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Development and validation of a LC-MS/MS method for simultaneous measurement of Tanespimycin and its active metabolite (17-AG) in human plasma.

Morse Faria[*1], *Omnia Ismaiel*[1, 2], *James Waltrip*[1], *Tom Mariannino*[1], *Moucun Yuan*[1], *William Mylott*[1], *Vikram Roongta*[3], *Jim X Shen*[3], *Pathanjali Kadiyala*[3]

[1]Biologics by LC-MS/MS, PPD Laboratories, Richmond, VA,USA

[2]Department of Analytical Chemistry, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt

[3]Bioanalytical Sciences, Research & Development, Bristol-Myers Squibb, Princeton, NJ, USA

*CONTACT INFORMATION: morse.faria@ppdi.com

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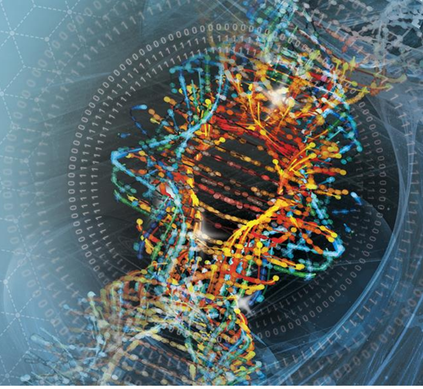
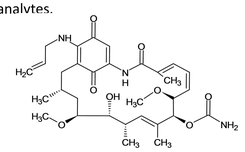
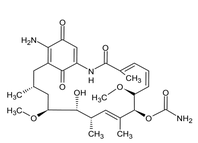


PURPOSE

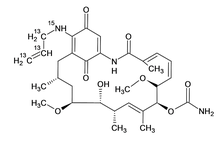
Tanespimycin (17-*N*-allylamino-17-demethoxygeldanamycin, 17-AAG) is a derivative of the antibiotic geldanamycin that is being studied in the treatment of cancer. 17-amino-17-demethoxygeldanamycin (17-AG) is an active metabolite of Tanespimycin [1-3]. During method development, an in-source reduction was observed arising due to the conversion of the quinone moiety in the analyte(s) and internal standard which impacted the quantitation of the active metabolite. This poster highlights the various steps undertaken to mitigate the impact of in-source reduction. A method was developed and validated for simultaneous measurement of Tanespimycin and its active metabolite from sodium heparin human plasma by LC-MS/MS using a stable isotope labeled Tanespimycin-$^{13}C_3$,^{15}N as an internal standard for both analytes.



Tanespimycin (17-AAG)



Tanespmycin metabolite (17-AG)



Tanespimycin-$^{13}C_3$,^{15}N (Internal Standard)

METHOD(S)

Extraction: Protein Precipitation with ACN (1:4) → Centrifuge → Supernatant diluted with 0.2% Acetic Acid (1:1)

LC Instrumentation

LC Pumps: HP 1100 Series (Eluting Pump) or Shimadzu LC-10AD VP
Analytical Column: Waters, XBridge C18 IS, 2.1 mm x 20 mm, 5 µm
Column Temperature: Room Temperature
Mobile Phase A: 0.1 % Acetic Acid, v/v
Mobile Phase B: 0.1 % Acetic Acid in ACN, v/v
Mobile Phase C: 90:10:0.1 MeOH / ACN / Acetic Acid, v/v
Injector Loop: 50 µL ; Injection Volume: 60 µL
Autosampler Wash 1: 50:50:0.1 ACN / MeOH / Acetic Acid, v/v/v
Autosampler Wash 2: 50:50:0.1 H_2O/ MeOH/ Acetic Acid, v/v/v

Eluting Pump Program (Pump 1 to Autosampler)

Elution at 20% Mobile Phase A/80% Mobile Phase B at a flow rate of 500 µL/min for 2.30-3.50 mins

