Validation of an LC-MS/MS Method for the Determination of Dronedarone (Multaq®) in Human Plasma

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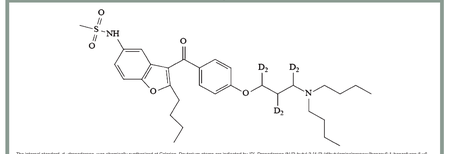
OBJECTIVES:

- Validation of a liquid-liquid extraction of dronedarone from plasma samples to provide clean samples compatible with LC-MS/MS analysis.
- Validation of a sensitive and selective LC-MS/MS method
- Synthesis of a deuterated internal standard to track dronedarone extraction and injection onto the LC-MS/MS for enhanced precision and accuracy (see Figure 1).
- Establish intraday and interday precision and accuracy for the method according to industrial guidelines.
- Establish stability of dronedarone in stock solutions, biological matrix, and injection solvent for the time periods and temperatures required for analysis of clinical samples.
- Obtain consistent extraction recovery and consistent peak responses from the LC/MS/MS instrument for multiple lots of plasma, including hemolyzed and lipemic lots.

Figure 1. Structure of the deuterated internal standard, d_6-dronedarone



The internal standard, d_6-dronedarone, was chemically synthesized at Celerion. Deuterium atoms are indicated by "D". Dronedarone (*N*-[2-butyl-3-[4-[3-(dibutylamino)propoxy]benzoyl]-1-benzofuran-5-yl] methanesulfonamide) is an antiarrhythmic agent, indicated for the treatment of patients with a history of atrial fibrillation or atrial flutter. Multaq® was approved for use in patients whose heart rhythms have returned to normal or patients who will undergo drug or electric shock therapy to restore normal heart rhythm. Dronedarone is an extremely hydrophobic compound with logP >7. The sulfonamide and tertiary amine functional groups both have pK_a values near 8.4. The drug is >98% bound in vitro to plasma proteins. Dronedarone is a moderate inhibitor of CYP 3A and CYP 2D6.

METHODOLOGY:

- Internal standard, d_6-dronedarone (86.12% potency, 99% isotopic purity).
- Dronedarone and d_6-dronedarone stock solutions were 100 µg/mL in methanol, stored at −20 °C.
- Plasma free of significant interference at the retention time and mass transitions of dronedarone and d_6-dronedarone (IS) was used for control matrix to prepare calibration standard and quality control (QC) samples.
- Human plasma (EDTA) containing the analyte and internal standard was extracted using a liquid-liquid extraction procedure.
  - 0.0500 mL of each plasma sample was aliquotted in 2 mL 96 well plate.
  - Calibration standards contained 1.00, 2.00, 5.00, 10.0, 25.0, 50.0, 100, 200, or 250 ng/mL dronedarone in human plasma.
  - Quality control (QC) samples contained 1.00 (LLOQ), 3.00, 60.0, 175, or 500 (dilution) ng/mL dronedarone in human plasma. Dilution QC samples were diluted 10-fold with blank control human plasma at the time of analysis.
  - Working internal standard (10.0 ng/mL d_6-dronedarone in acetonitrile, 0.0500 mL) was spiked into each sample. A spike of acetonitrile (ACN) was substituted for double Blank samples.
  - Added 0.3 mL of ACN to each sample.
  - Centrifuged and transferred 0.2 mL of supernatant to a clean 2 mL 96 well plate.
  - Added 0.2 mL of a NaOH solution, mixed, and added 0.8 mL of *n*-butyl chloride to each sample.
  - After mixing, transferred 0.2 mL of the organic layer to a 1.2 mL 96 well plate.
  - Dried the samples under nitrogen at 40 °C and reconstituted samples in ACN.
  - Extracted samples were stored at 5 °C before and during injection on the LC-MS/MS.
- Extracted samples were analyzed by an HPLC equipped with an AB SCIEX 4000 triple quadrupole mass spectrometer using an ESI source.
  - Stationary Phase was Agilent Zorbax 300-SCX, 3.0 x 50 mm column with 5 µm particles, run at ambient temperature.
  - Mobile Phase was a mixture of acetonitrile and low pH ammonium formate, isocratic, and pumped at 1 mL/min.
  - Needlewash solutions: (1) TFA in MeOH, (2) ACN: water mixture
  - Injection volume on Leap Technologies CTC PAL autosampler was 5 to 10 µL.
  - Multiple reaction monitoring (MRM) of positive ions for dronedarone (557.3 ➔ 435.4) and d_6-dronedarone (563.4 ➔ 441.3).
  - Acquisition time for chromatograms was 2 minutes.
- Quantitation was performed using a weighted (1/concentration^2) linear regression analysis of instrument response (ratio of peak areas of the analyte and internal standard) versus concentration.

