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High-throughput assay for simultaneous quantification of the plasma concentrations of morphine, fentanyl, midazolam and their major metabolites using automated SPE coupled to LC–MS/MS

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ABSTRACT

A rapid LC–MS/MS assay method for simultaneous quantification of morphine, fentanyl, midazolam and their major metabolites: morphine-3-β-D-glucuronide (M3G), morphine-6-β-D-glucuronide (M6G), norfentanyl, 1′-hydroxymidazolam (1-OH-MDZ) and 4-hydroxymidazolam (4-OH-MDZ) in samples of human plasma has been developed and validated. Robotic on-line solid phase extraction (SPE) instrumentation was used to elute the eight analytes of interest from polymeric SPE cartridges to which had been added aliquots (150 μL) of human plasma and aliquots (150 μL) of a mixture of two internal standards, viz. morphine-d3 (200 ng/mL) and 1′-hydroxymidazolam-d5 (50 ng/mL) in 50 mM ammonium acetate buffer (pH 9.25). Cartridges were washed using 10% methanol in ammonium acetate buffer, pH 9.25 (1 mL, 2 mL/min) before elution with mobile phase comprising 0.1% formic acid in water (A) and acetonitrile (B) with a flow rate of 0.6 mL/min using an 11.5 min run time. The analytes were separated on a C18 X-Terra® analytical column. The linear concentration ranges were 0.5–100 ng/mL for fentanyl, norfentanyl and midazolam; 1–200 ng/mL for 4-hydroxymidazolam, 2.5–500 ng/mL for 1′-hydroxymidazolam and 3.5–700 ng/mL for morphine, M3G, and M6G. The method showed acceptable within-run and between-run precision (relative standard deviation (RSD) and accuracy <20%) for quality control (QC) samples spiked at concentrations of 80% and 50% of the ULOQ, 3 times higher than the LLOQ, and also at the LLOQ. Furthermore, analytes were stable in samples (after mixing with internal standard) for at least 48 h in the autosampler (except for 4-hydroxymidazolam which decreased by 22% after 24 h), 5 h at room temperature and after three cycles of freeze and thaw. No autosampler carry-over was observed and the absolute recovery (the area ratio of analyte in plasma relative to that in ammonium acetate buffer 50 mM, pH 9.25) was in the range 40% (midazolam) to 110% (morphine). The assay was applied successfully to the measurement of the analytes of interest in plasma samples from patients on extracorporeal membrane oxygenation (ECMO).

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1. Introduction

LC–MS/MS provides the opportunity for simultaneous quantification of tens of compounds with the current limiting step being that of adequate sample clean-up. Protein precipitation and solid phase extraction (SPE) in combination [1,2], or separately [3] have been used for this purpose; however, SPE alone is the method of choice. Subramanian et al. [4] separated and quantified nine antiepileptic drugs using a single SPE. Low-speed centrifugation was used to force solutions and samples through the cartridges. Likewise, 21 benzodiazepines were separated from urine and quantified using a single SPE method by Quintela et al. [5]. Rate-limiting steps for these methods were manual analyte extraction as well as evaporation of sample eluants, and dried sample reconstitution. Ghassabian et al. [2] used an automated SPE instrument (off-line) to simultaneously extract 8 analytes from samples of human plasma following protein precipitation with acetonitrile. This was followed by an eluent evaporation step that limited the overall speed of the method. On-line SPE facilitates high-throughput analyte extraction

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from the biological matrix by eliminating the eluent evaporation and reconstitution steps. For example, an on-line SPE method was used to quantify the concentrations of 20 drugs of abuse in sewage water [6], and 14 antidepressant drugs and their major metabolites in human plasma samples [7]. LC–MS/MS bioanalytical methods that utilize manual SPE for analyte extraction lend themselves to ready conversion to on-line robotic SPE for sample preparation as a means of enhancing sample throughput and improving sample analysis speed.

The aim of this research was to develop and validate an LC–MS/MS method utilizing on-line robotic SPE to quantify the plasma concentrations of two analgesic agents and one sedative drug, viz. morphine, fentanyl and midazolam, and their major metabolites: M3G, M6G, norfentanyl, 1-OH-MDZ and 4-OH-MDZ, respectively, in plasma samples collected from patients receiving extracorporeal membrane oxygenation (ECMO). The impact of ECMO on the pharmacokinetics of sedative and analgesic drugs is poorly understood [8]. Hence, this bioanalytical method was developed to address this knowledge gap.

