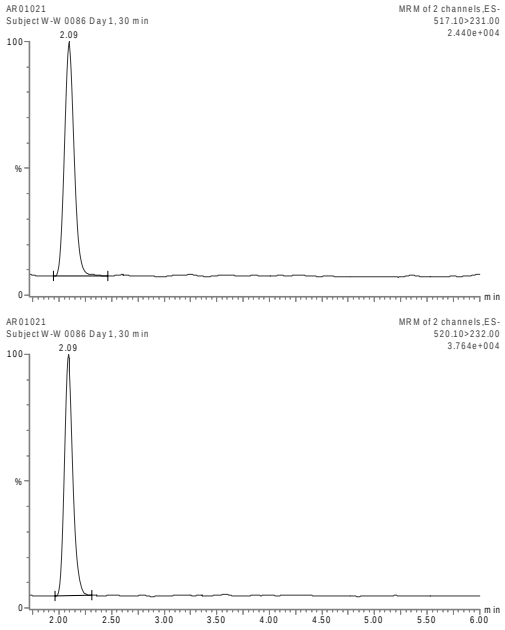

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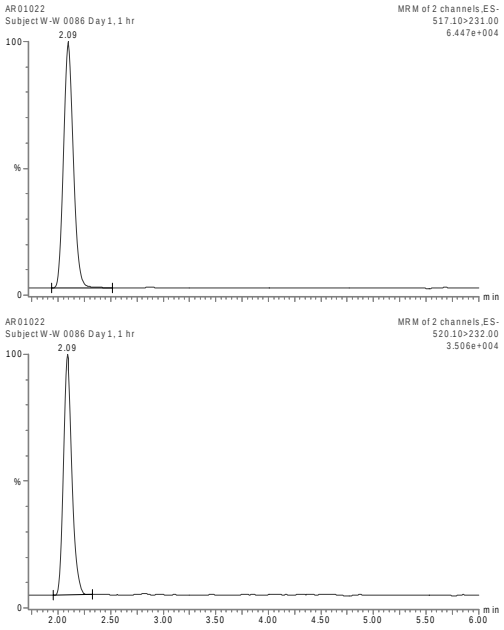

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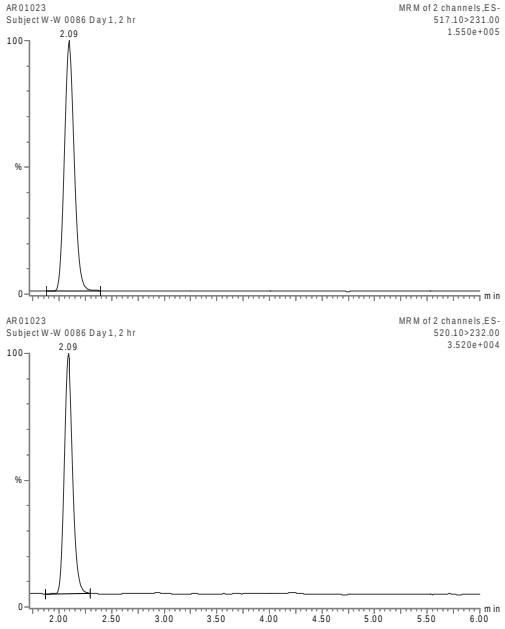

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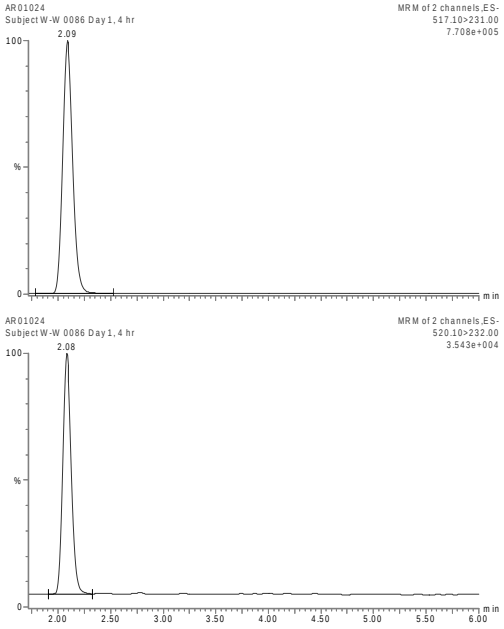

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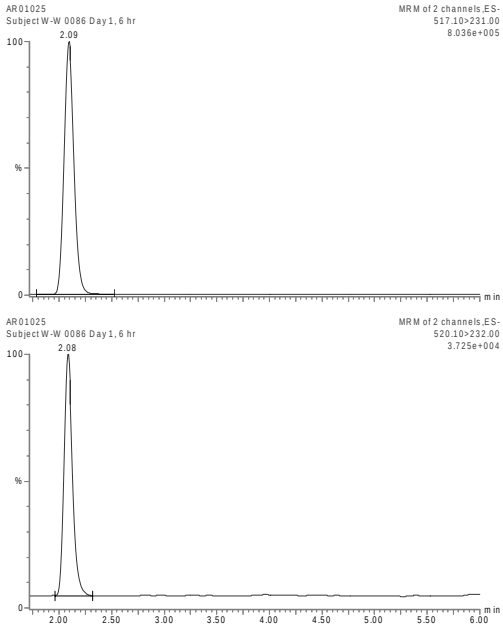

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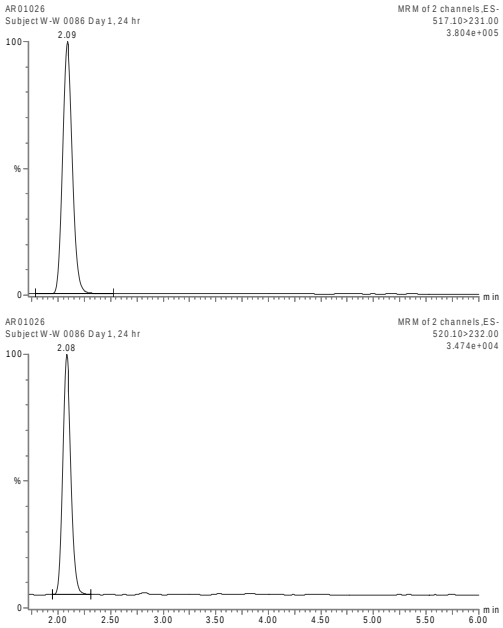

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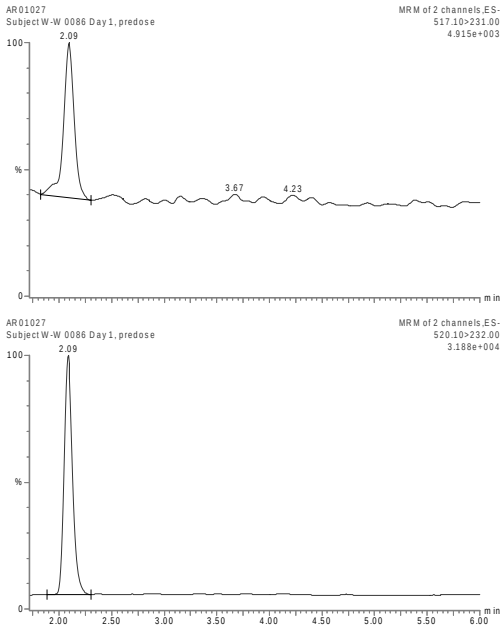

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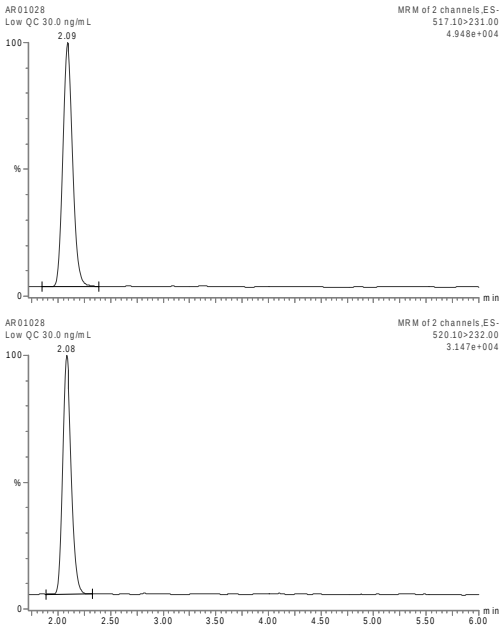

