Elimination of Ionization Suppressing Dosing Excipient from LC-MS Method for Bendamustine and M3 in Human Plasma

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Introduction

Intravenously administered chemotherapy drug Bendamustine is readily hydrolyzed *in vivo* to mainly inactive metabolites, and partly to cytotoxic M3. Because Bendamustine is also unstable in aqueous solution, different dosing formulations have been tested to reduce this instability.

Some of these excipients remain in the extracts following protein precipitation of the plasma samples and caused severe ionization suppression when using HILIC chromatography coupled with positive mode electrospray ionization. Labeled internal standards (ISTDs), Bendamustine-D11 and M3-D8, didn't sufficiently track the target analytes during this suppression.

To enable accurate quantitation and reduce ionization suppression, a supported liquid extraction was developed to remove the excipients. The LC-MS/MS method was successfully validated, and used to support the pharmacokinetic portion of numerous clinical trials with various dosing formulations.

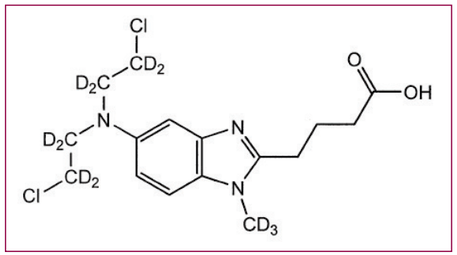

Figure 1. Structure of Bendamustine-D11.

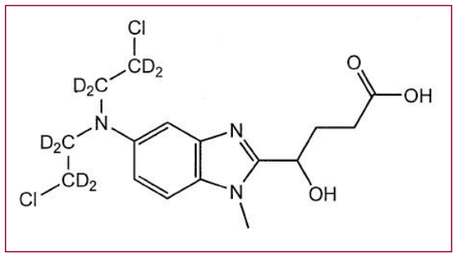

Figure 2. Structure of M3-D8.

Methods

The LC-MS/MS conditions used an Atlantis HILIC Si 50x2 mm, 5 µm column, mobile phase at 0.8 mL/min, consisting of a acetonitrile/water gradient with ammonium formate and formic acid. Detection was performed on an API-4000 triple quadrupole mass spectrometer, using positive mode electrospray. MRM mass transitions of 358/228, 374/198, 369/233 and 382/200 were used for Bendamustine, M3, Bendamustine-D11 and M3-D8, respectively.

Human plasma high QC samples (HQC) were prepared with both Bendamustine and M3, with some sub lots also fortified with various combinations and concentrations of potential dosing excipients; PEG400, propylene glycol (PG) and monothioglycerol (MTG).

Matrix effect and post-column infusion experiments were performed to compare two extraction mechanisms: (1) protein precipitation with acidified acetonitrile, and (2) supported liquid extraction (SLE+) using MTBE under acidic conditions.

Protein Precipitation	Supported Liquid Extraction
0.05 mL of human plasma sample	0.05 mL of human plasma sample
	0.2 mL 5% Formic Acid in Water
0.3 mL of 1% Formic Acid in ACN, with ISTDs	0.05 mL Methanol containing ISTDs
Vortex mix	Vortex mix
Centrifuge	Load 0.2 mL onto Biotage SLE+
Transfer supernatant	Elute with 1 mL MTBE
	Evaporate
	Reconstitute 0.3 mL 1% Formic Acid in ACN
Inject on LC-MS/MS	Inject on LC-MS/MS
Not suitable for samples containing PEG-400	**Suitable for samples containing PEG-400**

Column: Waters, Atlantis HILIC Si, 50 x 2.1 mm, 5 µm particle size

Mobile Phase: A: Water with 30 mM ammonium formate and 0.1% formic acid
B: Acetonitrile with 0.1% formic

Gradient Program:

Time (minute)	Function	Value (%)
0.01	Pump B Conc.	94
0.20	Pump B Conc.	94
1.80	Pump B Conc.	85
1.90	Pump B Conc.	70
2.50	Pump B Conc.	70
2.60	Pump B Conc.	94
3.50	Stop	

Table 1. MRM Mass Transitions on API-4000 Triple Quadrupole Using Positive Mode Electrospray

Compound Name	Transition Monitored	Collision Energy (eV)
Bendamustine	358.2→228.2	49
M3	374.2→198.1	64
Bendamustine-D11	369.2→233.2	49
M3-D8	382.2→200.1	64

Results

Table 2. Bendamustine Extracts from Protein Precipitation

Sample Name	Analyte Peak Area (counts)	IS Peak Area (counts)	% Accuracy	% from Normal Drug Response	% from Normal ISTD Response
HQC, no excipients	9219818	526905	98.4	102.4	105.4
HQC + PG 0.8 mL/L	9264524	514058	101.0	102.9	102.8
HQC + MTG 34 mg/L	9016005	501492	101.0	100.2	100.3
HQC, no excipients	8815378	487548	102.0	97.9	97.5
HQC + PEG400 6.4 mL/L	**1267746**	**81479**	**87.5**	**14.1**	**16.3**
HQC, no excipients	8851445	500312	99.5	98.3	100.1
HQC + PEG400 3.2 mL/L	**1647855**	**124280**	**74.6**	**18.3**	**24.9**
HQC, no excipients	8817856	509745	97.3	98	101.9
HQC + PEG400 1.28mL/L	**2727883**	**208684**	**73.5**	**30.3**	**41.7**
HQC, no excipients	9005789	500673	101.0	100.1	100.1
HQC + PEG400 0.64 mL/L	**3611123**	**265488**	**76.5**	**40.1**	**53.1**
HQC, no excipients	9023246	507865	99.9	100.3	101.6
HQC + PEG400 0.32 mL/L	**4889251**	**329294**	**83.5**	**54.3**	**65.9**

