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An improved liquid chromatography tandem mass spectrometry (LC-MS/MS) method for quantification of dexmedetomidine concentrations in samples of human plasma

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Abstract

Dexmedetomidine (DMET) is a sedative, analgesic and anxiolytic with minimum adverse respiratory effects. An LC-MS/MS bioanalytical method has been developed and validated to accurately measure DMET concentrations in samples of human plasma. The method overcomes difficulties in the extraction and quantification of DMET due to the fact that it binds strongly to glass and plastic tubes, as well as solid phase extraction (SPE) cartridges. Human plasma (50 µL) was mixed with the internal standard (IS) (DMET-d4) solution (100 µl) and 0.1% formic acid (50 µL) and extracted using Oasis HLB 1 CC (30 mg) solid phase extraction (SPE) cartridges (Waters®). The glass tubes were coated with bovine serum albumin (BSA) 0.5% (20 µL) before eluting DMET and the IS. After evaporation under nitrogen at room temperature, the analytes were reconstituted in 20% acetonitrile in 0.1% formic acid in water and transferred to silanized glass vials. An electrospray ionisation (ESI) mass spectrometry method in positive mode was created and the precursor/product transitions (m/z) were 201.1 → 95.0 (DMET) and 204.9 → 99.0 (IS). The method was robust and fully validated based on the 2012 EMEA guideline for bioanalytical method validation in the concentration range of 0.5-20 ng/mL. Using this assay, we showed that DMET binds strongly to Extracorporeal Membrane Oxygenation (ECMO) circuits, consistent with expectations for small lipophilic compounds.

1. Introduction

Dexmedetomidine (DMET) is a potent sedative and analgesic drug with high affinity for α-2-receptors which has been approved by the United States Food and Drug Administration (FDA) for use in Intensive Care Units [1]. DMET is a lipophilic small molecule (Mw=200 g/mole, Log P=3.2) with a large volume of distribution (1.0 L/kg) and it has been reported to be adsorbed to polyvinyl chloride (PVC) tubing during circulation of patients´ blood through extracorporeal membrane oxygenation (ECMO) circuits [2].

There are several published LC-MS/MS methods for the quantification of DMET with the focus of these methods being on reducing sample volume and improving the lower limit of quantification (LLOQ) [3-9]. Most of these assays require evaporation of extracted samples after Liquid-Liquid Extraction [3-4] or SPE [5-7] necessitating long periods of exposure to the plastic or glass surfaces which can potentially adversely affect the performance of the assay. More recently, a protein precipitation method using 200 µL acetonitrile added directly to plasma samples (100 µL) without any evaporation step and a very short run-time, was published [8]. However, this approach produces 'dirty' samples and so is only suitable for low turn-over laboratories with small batches as it requires extensive washing of the HPLC system between sample analysis batches.

The aim of this study was to develop and fully validate an LC-MS/MS bioanalytical method to improve the quantification of DMET in samples of human plasma. This assay was used to estimate the extent of adsorption of DMET onto ECMO circuits. In this paper, we explain the main issues overcome during method development and validation for quantification of DMET concentrations in samples of human plasma.

2. Experimental

2.1 Materials and Reagents

DMET and DMET-d4 were obtained from Toronto Research Chemicals (North York, Ontario, Canada). Formic acid (99%) was purchased from Univar (Sydney, NSW, Australia). HPLC grade methanol and acetonitrile were bought from Lab Scan (Brisbane, QLD, Australia). Human blank plasma was obtained from BioCore Pty Ltd (Sydney, NSW, Australia). SPE cartridges; Oasis HLB 1 CC (30 mg) were purchased from Waters (Rydalmere, NSW, Australia).

2.2 Chromatographic Conditions

Liquid chromatography and pump gradients were created using Symbiosis Pro software for analyst (V 2.1.0.0) and the mass spectrometer controlling software (Analyst 1.6.1). An HPLC column (X-Terra® MS C18, 2.1 mm x150 mm, 5 µm; Waters, Sydney, NSW, Australia), and Phenomenex Security Guard C18 column (Phenomenex, Sydney, Australia) were used for chromatographic separation of the DMET and DMET-d4. The column and autosampler temperatures were set at 50°C, and 4 °C, respectively. The mobile phase comprised solvent A (0.1% formic acid in water) and solvent B (acetonitrile) and the mobile phase flow rate was 0.5 mL/min. The mobile phase elution program was started at 20% mobile phase B and continued until 1:00 min. Then, the flow increased to 90% B at 2:50 min and stayed constant for 1 min before dropping to 20% B at 3:40 min, followed by a 2 min equilibrium period.