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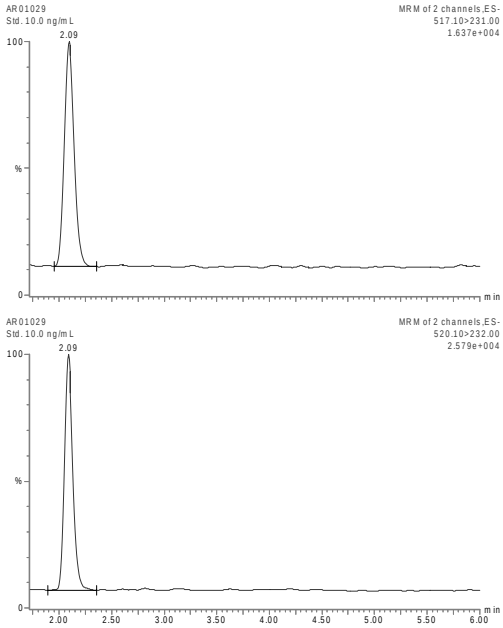

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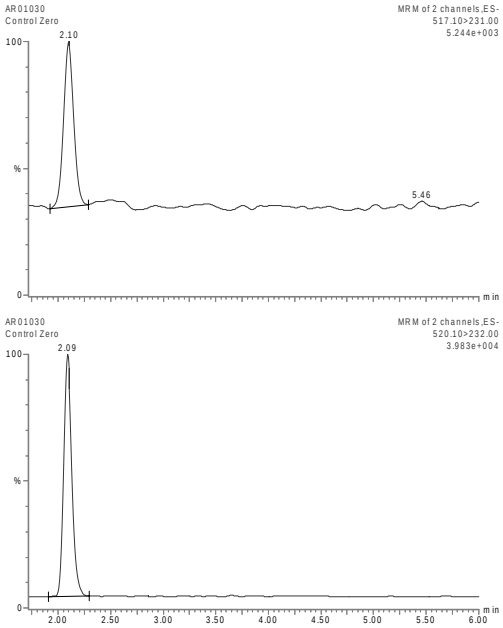

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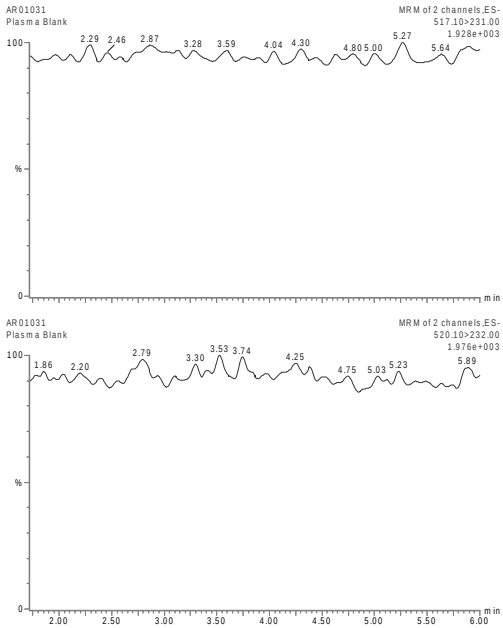

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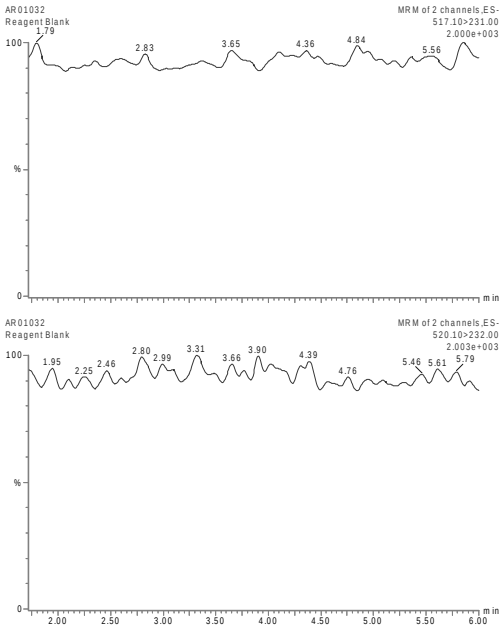

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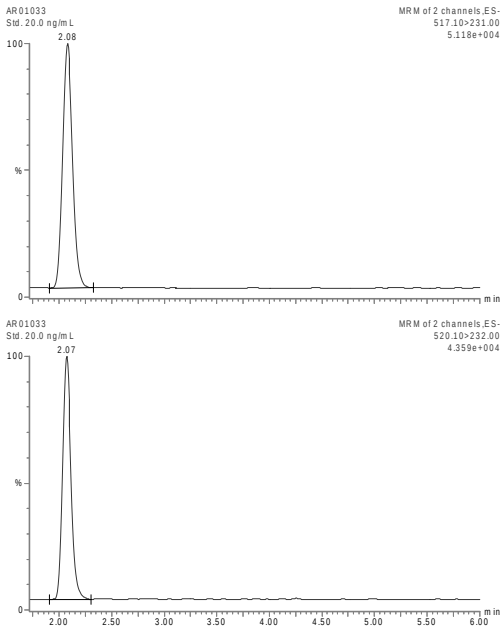

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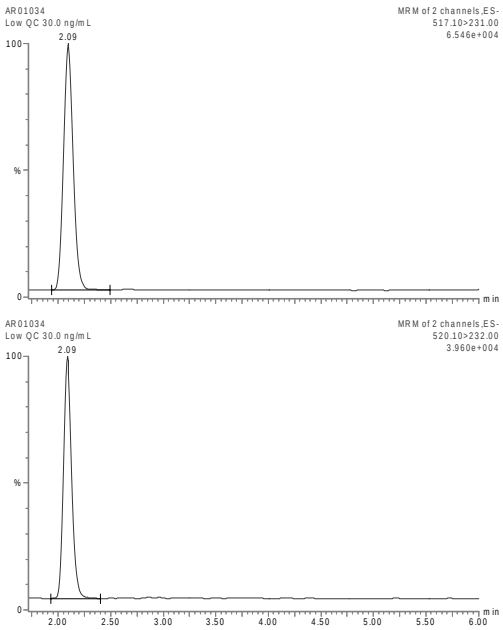

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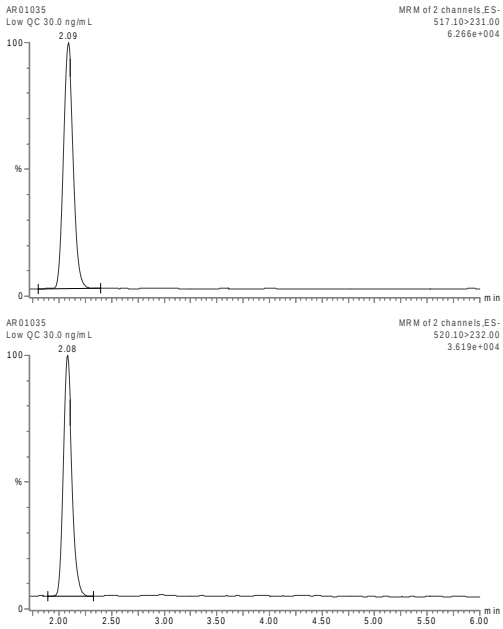

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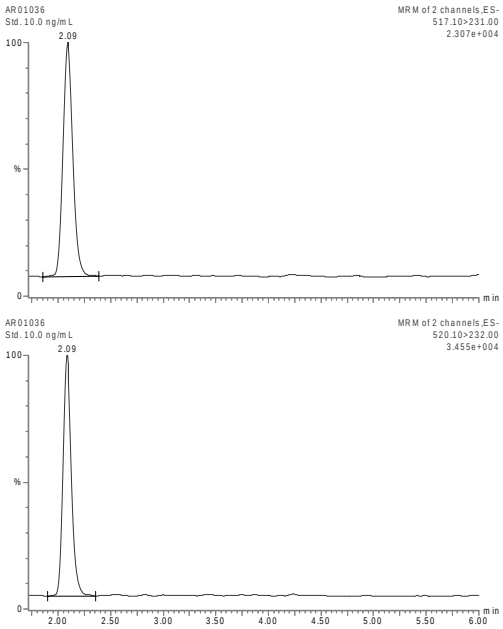

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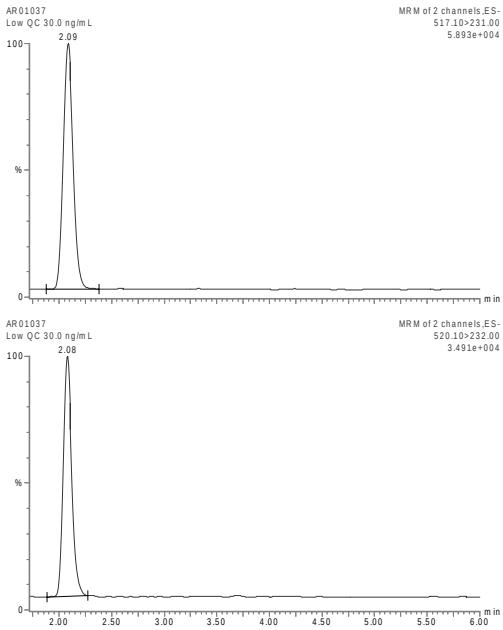