Previously, separate LC–MS/MS assay methods for quantification of the plasma concentrations of morphine, M3G and M6G [9–13]; fentanyl and norfentanyl [14–19]; midazolam, 1-OH-MDZ and 4-OH-MDZ [20–25] have been described in the literature. Here, we have successfully extracted all eight of these analytes of interest from a single 150 μL plasma sample using a single polymeric cartridge and on-line SPE with subsequent LC–MS/MS quantification. The main advantages of the present method include (i) replacement of at least three separate assays, (ii) high sensitivity and selectivity, (iii) minimum sample manipulation (reducing the potential for human error), (iv) minimum sample volume, (v) minimum use of organic solvents, and (vi) elimination of time-consuming solvent evaporation and reconstitution steps.

2. Experimental

2.1. Materials and reagents

Morphine, morphine-d3, M3G, M6G, midazolam, 1-OH-MDZ, 1-OH-MDZ-d5, and 4-OH-MDZ were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Fentanyl and norfentanyl were purchased from Cerillant-Kinesis (Redland Bay, QLD, Australia). HPLC grade methanol and acetonitrile were obtained from Lab Scan (Brisbane, QLD, Australia). Formic acid (99%) was bought from Univar (Sydney, NSW, Australia). Ammonium acetate (97%) was purchased from Chem-Supply (Gillman, SA, Australia). Human blank plasma was purchased from BioCore Pty Ltd (Sydney, NSW, Australia). SPE cartridges; HySphere Resin GP; were purchased from SPARK Holland (VE, Emmen, Netherlands).

2.2. Chromatographic conditions

Liquid chromatography and extraction methods were created by Symbiosis Pro software for analyst (V 2.1.0.0) and submitted to the MS controlling software (Analyst 1.5.1). An X-Terra® MS C18 column (2.1 mm × 150 mm, 5 μm; Waters, Sydney, NSW, Australia), and Phenomenex Security Guard C18 column (Phenomenex, Sydney, Australia) were used for chromatographic separation of the analytes. The column was heated to 50 °C, and the autosampler temperature was set at 4 °C. The injection volume for all samples was 10 μL. The mobile phase comprised solvent A (0.1% formic acid in water) and solvent B (acetonitrile) and the mobile phase flow rate was 0.6 mL/min. The mobile phase gradient is shown in Fig. 1.

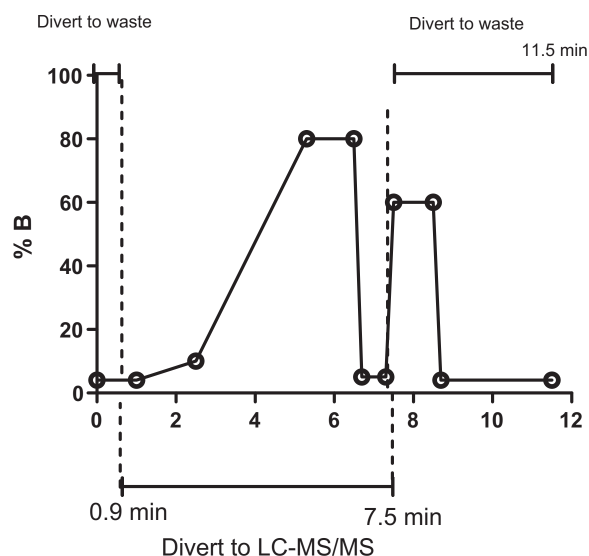

Fig. 1. Gradient elution profile for the mobile phase. Mobile phase A: 0.1% Formic acid in water. Mobile phase B: acetonitrile.

2.3. Mass spectrometry conditions

Mass spectrometry detection was carried out in ESI mode using an API 5500 (AB-Sciex, Concord, Ontario, Canada) triple quadrupole system. The highest abundant product ions were selected for each analyte. Positive ionization mode was chosen for all analytes of interest. The first 7.5 min of the run time were acquired by the mass spectrometer (Table 1).

2.4. SPE method development

The on-line SPE Symbiosis Pharma System (SPARK Holland, Emmen, The Netherlands) was used to extract the 8 compounds of interest and the two internal standards from samples of human plasma.