2.3 Mass Spectrometry Conditions

Mass spectrometry detection was carried out in ESI positive mode using a Q-Trap 5500 (AB-Sciex, Concord, Ontario, Canada) LC-MS/MS instrument. The highest abundant product ions which had no interference by plasma matrix components were selected for the quantification of DMET and the internal standard (IS).

2.4 Preparation of standards, calibration curves and quality control samples

Stock solutions of DMET and DMET-d4 were prepared in 50% methanol in water at a concentration of 1 mg/mL and were stored frozen at −20 °C. Working solutions were made in 50% methanol in water to achieve a concentration of 10 µg/mL. For making calibration standards, the working solution (10 µg/mL) was diluted in 50% methanol in water to make standard solutions of 20, 16, 10, 5, 3, 1, 0.5 ng/mL. Aliquots of the standard solutions (50 µL) were spiked in 50 µL aliquots of pooled human plasma and mixed with IS solution 10 ng/mL (100 µL) and 0.1% formic acid in water (50 µL).

For QC preparations, aliquots of the stock solution of DMET in 50% methanol in water (100 µg/mL) were serially diluted using 50% methanol in water to prepare four QC solutions. Aliquots of 100 µL of those solutions were spiked in 5 mL of pooled human plasma to achieve final concentrations of 15 (QCH), 10 (QCM), 1.5 (QCL) and 0.5 (QCLLOQ) ng/mL. QCs prepared in pooled human plasma were aliquoted in small volumes (200 µL) and stored in frozen at -20 °C.

2.5 Sample preparation

Plasma samples and QCs (50 µL) were mixed with 100 µL aliquots of the IS (DMET-d4) solution at 10 ng/mL in 0.1% formic acid in water) and 50 µl aliquots of 0.5% formic acid in water, and vortexed before loading onto SPE cartridges. The SPE cartridges were conditioned with 1 mL aliquots of methanol and 1 mL aliquots of 0.1% formic acid in water prior to loading of the plasma samples. After plasma sample loading, the cartridges were washed with 30% methanol in water (1 mL). Before elution of the analytes of interest, aliquots of BSA 0.5% in water were added to each tube (20 µL) and vortexed. Then, DMET and the IS were eluted using 1 mL of 5% isopropanol, 10% acetonitrile and 85% methanol. Samples were evaporated using nitrogen at room temperature and reconstituted in 20% acetonitrile in 0.1% formic acid in water and transferred to silanized glass tubes with crimped

caps and placed in the autosampler. Thereafter, 10 µL aliquots were injected into the LC-MS/MS system.

2.6 Assay performance

The method was validated based upon the criteria specified by EMEA guideline on bioanalytical method validation, February 2012 [10].

3. Results and Discussion

3.1 Method development

SPE is preferable to liquid-liquid extraction of plasma samples in terms of minimising the use of toxic organic solvents. Protein precipitation is good when only small numbers of samples need to be analysed quickly because it negatively affects the life-time of the HPLC column and the whole LC-MS/MS system. In the present method, a relatively high percentage of methanol in water (30% methanol in water) was used to wash the SPE cartridges prior to analyte elution and this resulted in very clean samples without losing the analytes. Acidification of plasma samples before SPE extraction can reduce the lipophilicity of the DMET (Log D= 2.3 at pH=3) and therefore make it easier for elution from the SPE cartridges.

Initially, we used tolazoline as IS but it was unsatisfactory because its elution pattern from the cartridges was differed from that of DMET. As noted by Lee et al [5], tolazoline needs to be added after completion of SPE which compromise the role of IS as a correction factor for loss of analytes during the extraction. Dissimilar physicochemical properties between tolazoline and DMET are also underscored by the very different retention time of these two analytes [5]. Cui et al [3] used testosterone as the IS for the validation of the DMET assay in ewe plasma samples. However, testosterone is not suitable for the analysis of human samples due to the endogenous levels of the hormone in human blood. Using DMET-d4 with

similar affinity for SPE cartridges and the HPLC column enabled a robust SPE extraction method to be devised.