Backflush Pump Program – Pump 2 to Valve 1

100% Mobile Phase C at a flow rate of 550 µL/min

Make-up Pump Program – Pump 3 to Valve 2

100% Mobile Phase B at a flow rate of 500 µL/min

Make-up Pump Program – Pump 4 to Tee Post-column

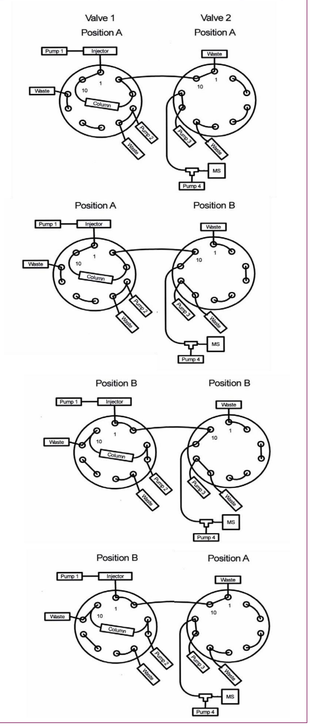
100% Mobile Phase B at a flow rate of 500 µL/min

MS Instrumentation

Mass Spectrometer: Sciex API 4000, Triple quadrupole LC/MS/MS
Ionization Mode: APCI, MRM, negative ion
CAD, CUR, NEB, AUX Gas: Nitrogen;
Source Temp: 425 °C
Nebulizer Current: -3 µA; Collision Gas Flow (CAD): 8.00
Curtain Gas Flow (CUR): 25.00; Nebulizer Gas Flow (GS1): 30.00

Analyte	~ t_R (min)	Q1 m/z	Q3 m/z	Dwell Time (ms)	DP	CE	CXP	EP
Tanespimycin	2.28	584.4	541.3	100	-75	-30	-13	-10
IS	2.28	588.4	545.3	100	-75	-30	-13	-10
17-AG	1.91	544.4	501.4	100	-80	-30	-13	-10

Fig 1. Online dilution (Valve Diagram)



RESULT(S)

In-Source Reduction: During method development, an insidious IS variability trend was observed. The IS response increased proportionally to the Tansepimycin concentrations. Initial observations showed that the quantitation of Tanespimycin and its active metabolite (17-AG) were not impacted. Further investigation revealed that the quantitation of 17-AG were impacted if the Tanespimycin / 17-AG ratio was varied. Recovery studies showed that the IS variability was not arising during extraction. When the Q1 spectra was compared in the presence of different protic mobile phases, a shift from the expected isotopic distribution was observed (See Fig 2). This shift in the isotopic pattern is potentially arising due to the reduction of Tanespimycin (as well 17-AG and IS) quinone moiety ($[M-H]^-$) to its semiquinone($[M]^-$) and hydroquinone ($[M+H]^-$) form [4-7]. As the Tanespimycin and its IS elute at the same retention time, the loss of IS ions due to reduction was compensated by preferential reduction of Tanespimycin at higher concentrations. The IS concentration used in the initial evaluation was comparatively lower i.e. 150 ng/mL to Tanespimycin concentrations (10 - 2500 ng/mL).

Fig 2: Impact of protic solvents on Tanespimycin ionization (similar trends were seen in the internal standard spectra)