RESULTS:

- Dronedarone response was linear from 1 – 250 ng/mL in K_2EDTA human plasma. Precision and accuracy statistics for standard concentrations back-calculated from the calibration curve are summarized in Table 1.
- Precision and accuracy of quality control (QC) samples were within acceptance criteria. A summary of statistics for intra-batch and inter-batch QCs is detailed in Table 2.
- No significant interference at the retention time and mass transition of dronedarone or d_6-dronedarone was observed from endogenous components in any of the 10 human plasma (EDTA) lots screened.
- No significant matrix effect was observed in any of the 10 human plasma (EDTA) lots that were fortified with dronedarone at the concentration of the LLOQ or the high QC (175 ng/mL). See Table 3.
- Accurate quantification and selectivity of the method was demonstrated in the presence of ibuprofen (20,000 ng/mL), nicotine (20.0 ng/mL), caffeine (20,000 ng/mL), and acetaminophen (25,000 ng/mL).
- Carryover was evaluated by injection of reconstitution solution immediately following extracted plasma samples fortified with dronedarone at the ULOQ. No significant carryover was observed.
- Blank plasma samples surrounded by plasma samples fortified with dronedarone at the ULOQ, and vice versa, were extracted in a 96-well plate to test for cross-well contamination. No significant contamination was observed.
- Integrity of quantification was established in three lots of plasma containing 0.5% hemolyzed whole blood as well as in three lots of lipemic plasma (see Table 4).
- Recovery of dronedarone from plasma was 88 - 92% over the analytical concentration range. The recovery of d_6-dronedarone was 101%, consistent with the analyte.
- Stability was demonstrated for dronedarone in stock solutions, in extracted samples, and in human plasma (EDTA), including hemolyzed and lipemic plasma. Sufficient stability was demonstrated for processing clinical samples (see Table 5).
- Dronedarone in fresh whole blood and fresh human plasma that is not kept under ice-cold conditions is apparently prone to degradation, particularly at low concentrations. Dronedarone, when spiked in fresh whole blood (EDTA) kept at room temperature, appeared to degrade to <80% of the initial concentration (at the low end of the standard curve range) even within 22 minutes of incubation at room temperature (see Table 6). The degradation significantly slowed down, falling slightly more to 75% of the initial concentration after 2 hours of incubation at room temperature. Due to evidence suggesting degradation, clinical samples were collected in pre-chilled containers. Plasma samples were thawed in an ice water bath and kept cold while out of frozen storage.
- 55 out of 56 (or 98.2%) reanalyzed incurred samples quantified to within ±20% of the mean of the original and reassay concentrations (see Table 7).

Table 1. Back-calculated Calibration Curve Standard Concentrations of Dronedarone in Human Plasma (EDTA)

	STD B 1.00 ng/mL	STD C 2.00 ng/mL	STD D 5.00 ng/mL	STD E 10.0 ng/mL	STD F 25.0 ng/mL	STD G 50.0 ng/mL	STD H 100 ng/mL	STD I 200 ng/mL	STD J 250 ng/mL
Mean	1.00	2.01	4.87	10.3	23.8	50.2	103	199	252
% CV	0.9	1.0	3.7	4.4	3.2	0.7	1.5	3.1	0.4
% Bias	0	0.5	-2.6	3.0	-4.8	0.4	3.0	-0.5	0.8
n	3	3	3	3	3	3	3	3	3

Table 2. Intra-batch and Inter-batch Precision and Accuracy of Dronedarone in Human Plasma (EDTA)

Batch		LLOQ QC 1.00 ng/mL	QC A 3.00 ng/mL	QC B 60.0 ng/mL	QC C 175 ng/mL
Intra-batch 1	Mean	0.845	2.74	55.7	164
	CV%	5.5	2.4	2.7	2.8
	%Bias	-15.5	-8.7	-7.2	-6.3
	n	12	12	12	12
Intra-batch 2	Mean	0.949	2.96	59.8	175
	CV%	10.3	5.2	1.3	2.8
	%Bias	-5.1	-1.3	-0.3	0
	n	6	12	12	12
Intra-batch 3	Mean	0.911	2.84	58.2	171
	CV%	3.6	4.5	2.2	3.1
	%Bias	-8.9	-5.3	-3.0	-2.3
	n	6	6	6	6
Inter-batch	Mean	0.888	2.85	57.8	170
	CV%	8.3	5.4	3.8	4.1
	%Bias	-11.2	-5.0	-3.7	-2.9
	n	24	30	30	30