A significant issue with extraction of DMET from samples of human plasma was its binding to all tubes with a higher binding tendency to plastic compared with glass tubes which this problem is exacerbated at low concentrations. Although QCs stored in the freezer (both -20 °C and -80 °C) for only a few weeks may pass the accuracy criteria, longer periods of frozen storage led to a significant reduction in the content of DMET in QCL and QCLLOQ. This is likely due to adsorption of the analyte to the plastic tubes during the storage. When the pure standards (0.5-10 ng/mL) were diluted in plastic tubes, evaporated to dryness at room temperature and reconstituted in 500 µL of 0.1% formic acid in water, the peak areas of the standards were 15-20% smaller than what was observed for the same solutions in glass tubes (this was most marked at low concentrations). Furthermore, for the Low standard (1.5 ng/mL) spiked in plasma and extracted, the peak area was 7-fold larger than that for the same standard in solution which may be due to the presence of plasma proteins in minimising the adsorption of the analyte to the tubes' surfaces.

To address this issue, we used glass tubes containing a small volume (20 µL) of BSA 0.5% in water to collect the SPE elution solvent containing DMET and DMET-d4. DMET working solutions were diluted in 50% methanol in water before spiking into the 'blank' plasma samples as it is well-known that organic solvents and plasma proteins can minimise analyte binding to tubes. After extraction and evaporation of the organic solvents, analytes were reconstituted in 20% acetonitrile in water and we used silanized glass vials to minimise binding.

DMET is an anaesthetic drug that is similar to thiopental and propofol [11] in that the analyte is lost during evaporation at high temperature. Herein, we found that the DMET concentrations eluted from the SPE cartridges were stable when elution solvent was evaporated under a stream of nitrogen and at room temperature.

3.2 MS conditions

For DMET and DMET-d4, positive ion electrospray ionisation in MRM mode produced the best results. The optimised MS parameters were as follows: spray voltage 4500 V, turbo gas temperature 300°C, curtain gas pressure 30 psi, nebulizer gas pressure 50 psi, heater gas pressure 50 psi and collision gas pressure set at Medium. The collision energy was optimised at 25 V for both DMET and DMET-d4. The cell exit potentials (CXP) were 14 for DMET and 55 for DMET. The declustering potential (DP) were set at 66 and 86 for DMET and DMET-d4, respectively. For quantification, the transitions of the parent ions to the product ions were 201.1 → 95.0 m/z for DMET and 204.9 → 99.0 for DMET-d4.

3.3 Assay performance

The calibration curves for DMET were linear when assessed on 6 separate occasions using a linear regression model weighted by 1/x using Analyst® software (AB Sciex, Concord, Ontario, Canada). The precision and accuracy of the assay of DMET was within the required ranges (all less than 10%) and the mean regression coefficients (r^2) were all greater than 0.9956 (Table 1).

The signal to noise ratio for DMET was 50 (average of 18 replicates of LLOQ). It is clear from the signal to noise ratio, and the accuracy and precision of the method at the LLOQ that the sensitivity of the method could potentially be improved 10-fold, if required. However, the proposed application of this assay to the quantification of DMET concentrations in samples collected from an *ex-vivo* model of ECMO, does not require such a high level of assay sensitivity. DMET is well ionised in the mass spectrometer detector and therefore low

concentrations at 5 pg/mL are easily detectable [5, 9]. By increasing the sample and injection volumes, and decreasing the reconstitution solvent volume, the sensitivity can be potentially increased by more than 100-fold although the binding of DMET to the tubes as such low concentrations may be problematic.

The within-run and between-run accuracy and precision were assessed using LLOQ, low, medium and high QCs (Table 2). The precision and accuracy of six replicates for each of the QCs were within the ±15% range and ± 20% at the LLOQ. The accuracy and precision of the diluted QCs (QCs at 5 times the highest standard concentration of DMET (100 ng/mL) that were diluted 1 in 10 with free blank plasma) were 90.5% and 2.7%, respectively.