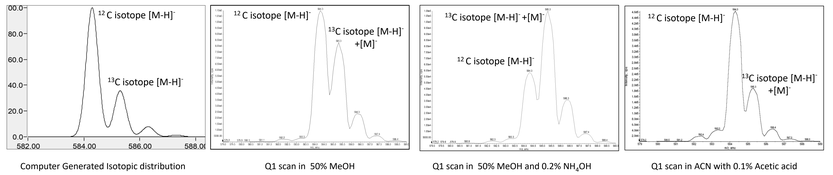
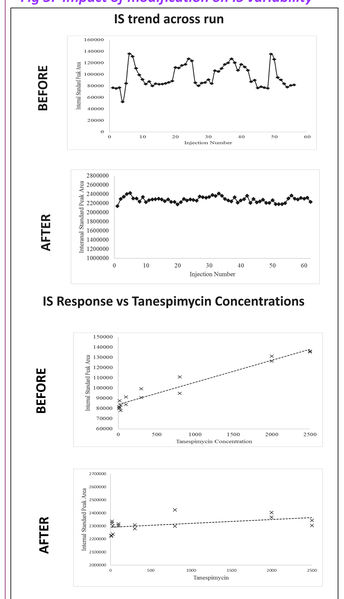


Table 1: Impact of IS concentration and injection volume on quantitation of 17-AG (n=4)

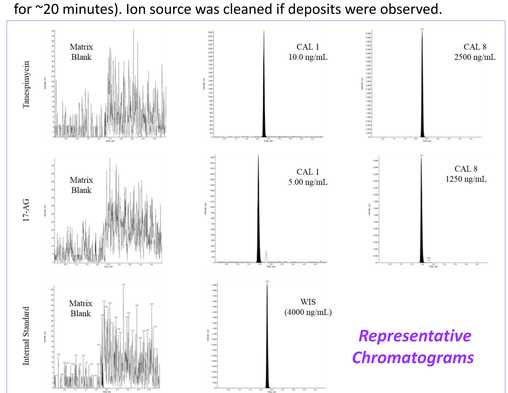
IS Conc. (ng/mL)	150						2500					
Injection Volume /Loop size	30 µL /50 µL Loop			60 µL / 50 µL Loop			30 µL /50 µL Loop			60 µL / 50 µL Loop		
QC level	Low	High	Mid	Low	High	Mid	Low	High	Mid	Low	High	Mid
Tanespimycin Conc. (ng/mL)	NA	NA	1250	NA	NA	1250	NA	NA	1250	NA	NA	1250
17- AG Theoretical Conc. (ng/mL)	12.5	950	110	12.5	950	110	12.5	950	110	12.5	950	110
%C.V.	5.45	2.26	5.48	4.88	1.93	3.28	-5.8	1.99	4.65	3.54	3.13	1.82
% DFT	8.2	*61*	*-24.7*	-0.8	15.4	-5.7	-6.8	5.53	0.23	-4	0.24	8.18

Fig 3: Impact of modification on IS variability



Addressing IS Variability: The following method modification minimized IS variability and improved 17-AG quantitation (see Fig 3 for impact evaluation):

- **ACN (aprotic solvent) with 0.1% acetic acid:** was used for elution to minimize the presence of protic solvent during ionization.
- **The concentration of internal standard:** was *increased* from 150 ng/mL to 2500 ng/mL (final method used 4000 ng/mL), so that the concentration of co-eluting Tanespimycin is comparatively lower and does not impact ionization of the internal standard (see Table 1).
- **The injection volume:** was *increased* from 30 µL to 60 µL to enable higher on- column amount, and in turn higher amount in ion source (see Table 1).
- **Ion Source maintenance** - Ion Source temperature was increased from 350 °C to 425 °C. The APCI source probe was heated in-between each run (700 °C for ~20 minutes). Ion source was cleaned if deposits were observed.