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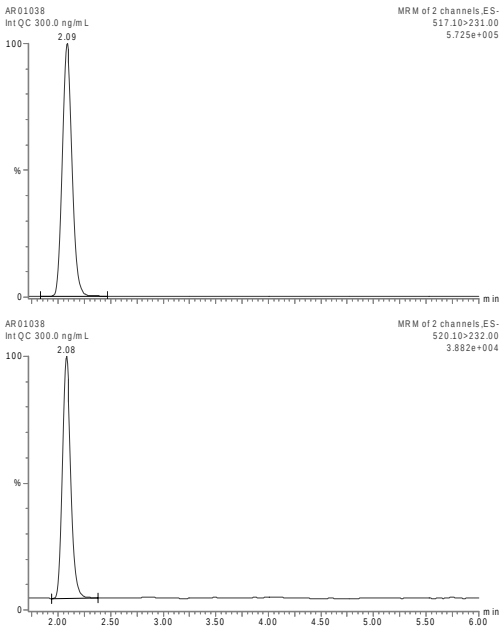

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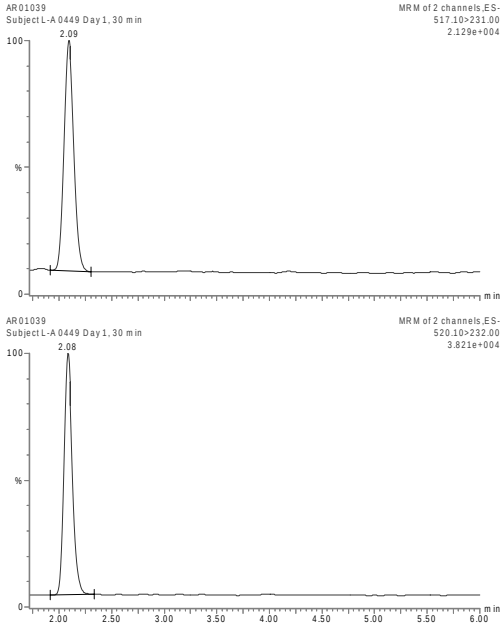

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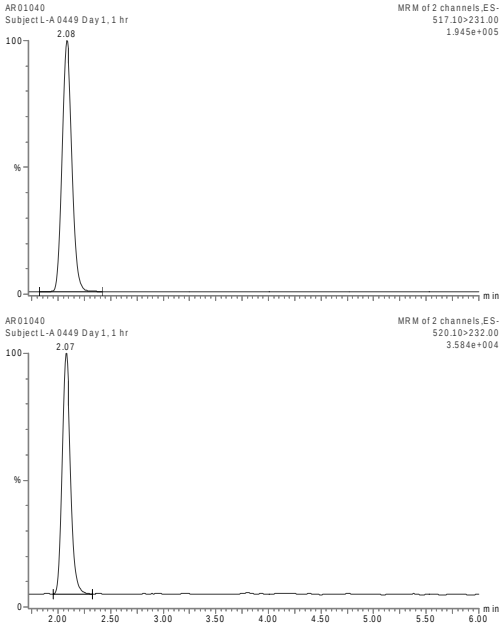

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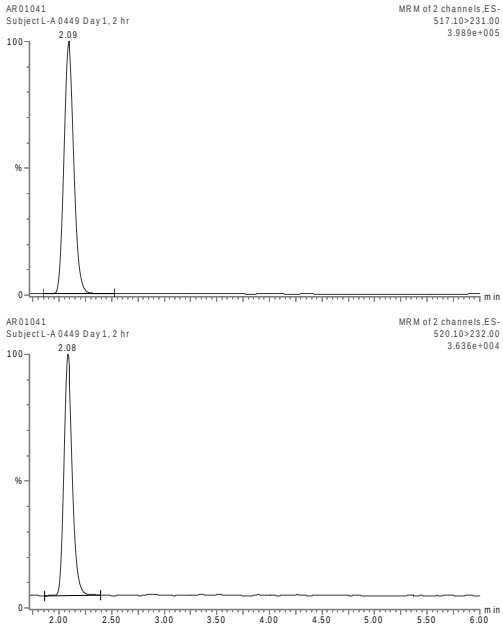

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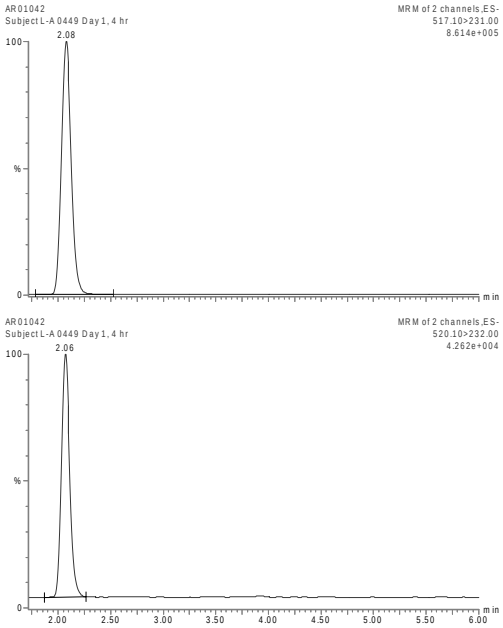

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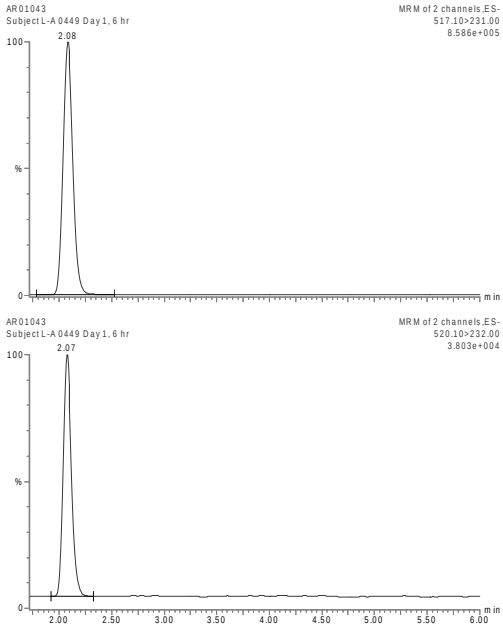

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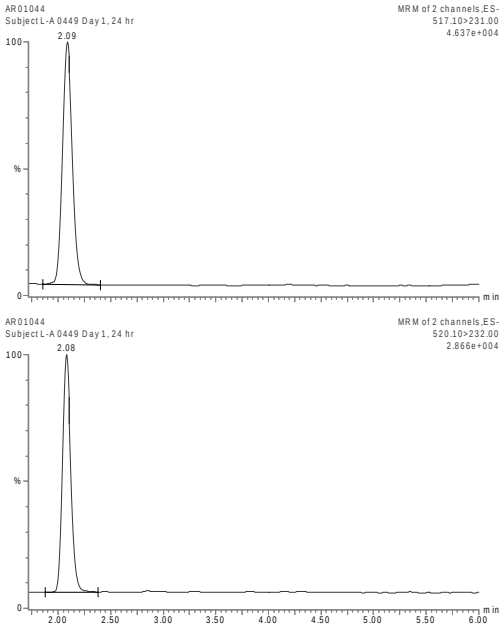

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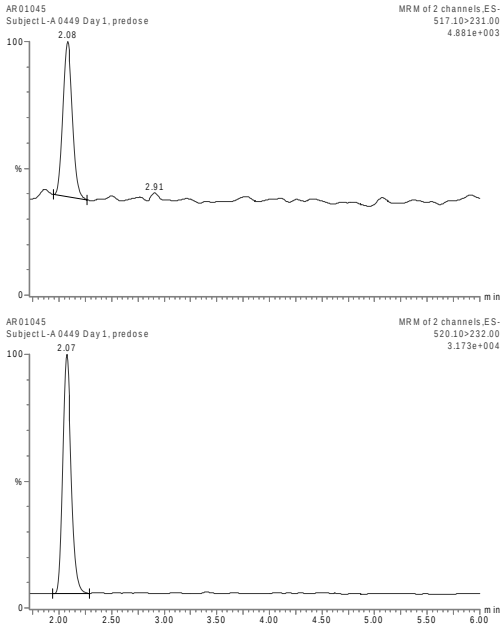

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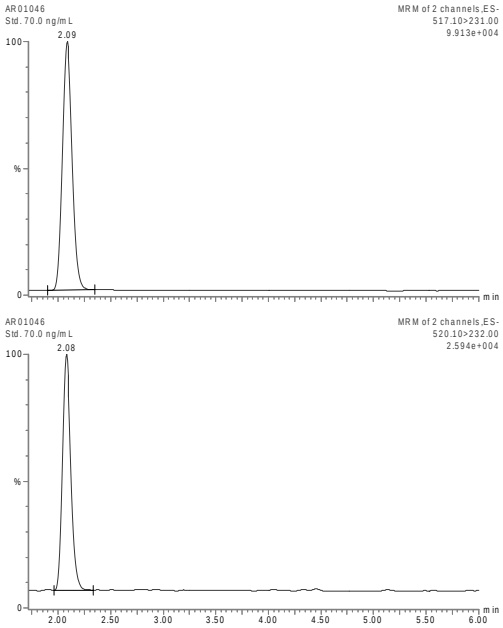