The recovery of DMET was high and reproducible in three spiked pooled plasma samples at concentrations of 1.5, 10 and 15 ng/mL compared with the same concentrations of pure DMET dissolved in aqueous solutions (0.1% formic acid in water). The average recoveries for DMET and the IS (10 ng/mL) were 86% and 85%, respectively.

The matrix factor (MF) (the ratio of the peak area in the presence of matrix (measured by analysing blank matrix after extraction and then spiked with DMET, relative to the peak area in the absence of matrix) for the individual human plasma samples spiked with DMET at 1.5 and 15 ng/mL, and DMET-d4 at 10 ng/mL was calculated. The IS normalised MF was calculated by dividing the MF of DMET by the MF of DMET-d4. The CV% of the IS-normalised MF calculated from 6 different lots of human plasma were 4% and 3.8%, respectively.

No significant peak (≥20% of the LLOQ and 5% of the IS) was detected in blank samples injected after the ULOQ samples. Adding 3 needle wash solvents effectively washed the system between samples and reduced the carry-over.

Selectivity was tested using 6 sources of human plasma samples (Li-heparin as the anti-coagulant) which were analysed using the described method. The resulting chromatograms were checked for peaks that might interfere with the detection of the analyte of interest or the IS. No interfering peak (less than 20% of the LLOQ response, and <5% of the IS response) was detected when 6 individual blank plasma and 6 individual blank plasma spiked with IS were analysed using the described method.

DMET QCs at low and high (n=6) concentrations were stable after 4 cycles of freeze and thaw from −20 °C to room temperature, 112 days at -20 °C and 5 h at room temperature. Samples stored in the autosampler at 4 °C were stable for at least 48 h (Table 3). Stock solutions of DMET in 50% methanol in water were stable at -20 °C for at least 77 days. Our stability data is comparable with studies in which samples were kept at -20 °C and had the 5 h short-term stability and 4 freeze and thaw cycles [6, 8].

3.4 Assay application

To study the sequestration of DMET in ECMO circuits, four identical ECMO circuits that were filled with human whole blood were spiked with DMET at therapeutic concentrations. Serial blood samples were collected from the ECMO circuits over 24 hours and the DMET concentrations were quantified using the described assay. Details of the *ex-vivo* experiment have been published previously [12]. The collected samples from ECMO circuits and controls were analysed using the assay developed and validated in this paper. The data are presented in Fig 2. Sequestration of DMET to both old and new ECMO circuits was reported previously [2] consistent with expectations for small lipophilic compounds [13].

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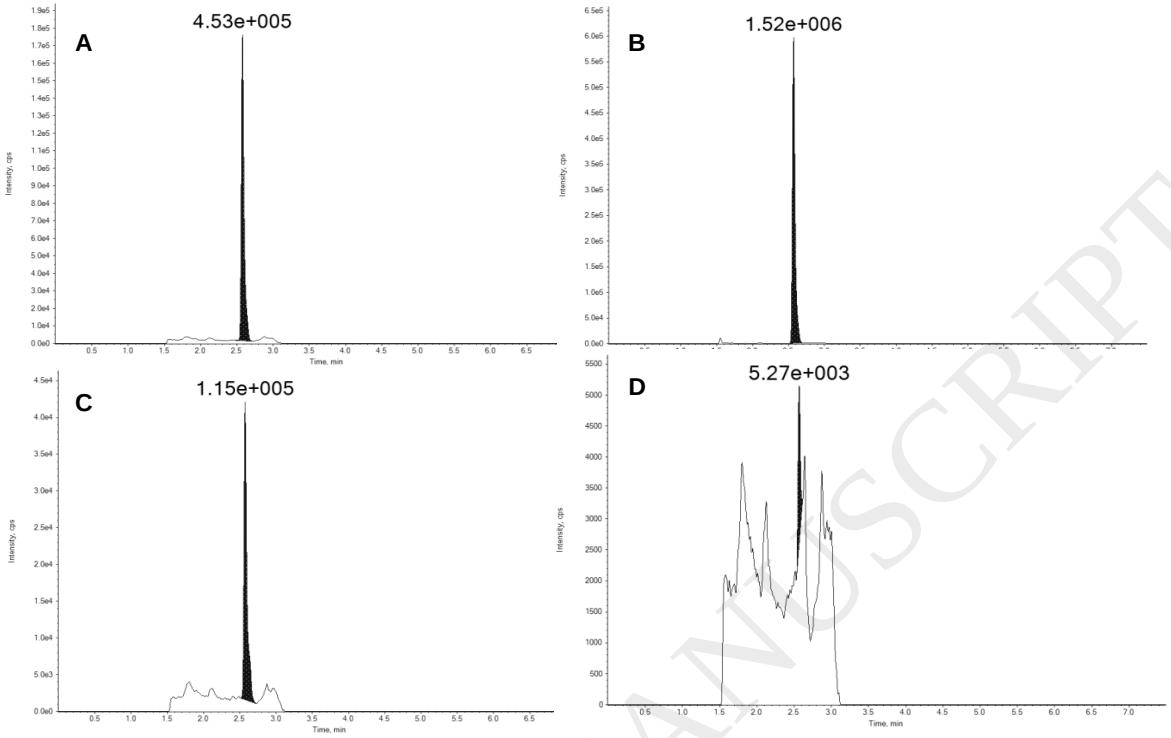