Representative Chromatograms

RESULT(S) — *Validation Summary*

Analyte	Tanespimycin (17-AAG)			17-AG		
Internal Standard (IS)	Tanespimycin-13C3,15N			Tanespimycin-13C3,15N		
Regression, Weighting	Linear, 1/concentration²			Linear, 1/concentration²		
Standard Curve	10.0 to 2500 ng/mL			5.00 to 1250 ng/mL		
QC Concentrations	10.0, 25.0, 180, 1250, 1900 and 10000 ng/mL			5.00, 12.5, 90.0, 625, 950, and 5000 ng/mL		
Intra-Assay Statistics (n=36)	**Conc. (µg/mL)**	**Precision**	**Accuracy**	**Conc. (µg/mL)**	**Precision**	**Accuracy**
LLOQ	10.0	2.1% to 3.6%	-8.7% to -1.8%	5.00	5.2% to 9.9%	-8.7% to -1.8%
Low	25.0	1.7% to 4.3%	-4.3% to 1.0%	12.50	2.8% to 11.1%	-6.3% to 9.7%
Geometric Mean	180	1.5% to 3.3%	-3.9% to 2.5%	90.00	3.5% to 7.1%	-6.4% to 12.6%
Mid	1250	1.7% to 3.9%	-3.7% to 0.4%	625.00	2.8% to 7.1%	-0.4% to 7.4%
High	1900	1.4% to 2.9%	-5.8% to 0.0%	950.00	3.6% to 4.8%	-5.0% to 5.9%
Over-the-curve (Diluted 10-fold)	10000	0.4% to 3.4%	-3.2% to 0.6%	5000.00	3.8% to 8.2%	-2.8% to 3.2%
Low (Only Tanespimycin)	25.0	1.1% to 3.9%	-5.3% to 0.1%	NA	NA	NA
High (Only Tanespimycin)	1900.0	1.8% to 4.5%	-4.9% to 2.0%	NA	NA	NA
Low (Only 17-AG)	NA	NA	NA	12.50	2.7% to 7.8%	-6.6% to 6.2%
High (Only 17-AG)	NA	NA	NA	950.00	4.5% to 7.2%	-0.1% to 7.0%
Inter-Assay Statistics (n=36)	**Conc. (µg/mL)**	**Precision**	**Accuracy**	**Conc. (µg/mL)**	**Precision**	**Accuracy**
LLOQ	10.0	3.7	-4.7	5.00	8.30	-4.21
Low	25.0	3.4	-2.2	12.5	5.97	3.26
Geometric Mean	180	3.3	-1.3	90.0	7.59	3.95
Mid	1250	3.2	-1.6	625	5.77	0.90
High	1900	2.9	-3.3	950	5.88	-0.64
Over-the-curve (Diluted 10-fold)	10000	3.0	-1.6	5000	6.14	1.10
Low (Only Tanespimycin)	25.0	3.3	-2.8	NA	NA	NA
High (Only Tanespimycin)	1900.0	4.1	-2.7	NA	NA	NA
Low (Only 17-AG)	NA	NA	NA	12.5	7.04	0.33
High (Only 17-AG)	NA	NA	NA	950	6.08	2.85
Freeze-thaw Stability (cycles)	Three cycles frozen at -20 °C and thawed at room temperature under reduced light			Three cycles frozen at -20 °C and thawed at room temperature under reduced light		
Extract Stability (hours)	115 hours at 2 to 8 °C			68 hours at 2 to 8 °C		
Frozen Matrix Storage Stability (days)	118 days at -20 °C and -70 °C			118 days at -20 °C and -70 °C		
Whole Blood Stability	Whole blood samples were stable at RT and on ice for at least for at least 0.5 hrs			Whole blood samples were stable at RT and on ice for at least for at least 0.5 hrs		
Reinjection Reproducibility	It is possible to re-inject runs.			It is possible to re-inject runs.		
Selectivity	No significant interfering peaks noted in blank human plasma samples.			No significant interfering peaks noted in blank human plasma samples.		
Matrix Factor	Lot-to-lot response consistency was demonstrated			Lot-to-lot response consistency was demonstrated		

CONCLUSION(S)

A robust method was developed and validated for simultaneous measurement of Tanespimycin and its active metabolite from sodium heparin human plasma for use in pharmacokinetic studies. Internal standard variation due to in-source reduction of quinone based moiety was addressed by selection of an appropriate mobile phases, internal standard concentration, injection volume, source temperature and continuous maintenance of the source between runs.

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