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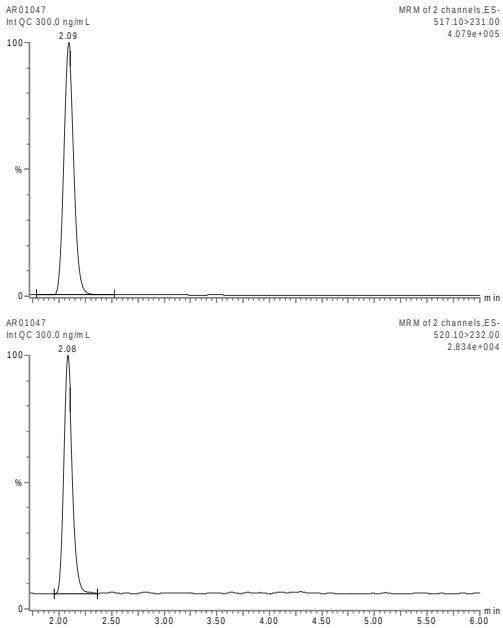

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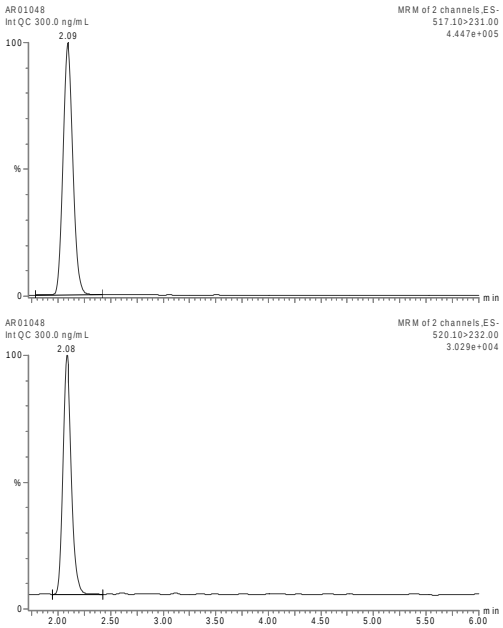

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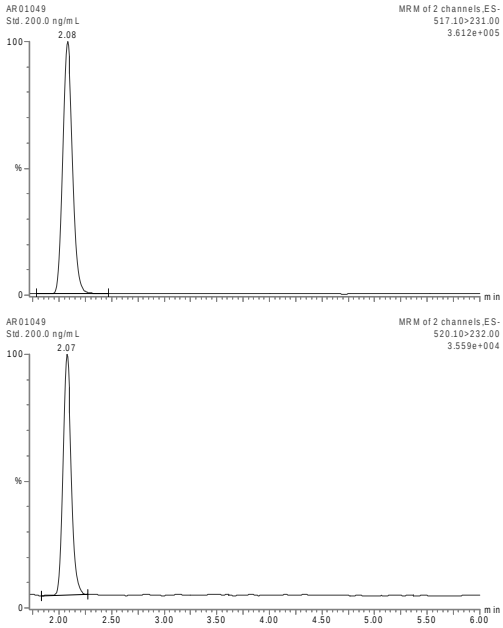

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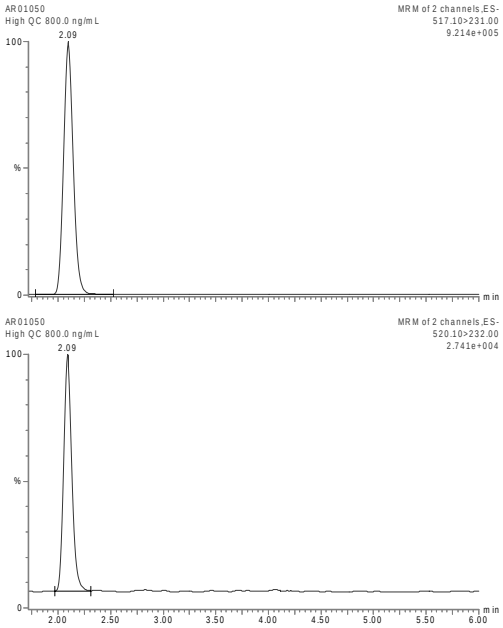

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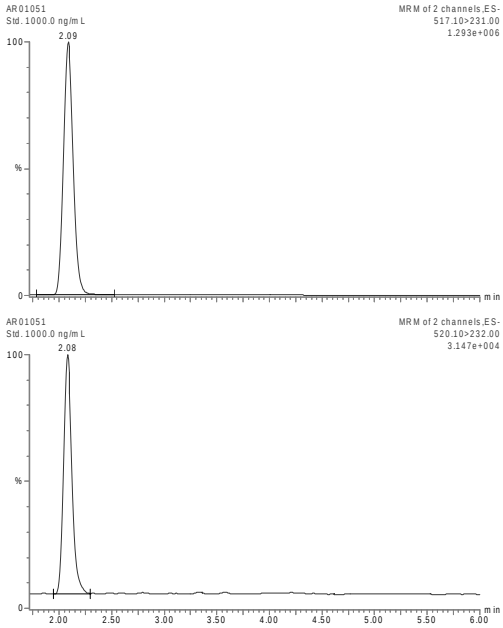

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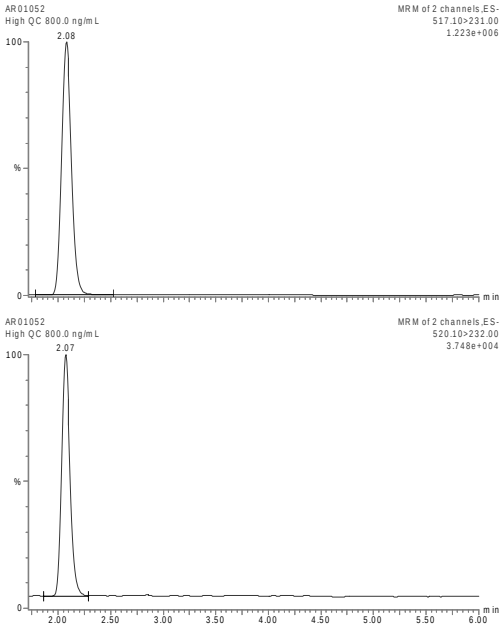

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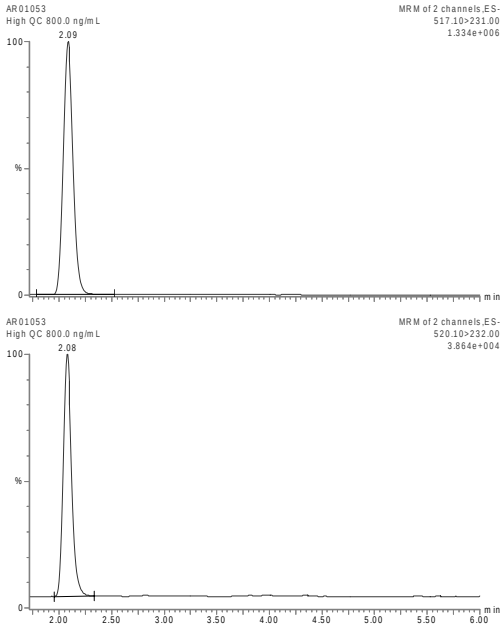

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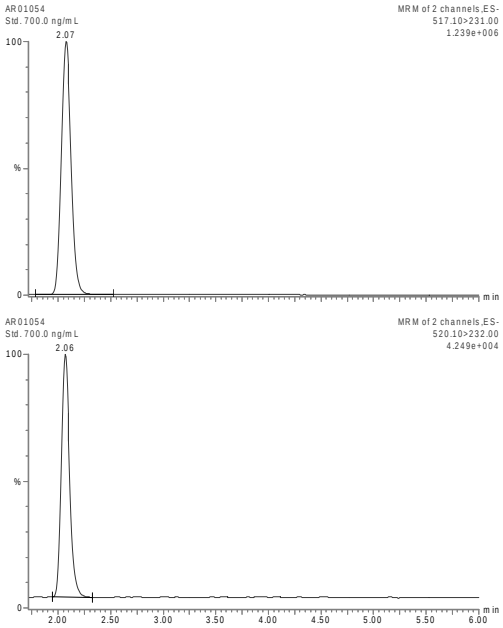

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Appendix B

Analytical Method MN05086

Analytical Method

Method for the Determination of R(-) Gossypol in Human Plasma using Racemic High-Performance Liquid Chromatography with Mass Spectrometric Detection

Prepared By:

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9050 Camino Santa Fe
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1.0 INTRODUCTION

The Bioanalytical Chemistry department at the San Diego facility of MicroConstants, Inc. has developed and validated a method for the determination of R(-) gossypol concentrations in human plasma using HPLC with MS/MS detection. The method is applicable for measuring concentrations of R(-) gossypol ranging from 10.0 to 1000 ng/mL using 100 μL of plasma for extraction. The results of the validation are contained in MicroConstants report MC05373.