Fig 1. Representative peaks of A. DMET standard 3 ng/mL, B. IS (10 ng/mL), C. LLOQ 0.5 ng/mL and D. double blank.

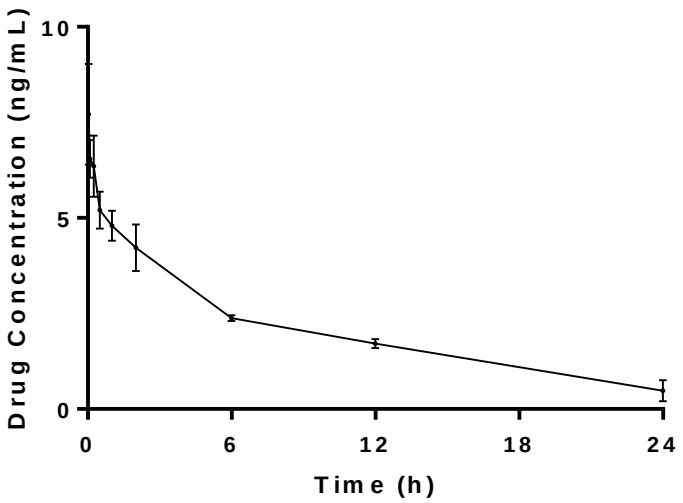

Fig 2. Temporal decrease in the concentration of DMET in ECMO circuits (concentration ± SEM)

Table 1. Linearity of DMET standard curves

Standard Concentration (nominal, ng/mL)	Measured concentrations (mean ± SEM, n=6)	Precision (%)	Accuracy (%)
0.5	0.53	9.220	93.67
1	0.97	7.601	103.15
3	2.95	3.842	101.69
5	4.98	4.436	100.36
10	10.05	2.606	99.53
16	16.00	2.910	100.01
20	20.05	1.356	99.74
a*	0.107		
r^2	0.9956		

*Y=ax+b

Table 2. The within-run and between-run accuracy and precision of the DMET QCs.

	Within-Run 1 (n=6)		Within-Run 2 (n=6)		Within-Run 3(n=6)		Between-Run (n=3)	
QC Concentration	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
LLOQ (0.5 ng/mL)	102.1	7.9	95.8	14.7	92.9	17.9	97.3	13.2
Low (1.5 ng/mL)	90.7	3.9	92.8	11.7	112.0	11.0	98.5	13.6
Med (10 ng/mL)	85.9	1.5	104.6	5.5	97.7	8.6	96.0	10.1
High (15 ng/mL)	87.0	4.6	97.4	4.9	94.8	4.1	93.1	6.5

Table 3. Stability of the DMET QCs after 4 cycles of freeze and thaw, 5h at room temperature and 112 days at -20 °C based on the accuracy and precision of calculated concentrations

		F&T Stability (4 cycles) (n=6)	Room Temperature Stability (5h) (n=6)	Frozen Storage Stability (112 days at -20 °C) (n=6)
QCL (1.5 ng/mL)	Accuracy	100.8	102.4	102.2
	Precision	5.9	6.8	3.7
QCH (15 ng/mL)	Accuracy	99.9	111.7	98.3
	Precision	5.9	4.9	3.3