Table 3. Matrix Effect for Dronedarone in Human Plasma

	Lot#	LLOQ 1.00 ng/mL	LLOQ % Dev.	High 175 ng/mL	High % Dev.
	1	0.863	-13.7	180	2.9
	2	1.07	7.0	181	3.4
	3	0.971	-2.9	178	1.7
	4	0.984	-1.6	175	0
	5	1.02	2.0	185	5.7
	6	0.938	-6.2	175	0
	7	1.03	3.0	167	-4.6
	8	0.937	-6.3	181	3.4
	9	1.08	8.0	173	-1.1
	10	0.922	-7.8	172	-1.7
Mean		0.982		177	
% CV		7.0		3.0	
% Theoretical		98.2		101.1	
n		10		10	

Table 4. Integrity for Dronedarone in Hemolyzed and Lipemic Human Plasma (EDTA)

	Hemolyzed		Lipemic	
	LLOQ 1.00 ng/mL	High 175 ng/mL	LLOQ 1.00 ng/mL	High 175 ng/mL
Mean	1.04	181	0.993	185
% CV	4.7	1.5	3.5	2.4
% Theoretical	104.0	103.4	99.3	105.7
n	3	3	3	3

Table 5. Stability of Dronedarone in Biological Matrix, Extracted Samples, and Stock Solutions

Stability Test	Results
Bench-Top Stability (Hrs)	Short-Term Stability in polypropylene tubes in an ice water bath under UV shielded light: 7 hours for control QCs, 24 hours for fresh plasma QCs, 28 hours for hemolyzed, lipemic plasma Cumulative Short-Term Stability (total of all thaw cycles) in polypropylene tubes in an ice water bath under UV shielded light: 37 hours for control QCs, 52 hours for fresh plasma QCs, 41 hours for hemolyzed, lipemic plasma
Freeze-Thaw Stability (Cycles)	Freeze (-20°C)-thaw (ice water bath) cycles in polypropylene tubes under UV shielded light: 6 cycles for control or fresh plasma QCs, 3 cycles for lipemic or hemolyzed plasma
Stock Stability (Days)	Approximately 100 µg/mL in methanol in a polypropylene container: 126 days long-term stability at -20°C, 17 hours short-term stability at ambient temperature under UV shielded light
Processed Stability (Hours)	Post-Preparative Stability: 107 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 102 hours in a polypropylene 96 well plate at 5°C
Long-Term Storage Stability (Days)	90 days in polypropylene tubes at -20°C (control QCs)
Sample Shipping Stability (Days)	3 days in polypropylene tubes at -80°C
Stability of Analyte During Sample Collection and Handling	Up to 120 minutes in human whole blood (EDTA) in polypropylene tubes in an ice water bath under white light
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.0500 mL, stored for 74 hours in a polypropylene 96 well plate at -20°C prior to extraction

Table 6. Evidence for the Degradation of Dronedarone During Sample Collection and Handling at Room Temperature

	SCHS A 3.00 ng/mL		SCHS D (DF 10) 500 ng/mL			SCHS A 3.00 ng/mL		SCHS D (DF 10) 500 ng/mL	
Time (min):	0	22	0	22		0	120	0	120
Mean	2.00	1.59	297	280		1.62	1.22	306	285
% CV	4.2	4.0	1.0	2.5		7.3	3.2	2.5	2.0
% of Control		79.5		94.3			75.3		93.1
n	6	6	6	6		6	6	6	6

CONCLUSIONS:

- A sensitive, precise, and accurate method has been developed and validated for the quantification of 1.00 to 250 ng/mL dronedarone in human plasma.
- The validated method reproducibly quantified dronedarone in clinical samples from a drug interaction study.
- Dronedarone has been demonstrated to require ice-cold temperatures while in fresh human plasma to remain stable.

Table 7. Summary of Incurred Sample Reproducibility Results

% Difference of Original vs. Reassay	Instances	% of Total Instances
0 - <2%	7	12.5
2% - <5%	13	23.2
5% - <10%	21	37.5
10% - <15%	13	23.2
15% - <20%	1	1.8
20% - <100%	0	0.0
Undefined *	1	1.8
Total	56	100.0

* Reassay concentration outside (above) analytical range of assay.

Figure 2. Representative chromatography of extracted double blank (no analyte, no IS) and blank (with IS) samples

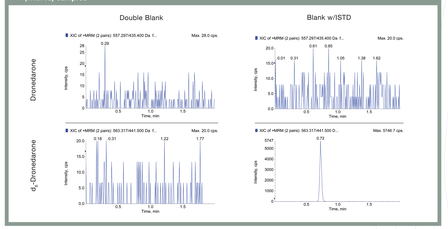


Figure 3. Representative chromatography of dronedarone at LLOQ (1.00 ng/mL) and ULOQ (250 ng/mL) concentrations, extracted from human plasma