2.0 PRINCIPLES OF THE METHOD

Human plasma samples containing R(-) gossypol, reduced glutathione and maleic anhydride as stabilizing agents and EDTA as the anticoagulant were precipitated with acetone, vortex mixed and centrifuged. Hypophosphorus acid was added to an aliquot of the supernatant. Gossypol-d_2 was added as an I.S. and the samples were vortex mixed and centrifuged. The samples were diluted with water and analyzed by reversed phase HPLC using a Thermo Electron BetaBasic-4 HPLC column maintained at 40°C. The mobile phase was nebulized using heated nitrogen in a Z-spray source/interface and the ionized compounds were detected using a tandem quadrupole mass spectrometer.

3.0 EXPERIMENTAL

3.1 Required Materials

3.1.1 Reagents

- Acetic Acid, glacial, Burdick & Jackson
- Acetone, HPLC grade, Burdick & Jackson
- Acetonitrile, HPLC grade, Burdick & Jackson
- Formic acid, 99%, EM Science
- Hypophosphorus Acid, 50%, Mallinckrodt
- Maleic Anhydride, ≥99%, Sigma
- Methanol, HPLC grade, Burdick & Jackson
- Reduced Glutathione, ≥99%, Fluka
- Tetrahydrofuran, HPLC grade, Burdick & Jackson
- Water, HPLC grade, EM Science
- 2,4-Pentaedione, 99%, Sigma-Aldrich

Note: Equivalent reagents from other vendors may be substituted.

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3.1.2 Apparatus

- Beakers, glass, various sizes, VWR
- Cryotube, screw cap, 1.8 mL, VWR
- Cylinders, graduated, VWR
- Disposable culture tubes, borosilicate glass, 13 x 100 mm, VWR
- Disposable culture tubes, polypropylene, 13 x 100 mm, VWR
- Disposable scintillation vials, 20 mL, polypropylene, VWR
- Micro-centrifuge tubes, 1.7 mL, VWR
- Pipettes, volumetric, various sizes, VWR
- Repeator tips, Combitip, Brinkmann Instruments, Inc.
- Vials, polypropylene, 750 μL, VWR
- Well Plate, 2-mL, polypropylene, Waters

Note: Equivalent apparatus from other vendors may be substituted unless noted otherwise.

3.1.3 Equipment

- Analytical balance, MT5, Mettler Toledo
- Analytical balance, AB204, Mettler Toledo
- Balance, top-loading, V-1mg, Acculab
- Centrifuge, 5415C, Eppendorf
- Centrifuge, T6000B, Sorvall Instruments
- Freezer, -20°C, Model U2020GA14, VWR/Revco
- Freezer, -70°C, Model 5463, VWR/Forma
- Nitrogen evaporator, TurboVap LV, Zymark
- Pipetman, Models P1000, P200 and P100, Rainin Instruments
- Pipette, Repeator, Eppendorf
- Pipette, variable volume, 10-100, 100-1000, Eppendorf
- Pipettes, volumetric, various sizes, VWR
- Refrigerator, 5°C, VWR/Revco
- Ultrasonic bath, Model 5210RDTH, Bransonic
- Vortex mixer, vertical multi-tube vortex, Model 945057, VWR

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- Vortex mixer, single tube, Model M16715, Barnstead Thermolyne

Note: Equivalent equipment from other vendors may be substituted unless noted otherwise.

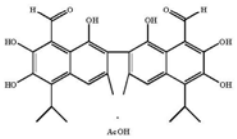
3.1.4 Analytical Instruments

- Analytical HPLC column, 50 x 2.1 mm, 3 μm, BetaBasic-4, Thermo Electron
- HPLC system, 1100 series, Agilent
- Tandem quadrupole mass spectrometer, Quattro Ultima, Micromass
- Data acquisition and processing hardware, Uppertek
- Data acquisition and processing software, Masslynx, Waters

Note: Equivalent analytical instruments from other vendors may be substituted unless noted otherwise.

3.1.5 Analytical Reference Standard

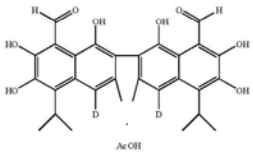
Compound Name: R(-) Gossypol Acetate (AT-101)



Supplier: Ascenta

3.1.6 Internal Standard

Compound Name: Gossypol-d_2 Acetate



Supplier: Ascenta

3.1.7 Control Matrix

Species: Human

Matrix: Plasma

Anticoagulant: EDTA

Note: Any additional lots should be tested for specificity prior to use.

3.2 Preparation of Reagents

3.2.1 Solvent A: 0.1% Acetic Acid in Water

Using a clean graduated cylinder, measure 1000 mL of HPLC grade water and transfer to a clean 1-L bottle. Pipette 1.00 mL of glacial acetic acid into the bottle and mix. Cap the bottle, loosen the cap, degas for several minutes, store at room temperature and assign an expiration date not longer than six months.

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3.2.2 Solvent B: Acetonitrile:Tetrahydrofuran (900:100), v/v

Using a clean graduated cylinder, measure 900 mL of acetonitrile and transfer to a clean 1-L bottle. Using a clean graduated cylinder, measure 100 mL of tetrahydrofuran and transfer to the same bottle. Cap the bottle and mix by inversion. Loosen the cap, degas for several minutes, store at room temperature and assign an expiration date not longer than six months.

3.2.3 Solvent 1: 0.1% Formic Acid, 0.05% 2,4-pentanedione in Methanol

Using a clean graduated cylinder, measure 1000 mL of methanol and transfer to a clean 1-L bottle. Pipette 1.00 mL of concentrated formic acid and 0.50 mL of 2,4-pentanedione into the bottle and mix by inversion. Store at room temperature and assign an expiration date not longer than six months.

3.2.4 Solvent 2: 0.1% Acetic Acid in Water

Same as Solvent A.

3.2.5 Stabilizing Solution A: 0.2 M Reduced Glutathione in Water

To a clean 20-mL polypropylene scintillation vial add 307 mg of reduced glutathione and 5.00 mL of HPLC grade water. Mix by inversion until a solution is obtained. Make fresh daily and store on ice.

3.2.6 Stabilizing Solution B: 250 mM Maleic Anhydride in Acetonitrile

To a clean 20-mL polypropylene scintillation vial add 490 mg of maleic anhydride and 20.0 mL of acetonitrile. Mix by inversion until a solution is obtained. Make fresh daily and store on ice.

3.2.7 Precipitated Stabilized Plasma

To a clean 250-mL polypropylene bottle add 200 μL of Stabilizing Solution B. Swirl in the bottom of the bottle and allow to evaporate (this may take several hours). Add 20.0 mL of blank human plasma and 2.00 mL of Stabilizing Solution A. Mix by inversion for five minutes. Add 198 mL of acetone. Mix by inversion for five minutes. Transfer the precipitated plasma in approximately 5 mL aliquots to 16 x 100 mm borosilicate culture tubes. Centrifuge the tubes at approximately 2500 rpm (575 xg) for approximately five minutes. Continue the supernatant of the tubes into a clean 250-mL polypropylene bottle. Transfer 200 mL of the supernatant to another clean 250-mL polypropylene bottle. Add 8.0 mL of hypohosphorus acid in the bottle. Mix by inversion for approximately 15 seconds. Store in a freezer set to maintain -70°C and assign an expiration date not longer than six months.

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Note: Calculation of Plasma Concentration:

Precipitated Stabilized Plasma is plasma diluted with 10% 0.2M reduced glutathione (1.00 mL plasma plus 0.100 mL 0.2 M reduced glutathione = 1.10 mL diluted plasma), followed by precipitation with acetone (1.10 mL diluted plasma plus 9.90 mL acetone = 11.0 mL precipitated diluted plasma) and acidified with hypophosphorus acid (10.0 mL precipitated diluted plasma plus 0.400 mL hypophosphorus acid = 10.4 mL precipitated stabilized plasma). Since the plasma standard is prepared in precipitated stabilized plasma, the actual concentration of the analyte in the plasma is calculated by multiplying the drug concentration in precipitated stabilized plasma by various dilution factors, 1.1/1 for reduced glutathione, 11/1.1 for acetone and 10.4/10 for hypophosphorus acid.