Table 3. M3 Extracts from Protein Precipitation

Sample Name	Analyte Peak Area (counts)	IS Peak Area (counts)	% Accuracy	% from Normal Drug Response	% from Normal ISTD Response
HQC, no excipients	5518996	1536035	98.8	102.0	103.2
HQC + PG 0.8 mL/L	5500593	1519081	99.5	101.6	102.1
HQC + MTG 34 mg/L	5540633	1507262	101	102.4	101.3
HQC, no excipients	5308561	1462483	99.8	98.1	98.3
HQC + PEG400 6.4 mL/L	**369075**	**89086**	**114**	**6.8**	**6.0**
HQC, no excipients	5374335	1514828	97.5	99.3	101.8
HQC + PEG400 3.2 mL/L	**751683**	**170095**	**121**	**13.9**	**11.4**
HQC, no excipients	5351473	1479098	99.5	98.9	99.4
HQC + PEG400 1.28mL/L	**1607532**	**364445**	**121**	**29.7**	**24.5**
HQC, no excipients	5532152	1533981	99.1	102.2	103.1
HQC + PEG400 0.64 mL/L	**2458005**	**617514**	**109**	**45.4**	**41.5**
HQC, no excipients	5550870	1524223	100	102.6	102.4
HQC + PEG400 0.32 mL/L	**3211170**	**804719**	**110**	**59.3**	**54.1**

Results of various combinations of excipients (PEG400 + PG + MTG) were dictated by the amount of PEG400 (data not shown), closely matching the response from the tables above as if PEG400 was the only excipient.

For the SLE+, no detectable impact on the intensities or accuracies could be detected (data not shown).

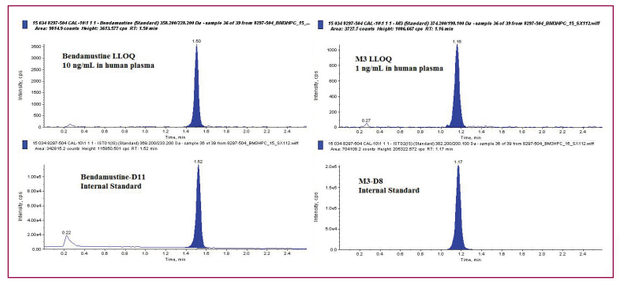

Figure 3. Chromatogram of extracted LLOQ, 10 ng/mL of Bendamustine and 1 ng/mL of M3 in human plasma, extracted with SLE+.

A post-column infusion experiment was performed to see the area of ion suppression caused by PEG400 when using the HILOC chromatography. A blank reagent sample was used as the control, and a neat solution with the PEG400 diluted down to the expected levels post-extraction. These samples were injected while infusing an ISTD post-column to observe the impact on the ionization.

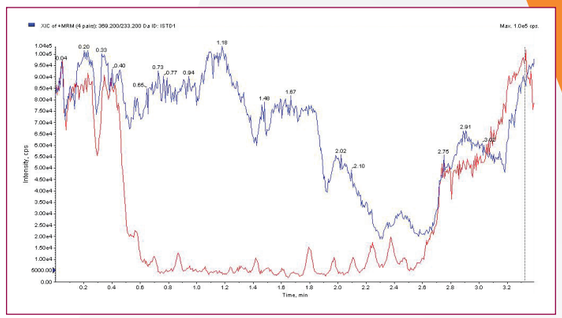

Figure 4. Post Column Infusion Experiment. Bendamustine-D11 infused post-column.

Blue scan = Injection of blank reagent neat solution showing normal effect from gradient.

Red scan = Injection of solution containing expected concentration of dosing excipient PEG-400, showing massive and long lasting suppression to the ionization.

Expected retention times: Bendamustine 1.5 minutes, M3 1.2 minutes.

Discussion

Results from plasma samples fortified with only Bendamustine and M3 (with no excipients) extracted via protein precipitation produced excellent results. For QC samples containing PEG400 at concentrations anticipated at the end of IV dosing, the protein precipitation extracts showed significant decrease in signal intensity (up to 90% lower). The analyte-to-ISTD ratios were also impacted by the use of PEG400, potentially affecting the measured concentrations. The post-column infusion experiment confirmed significant ion suppression caused by PEG400 at the retention times of Bendamustine and M3. The stable-labeled ISTDs, Bendamustine-D11 and M3-D8, had slightly different retention times (deuterium effect) from their corresponding analytes, which likely prohibited them from adequately compensating for the ion suppression.

The other tested excipients (PG and MTG) had no significant impact on the signal intensity from the protein precipitation extracts.

The extracts from the SLE+ likely removed most or all of the PEG400 from the final extracts, and thus displayed no observable signal suppression such that a sample without PEG400 would be indistinguishable from samples containing PEG400. PEG400, PG and MTG had no observable adverse impact on the method when using the SLE+ with the LC-MS/MS parameters defined.

The LC-MS/MS method was successfully validated and used to support the pharmacokinetic portion of numerous clinical trials with various dosing formulations including some containing high levels of PEG400.

Presented at ASMS 2017

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