For Example: The concentration of R (-) gossypol in Plasma Standard P9 is calculated by:

$$\frac{0.02 \text{ mL stock} * 100\ \mu\text{g/mL}}{22.88 \text{ mL of precipitated stabilized plasma}} \times \frac{1.1}{1} \times \frac{11}{1.1} \times \frac{10.4}{10} = 1000.0 \text{ ng/mL}$$

3.3 Preparation of Stock Standard Solutions

3.3.1 R(-) gossypol Standard Stock Solution (100 μg/mL)

Weigh approximately 1.00 mg of R(-) gossypol, accounting for purity and corrections and record the actual weight. Note: Different reference standard lots may require different correction factors. Transfer to a 20-mL polypropylene scintillation vial and dissolve in the appropriate amount of acetonitrile to make a 100 μg/mL solution (approximately 10.0 mL). Store in a freezer set to maintain -20°C.

3.4 Preparation of Precipitated Stabilized Plasma Standards

3.4.1 PSP Standard P9 (87.41 ng/mL or 1000 ng/mL Plasma Standard)

Using a pipette, transfer 20.0 μL of Standard Stock Solution (100 μg/mL) into a 20-mL polypropylene scintillation vial and add 22.86 mL of Precipitated Stabilized Plasma. Tightly cap the vial and mix by inversion for two minutes. Store in a freezer set to maintain -70°C.

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3.4.2 PSP Standard P8 (61.18 ng/mL or 700 ng/mL Plasma Standard)

Using a pipette, transfer 3.50 mL of PSP Standard P9 (1000 ng/mL) into a 20-mL polypropylene scintillation vial and add 1.50 mL of Precipitated Stabilized Plasma. Tightly cap the vial and mix by inversion for two minutes. Store in a freezer set to maintain -70°C.

3.4.3 PSP Standard P7 (34.96 ng/mL or 400 ng/mL Plasma Standard)

Using a pipette, transfer 5.00 mL of PSP Standard P9 (1000 ng/mL) into a 20-mL polypropylene scintillation vial and add 7.50 mL of Precipitated Stabilized Plasma. Tightly cap the vial and mix by inversion for two minutes. Store in a freezer set to maintain -70°C.

3.4.4 PSP Standard P6 (17.48 ng/mL or 200 ng/mL Plasma Standard)

Using a pipette, transfer 6.00 mL of PSP Standard P7 (400 ng/mL) into a 20-mL polypropylene scintillation vial and dilute to 12.0 mL final volume with 6.00 mL of Precipitated Stabilized Plasma. Tightly cap the vial and mix by inversion for two minutes. Store in a freezer set to maintain -70°C.

3.4.5 PSP Standard P5 (8.741 ng/mL or 100 ng/mL Plasma Standard)

Using a pipette, transfer 7.00 mL of PSP Standard P6 (200 ng/mL) into a 20-mL polypropylene scintillation vial and add 7.00 mL of Precipitated Stabilized Plasma. Tightly cap the vial and mix by inversion for two minutes. Store in a freezer set to maintain -70°C.

3.4.6 PSP Standard P4 (6.118 ng/mL or 70.0 ng/mL Plasma Standard)

Using a pipette, transfer 3.50 mL of PSP Standard P5 (100 ng/mL) into a 20-mL polypropylene scintillation vial and add 1.50 mL of Precipitated Stabilized Plasma. Tightly cap the vial and mix by inversion for two minutes. Store in a freezer set to maintain -70°C.

3.4.7 PSP Standard P3 (3.496 ng/mL or 40.0 ng/mL Plasma Standard)

Using a pipette, transfer 4.00 mL of PSP Standard P5 (100 ng/mL) into a 20-mL polypropylene scintillation vial and add 6.00 mL of Precipitated Stabilized Plasma. Tightly cap the vial and mix by inversion for two minutes. Store in a freezer set to maintain -70°C.

3.4.8 PSP Standard P2 (1.748 ng/mL or 20.0 ng/mL Plasma Standard)

Using a pipette, transfer 5.00 mL of PSP Standard P3 (40.0 ng/mL) into a into a 20-mL polypropylene scintillation vial and add 5.00 mL of Precipitated Stabilized Plasma. Tightly cap the vial and mix by inversion for two minutes. Store in a freezer set to maintain -70°C.

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3.4.9 PSP Standard P1 (0.8741 ng/mL or 10.0 ng/mL Plasma Standard)

Using a pipette, transfer 5.00 mL of PSP Standard P2 (20.0 ng/mL) into a 20-mL polypropylene scintillation vial and add 5.00 mL of Precipitated Stabilized Plasma. Tightly cap the vial and mix by inversion for two minutes. Store in a freezer set to maintain -70°C.

3.5 Preparation of Internal Standard Solutions

3.5.1 I.S. Stock Solution (10.0 μg/mL Gossypol-d_2)

Weigh approximately 0.100 mg of gossypol-d_2, accounting for purity and corrections and record the actual weight. Note: Different reference standard lots may require different correction factors. Transfer to a 20-mL polypropylene scintillation vial and dissolve in the appropriate amount of acetonitrile to make a 10.0 μg/mL solution (approximately 10.0 mL). Store in a freezer set to maintain -20°C.

3.5.2 Working I.S. Solution (30.0 ng/mL)

Using a pipette, transfer 30.0 μL of I.S. Stock Solution (10.0 μg/mL) into a 20-mL polypropylene scintillation vial and dilute to 10.0 mL final volume with 9.97 mL of acetonitrile. Store in a freezer set to maintain -20°C.

3.6 Preparation of Quality Control Samples

3.6.1 Over-the-Curve PSP QC (174.8 ng/mL or 2000 ng/mL Plasma Standard)

Using a pipette, transfer 20.0 μL of Standard Stock Solution (100 μg/mL) into a 20-mL polypropylene scintillation vial containing 11.42 mL of Precipitated Stabilized Plasma. Mix by inversion for approximately two minutes. Cap and store appropriately.

3.6.2 High PSP QC (69.93 ng/mL or 800 ng/mL Plasma Standard)

Using a pipette, transfer 2.00 mL of Over-the-Curve PSP QC (174.8 ng/mL) into a 20-mL polypropylene scintillation vial containing 3.00 mL of Precipitated Stabilized Plasma. Mix by inversion for approximately two minutes. Cap and store appropriately.

3.6.3 Intermediate PSP QC (26.22 ng/mL or 300 ng/mL Plasma Standard)

Using a pipette, transfer 900 μL of Over-the-Curve PSP QC (174.8 ng/mL) into a 20-mL polypropylene scintillation vial containing 5.10 mL of Precipitated Stabilized Plasma. Mix by inversion for approximately two minutes. Cap and store appropriately.

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3.6.4 Low PSP QC (2.62 ng/mL or 30.0 ng/mL Plasma Standard)

Using a pipette, transfer 500 µL of Intermediate PSP QC (26.22 ng/mL) into a 20-mL polypropylene scintillation vial containing 4.50 mL of Precipitated Stabilized Plasma. Mix by inversion for approximately two minutes. Cap and store appropriately.

3.7 Sample Preparation Procedure

Note: All samples, standards and QC samples should be thawed in an ice bath. All solutions should be kept on ice.

3.7.1 If a sample has not been previously precipitated, thaw on ice and immediately precipitate by pipetting a 110 µL aliquot of sample into a properly labeled 13 x 100 mm polypropylene tube. Add 990 µL of acetone to the tube and vortex approximately 10 seconds. Centrifuge the tubes at approximately 2500 rpm (575 xg) for approximately five minutes. Using a pipette transfer 1.00 mL of the supernatant to a properly labeled 1.8 mL cryotube. Add 40.0 µL of hypophosphorus acid to the cryotube. Mix the cryotube by inversion for approximately 15 seconds. Store the cryotube in a freezer set to maintain -70°C. The precipitated sample is used for analysis and possible reanalysis. If the quantity of the prepared sample is insufficient for reanalysis, ask a supervisor for instructions.

3.7.2 Using an Eppendorf repeating pipette, aliquot 200 µL of each sample prepared in step 3.7.1 or QC sample to labeled 13 x 100 mm polypropylene tubes. If a partial volume aliquot is needed, add the aliquot to the tube and dilute to full volume with blank precipitated stabilized plasma.

3.7.3 Prepare the calibration curve by aliquoting 200 µL of each PSP Standard. The corresponding plasma standard concentration should be used in the construction of the calibration curve. Include one reagent blanks (200 µL of water substituted for blank precipitated stabilized plasma), a plasma blank (200 µL of blank precipitated stabilized plasma) and a control zero (200 µL of blank precipitated stabilized plasma fortified with I.S.).

3.7.4 Using an Eppendorf repeating pipette, aliquot 50.0 µL of I.S. Working Solution (30.0 ng/mL) to all tubes except the blanks, resulting in 72.1 ng/mL I.S. in plasma.

3.7.5 Vortex the tubes for approximately 5 seconds on a multitube vortex.

3.7.6 Centrifuge the tubes at approximately 2500 rpm (575 xg) for no more than two minutes.

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3.7.7 Add 200 µL of water to the tubes.

3.7.8 Vortex the tubes for approximately five seconds on a multi-tube vortex.

3.7.9 Transfer the contents of the tubes to autosampler vials if appropriate, a well plate.

3.8 Chromatographic Conditions

Note: Conditions may be modified to obtain optimum chromatography and/or response.

3.8.1 HPLC Conditions

	HPLC-10
Sample Loop (µL)	10 µL
Sample Volume (µL)	20 µL
Sample Temp. (°C)	5°C
Flow Rate (mL/min)	0.300
Solvent A	40%
Solvent B	60%
Solvent Temp. (°C)	N/A
Auto Sampler program	Post clean Solvent 1 (3x) Valve Clean Solvent 1 (3x) Valve Clean Solvent 2 (3x)
Column Manufacturer	Thermo Electron
Column Packing	BetaBasic 4
Column Dimensions	50x2.1 mm, 3µm
Column Temp. (°C)	40
Column Switch (min)	Initial to waste 1.8 to mass spectrometer
Analysis Time (min)	~6

3.8.2 HPLC Gradient Conditions

% Solvent A	**% Solvent B**	**HPLC Time, min**
5	95	1.5
5	95	3.0
40	60	3.1

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3.8.3 Mass Spectrometer Conditions

	MSMS-05	MSMS-03	MSMS-12	MSMS-14
R(-) gossypol				
Mass Transition	517.10 > 231.45	517.25 > 231.20	517.25 > 231.20	517.10 > 231.00
Cone (V)	0	0	45	0
Collision (eV)	40	43	40	40
Dwell Time (secs)	0.2	0.2	0.2	0.2
Delay Time (secs)	0.02	0.02	0.02	0.02
I.S.				
Mass Transition	520.10 > 232.45	519.25 > 232.20	519.25 > 232.20	520.10 > 232.00
Cone (V)	0	0	45	0
Collision (eV)	40	43	40	40
Dwell Time (secs)	0.2	0.2	0.2	0.2
Delay Time (secs)	0.02	0.02	0.02	0.02
Ionization Mode	ESP-	ESP-	ESP-	ESP-
Source Temp. (°C)	130	130	130	130
Desolvation Temp. (°C)	350	350	350	350
Cone Gas (L/Hr)	~100	~50	~50	~120
Capillary (kV)	2.50	1.00	2.5	3.00
Hex 1	25.0	25.0	N/A	40.0
Aperature	0.0	0.0	N/A	0.0
Hex 2	1.0	1.0	N/A	0.0
LM/HM Resolution 1	13.5/13.5	13.5/13.5	13.5/13.5	13.5/13.5
Ion Energy 1 (V)	1.5	0.5	1.0	1.0
Multiplier (V)	650	650	650	650
Entrance	-2	-2	-2	-2
Exit	2	2	2	2
LM/HM Resolution 2	13.5/13.5	13.5/13.5	13.5/13.5	13.5/13.5
Ion Energy 2 (V)	1.5	1.0	1.5	1.0
Extractor	N/A	N/A	1.0	N/A
RF Lens	N/A	N/A	0.0	N/A

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3.8.4 System Suitability Conditions

Calculation of peak height response/ng on column:

$$\frac{\text{Height x Total Volume after Initial Dilution (0.450 mL)}}{\text{Theor. Conc. (ng/mL) x Aliquot Volume (0.2 mL) x Inj. Volume (0.01 mL)}}$$

	HPLC-10/ MSMS-05	HPLC-07/ MSMS-03	HPLC-04/ MSMS-12	HPLC-18/ MSMS-14
R(-) gossypol				
Retention Time (min)	2.1 ± 0.5	2.1 ± 0.5	2.1 ± 0.5	2.1 ± 0.5
Minimum Height/ng on column	100,000	50,000	5,000	100,000
Gossypol-d_2				
Retention Time (min)	2.1 ± 0.5	2.1 ± 0.5	2.1 ± 0.5	2.1 ± 0.5
Minimum Height/ng on column	35,000	35,000	2,500	35,000
Column Back Pressure (bar)	65 ± 30	65 ± 30	90 ± 30	90 ± 30

3.9 Calculations

Peak heights of R(-) gossypol, gossypol-d_2 were acquired using MassLynx™ v. 4.0 (Waters, Milford, MA). The calibration curves were obtained by fitting the peak height ratios of R(-) gossypol/gossypol-d_2 and the plasma standard concentrations to a power regression equation using MassLynx. The equations of the calibration curves were then used to interpolate the concentration of R(-) gossypol in the samples using their peak height ratios.

4.0 STABILITY

PSP QC Sample Storage Stability (Thawed on Ice): 28 hours

PSP QC Sample Storage Stability (Freeze/Thaw): 3 cycles

PSP QC Sample Storage Stability (Frozen at -70°C): 66 days

Processed Sample Stability (Refrigerated at 5°C): 102 hours

Stock Standard Stability (Frozen at -20°C): 308 days

5.0 TERMS AND DEFINITIONS

ESP: Electrospray Ionization

HPLC: High-Performance Liquid Chromatography

I.S.: Internal Standard

MS/MS: Tandem Quadrupole Mass Spectrometer

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N/A: Not Applicable

PSP: Precipitated Stabilized Plasma

QC: Quality Control

6.0 REFERENCES

MC05373
Validation of a Method for the Determination of R(-) Gossypol in Human Plasma using Racemic High-Performance Liquid Chromatography with Mass Spectrometric (MS/MS) Detection, MicroConstants, Inc., San Diego, CA.

7.0 REVISION HISTORY

7.1 MN05086.01

- Section 2.0
  Changed "centrifuged." to "aliquoted to a 96-well hydrophobic PTFE filter plate."
  Changed "An aliquot of the supernatant was diluted with a phosphoric acid solution" to "The samples where collected in a 2 mL well plate"
- Section 3.1.1
  Deleted "Phosphoric Acid, 85%, Mallinckrodt"
- Section 3.1.2
  Added "Filter plates, 96-well, Hydrophobic PTFE, Millipore"
- Section 3.2.5 Deleted
- Section 3.2.8
  Changed "180.0 mL" to "198.0 mL"
- Section 3.7.6 Updated
- Section 3.7.7 Updated
- Section 3.7.8 Added
- Section 3.8.1
  Updated Sample Loop (µL)
  Updated Sample Volume (µL)

7.2 MN5086.02

- Section 2.0
  Changed "aliquoted to a 96-well hydrophobic PTFE filter plate." to "centrifuged."
  Changed "The samples where collected in a 2 mL well plate" to "An aliquot of the supernatant was diluted with a phosphoric acid solution"

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- Section 3.1.1
  Added "Phosphoric Acid, 85%, Mallinckrodt"
- Section 3.1.2
  Changed "Filter plates, 96-well, Hydrophobic PTFE, Millipore" to "Micro-centrifuge tubes, 1.7 mL, VWR"
- Section 3.1.3
  Added "Centrifuge, 5415C, Eppendorf"
- Section 3.2.5-3.2.7
  Changed to 3.2.6-3.2.8
- Section 3.2.5 Added
- Section 3.7.2
  Changed "13 x 100-mm polypropylene culture tubes." to "1.7 mL micro-centrifuge tubes."
- Section 3.7.6-3.7.8 Updated
- Section 3.7.9 Added
- Section 3.8.1
  Updated Sample Loop and Sample Volume

7.3 MN05086.03

- Section 2.0
  Added "vortex mixed and centrifuged. Hypophosphorus acid was added to an aliquot of the supernatant."
  Changed "An aliquot of the supernatant" to "The samples"
  Changed "a phosphoric acid solution" to "water"
- Section 3.1.1
  Added "Hypophosporus Acid, 50%, Mallinckrodt"
  Deleted "Phosphoric Acid, 85%, Mallinckrodt"
- Section 3.1.2
  Changed volume of polypropylene vials from 0.3 mL to 750 µL
- Section 3.2.5 Deleted
- Sections 3.2.6-3.2.8
  Changed to Sections 3.2.5-3.2.7
- Section 3.2.7 Updated
- Section 3.2.8 Updated
- Section 3.4.20 Added

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- Sections 3.4.20-3.4.23
  Changed to Sections 3.4.21-3.4.24
- Sections 3.4.21-3.4.24
  Updated concentrations
  Changed "the R(-) gossypol Stock Standard Solution (100µg/mL)" to "Control Spiking Solution C5 (40.0 µg/mL)"
- Section 3.5.2
  Changed concentration of Working I.S Solution to 30.0 ng/mL
  Changed final volume of Working I.S. Solution to 9.9.7 mL
- Section 3.6
  Updated concentrations
- Section 3.7 Updated
- Section 8.8.1
  Updated auto sampler program
- Section 3.8.3
  Updated Mass Transition

7.4 MN05086.04

- Section 3.4.1-3.4.10 Deleted
- Section 3.4.11-3.4.19 Updated
- Section 3.4.20-3.4.24 Deleted
- Section 3.6.1-3.6.4 Updated

7.5 MN05086.05

- Section 3.8.3
  Updated Mass Transition in I.S. Condition
- Section 3.8.4
  Added "(ng)" to Minimum Height(s)
  Updated Minimum Height's

7.6 MN05086.06

- Section 1.0
  Added validation MC05373, 2006
- Section 3.1.5 and 3.1.6

  Deleted lot number, purity, MW salt, MW free, salt correction, solvent correction, overall correction, storage correction, and retest date

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- Section 3.7.3
  Changed to include one reagent instead of two reagent balnks
- Section 4.0
  Added stabilities
- Section 6.0
  Added reference MC05373, 2006

7.7 MN05086.07

- Section 3.8.3
  Added conditions for MSMS-03
- Section 3.8.4
  Added conditions for HPLC-07/MSMS-03

7.8 MN05086.08

- Section 3.8.3
  Added MSMS-12 column
- Section 3.8.4
  Added HPLC-04/MSMS-12 column
- Section 4.0
  Added stock standard stability

7.9 MN05086.09

- Section 3.2.7
  Updated section
  Added note
- Section 3.7.3
  Removed table
- Section 4.0
  Updated stabilities
- Section 5.0
  Added term "PSP"

7.10 MN05086.10

- Section 3.2.7
  Added paragraph "To a clean 250-mL…"

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- Section 3.4
  Changed name "Preparation of Plasma Standard Dilution" to Preparation of Precipitated Stabilized Plasma Standards"
  Small edits

7.11 MN05086.11

- Modified signature page
- Updated numbers accordingly to three significant figures
- Section 3.1.7
  Added to lot numbers
- Section 3.3
  Removed "If possible…standard solutions"
- Section 3.8
  Added to chromatographic conditions

7.12 MN05086.12

- Modified signature page
- Section 1.0
  Removed "high-performance liquid chromatography", "mass spectrometric" and "2006"
- Section 2.0
  Replaced "internal standard" with "I.S."
  Replaced "high-performance liquid chromatography" with "HPLC"
- Section 3.3
  Removed "Stock standard…3.0%"
- Section 3.8.3
  Added Cone Gas to mass spectrometer conditions
- Section 3.8.4
  Added system suitability equation
  Replaced "(ng)" with "/ng on column" in conditions table
  Replaced last column "HPLC-04/MSMS-12" with "HPLC-18/MSMS-14"
- Section 4.0
  Removed "and integrated"
- Section 5.0
  Modified definition for MS/MS
- Section 6.0
  Removed all "2006"

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Appendix C

Certificate of Analysis for R(-)Gossypol and Gossypol-d_2

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CERTIFICATE OF ANALYSIS

Compound Name: R-(-)-Gossypol Acetate

Storage Conditions: 2 – 8°C

Lot No. 3146.D.05.601	**COA Issue Date** August 15, 2005	**Intended Use** Reference Standard	**Retest Date** 08/02/06
Stock Number 37700	**Molecular Weight** 578.6 g/mol	**Empirical Formula** $C_{32}H_{34}O_{10}$	**Date of Mfg.** N/A

We hereby certify that the following analytical tests have been performed and results were found as noted.

TEST	RESULTS
Physical Description (TM 101.000)	Yellow solid No visible contaminants
^{1}H NMR ($CDCl_3$) (TM 110-003)	19343-330 conforms with 13613-270
^{13}C NMR ($CDCl_3$) (TM 110-004)	19343-331 conforms with 13923-040
FTIR (KBr) (TM 108-001)	FT2-2631 conforms with FT2-2177
X-ray powder diffraction (Sent out)	The sample is a crystalline phase or mixture of crystalline phases.
DSC (TM 137-000)	Onset 139.31°C Max 150.45°C Onset 160.14°C Max 167.09°C Onset 179.86°C Max 195.32°C
Karl Fischer (Sent out)	0.17%
Specific Rotation (TM 111-000)	α^{20}_{589} = - 342.5

Johnson Matthey
Pharma Services

Compound Name: R-(-)-Gossypol Acetate

Storage Conditions: 2 – 8°C

Lot No.	**COA Issue Date**	**Intended Use**	**Retest Date**
3146.D.05.601	August 15, 2005	Reference Standard	08/02/06
Stock Number	**Molecular Weight**	**Empirical Formula**	**Date of Mfg.**
37700	578.6 g/mol	$C_{32}H_{34}O_{10}$	N/A

We hereby certify that the following analytical tests have been performed and results were found as noted.

TEST	RESULTS
LC-MS (TM 135-000)	$[M+Na]^+ = 541.2$ Consistent with the structure
HPLC (chemical purity) (TM 113-316)	99.2% AUC, 99.3% w/w
HPLC (chiral purity) (TM 113-317)	100% R-(-)- gossypol
GC- Residual solvents (TM 112-121)	Acetone: 900 ppm Ethyl acetate: < 1000 ppm THF: ND Acetic acid: 106% Heptane: < 1000 ppm
Elemental Analysis (Sent Out)	Actual: C: 66.08% H: 5.86% Theoretical: C: 66.43% H: 5.92%
ROI (Sent Out)	0.22%

Approval: _____ Date: 8/15/05
Quality Assurance

Johnson Matthey
Pharma Services

Rev 0

RESEARCH CERTIFICATE OF ANALYSIS

Compound Name: d_2-Racemic Gossypol Acetic Acid, Recrystallized

Storage Conditions: - 20°C

Lot No.	**COA Issue Date**	**Intended Use**	**Retest Date**
GR-1350-166-2	June 20, 2005	Research	NA
Stock Number	**Molecular Weight**	**Empirical Formula**	**Date of Mfg.**
NA	580.7 g/mol	$C_{30}H_{30}O_8 \cdot C_2H_4O_2$	June 6, 2005

We hereby certify that the following analytical tests have been performed and results were found as noted.

TEST	Method	SPECIFICATION	RESULTS
Physical Description	101.000	Yellow crystalline powder No visible contaminants	Yellow crystalline powder No visible contaminants
FTIR (KBr)	108.001	For Information	FT2-2582 conforms to reference spectrum FT2-1811
Assay (HPLC)	113.316	95.0 to 105.0% w/w	101.4%
Impurities (HPLC)	113.316	Report Total	0.63 %
Enantiomeric Ratio	113.317	45 to 55 % each enantiomer	R (-) = 49.7 S(+) = 50.3
Acetic Acid ID and Content (GC)	112.121	Report Content (% Wt.)	11.7 %
Residual Solvents (GC)	112.121	Acetone: Report	Acetone = 1.53%
Water Content (KF)	USP <921> Sent Out	Report (% Wt.)	<0.5%
Mass Spectrometry	NA	For Information	[M+1-H_2O-Acetic Acid] = 503.3

Approval: _____ Date: 6/20/05
Quality Assurance

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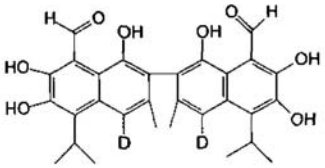
RESEARCH SHIPMENT DATA SHEET

PROJECT NUMBER: 55411Z

COMPOUND INFORMATION:

Product No.	Lot No.	Compound Name:	Net Wt.	Retain
R-3497 B	55411z GR-1350-166-2	Di-deuterogossypol.AcOH	0.506 g	0 g



Molecular Formula: $C_{30}H_{28}D_2O_8$
Molecular Weight: 520.58
Physical Description: Yellow to Yellow orange solid
Final Solvent: Acetone
^{1}H NMR Spectrum #:
Melting Point: N/A
GC:
Mass Spec:
HPLC (assay):
KF:

Elemental Analysis

Element	Calc.	Obs.
C		
H		
N		
Other		

Hazard Warning: Avoid Contact. See attached MSDS

Solubility: S = soluble, MS = moderately soluble, I = insoluble.
For analysis results see research certificate of analysis.

SHIPMENT INFORMATION:

Client:	Ming Guo, PhD Ascenta Therapeutics, Inc. 12750 High Bluff Drive, Suite 320 San Diego, CA 92130	**Ship To:**	Gil Lam, PhD Microconstants, Inc. 10150 Sorrento Valley Rd Suite 316 San Diego, CA 92121
Phone:	858.436.1230	Phone:	858-362-5699
Fax:	858.436.1201	Fax:	

Shipping Instructions:
Ship to arrive by : **06/21/05** Other Client Instructions: **Ship with C of A**
Documentation:

Packaging Instructions:
Ship Cold? [x] Yes [ ] No [x] Cool Packs [ ] 2-8°C [ ] −10°C [x] Dry Ice
Is desiccant required? [ ] Yes [x] No

Prepared By: ELR
Date: 06/17/05

Approved By (Team Leader or Designee): ELR
Date: 06/17/05

Shipped By: D Ball
Date: 06/20/05

Shipped via: Fed Ex
#Pcs & Wt:. 1 @ 4lbs
AWB# 7916 5607 0878

25 Patton Road, Devens, MA 01434 phone (978) 784-5000 fax (978) 784-5500