The on-line SPE Symbiosis System comprised two integrated units: the Reliance autosampler with a pair of binary LC pumps and the on-line SPE unit with two high pressure solvent delivery pumps or HPDs. The entire system was controlled by the Symbiosis Pro for Analyst V. 2.1.0.0 and MS controlling software (Analyst 1.5.1). LC, extraction methods, and batch tables were created with the Symbiosis Pro for Analyst software and submitted to the MS controlling software (Analyst 1.5.1). After completion of the SPE step, the analytes were eluted to the analytical column using the chromatographic mobile phase.

Plasma samples (150 μL) were mixed with 150 μL of the internal standards (morphine-d3 at 200 ng/mL and 1-OH-MDZ-d5 at 50 ng/mL) dissolved in ammonium acetate buffer (50 mM, pH 9.25), and shaken vigorously for 5 min before placing in the autosampler.

The compounds of interest are weakly basic with maximum hydrophobicity in the pH range 9–11 (Table 2) and so should be retained on the analytical and SPE column at pH values in the range 9–11, with elution at acidic pH. Also, M3G and M6G are not separated by the mass spectrometer due to the similarity in their ionization patterns, and so the mobile phase gradient had to be optimized to achieve optimal chromatographic separation of these two analytes. A method development tray including eight different types of SPE cartridges was used to facilitate selection of the optimal cartridge based upon the best retention time, analyte recovery and peak shape.

Table 1
MS parameters for monitoring of the eight analytes of interest and the internal standard.

	CUR[a] (psi)	CAD[b]	IS[c] (V)	TEM[d] (°C)	GS1[e] (psi)	GS2[f] (psi)	DP[g] (V)	EP[h] (V)	CE[i] (V)	CXP[j] (V)	MRM[k] (amu)
Fentanyl	35	Med	3000	550	40	60	136	10	31	14	337.1/188.1
Norfentanyl	35	Med	3000	550	40	60	71	10	17	10	233.1/84.0
Midazolam	35	Med	3000	550	40	60	136	10	39	22	326.0/291.1
1-OH-MDZ	35	Med	3000	550	40	60	111	10	37	20	342.3/203.0
4-OH-MDZ	35	Med	3000	550	40	60	106	10	31	28	342.0/325.0
Morphine	35	Med	3000	550	40	60	71	10	77	12	286.0/151.9
M3G	35	Med	3000	550	40	60	91	10	43	26	462.1/286.1
M6G	35	Med	3000	550	40	60	91	10	43	26	462.1/286.1
Morphine-d3	35	Med	3000	550	40	60	76	10	81	12	289.0/152.2
1-OH-MDZ-d5	35	Med	3000	550	40	60	106	10	55	16	347.0/173.1

[a] Curtain gas.
[b] Collision gas.
[c] Ion spray voltage.
[d] Temperature.
[e] Ion source gas 1.
[f] Ion source gas 2.
[g] Declustering potential.
[h] Entrance potential.
[i] Collision energy.
[j] Collision cell exit potential.
[k] Multiple Reaction Monitoring.

Table 2
Log *D* values for the analytes of interest at different pH values [27].

pH	Midazolam	Fentanyl	Morphine	Norfentanyl	1-OH-MDZ	4-OH-MDZ	M3G	M6G
0	-1.98	-0.05	-2.59	-1.81	-2.83	-0.82	-4.84	-4.30
1	-1.97	-0.05	-2.59	-1.62	-2.81	0.13	-4.85	-4.30
2	-1.93	-0.05	-2.59	-1.60	-2.68	0.88	-4.93	-4.35
3	-1.63	-0.04	-2.59	-1.59	-2.08	1.16	-5.33	-4.65
4	-0.84	0.00	-2.57	-1.59	-1.02	1.26	-6.15	-5.37
5	0.30	0.28	-2.44	-1.57	0.10	1.64	-6.90	-5.92
6	1.59	1.04	-1.87	-1.41	1.10	2.31	-7.12	-6.04
7	2.57	2.00	-0.96	-0.79	1.90	2.66	-6.72	-5.89
8	3.03	2.94	0.00	0.14	2.22	2.72	-5.88	-5.31
9	3.12	3.57	0.78	1.08	2.27	2.73	-5.10	-4.60
10	3.13	3.75	0.93	1.77	2.28	2.72	-4.76	-4.46
11	3.13	3.77	0.34	1.98	2.28	2.62	-4.73	-5.07
12	3.13	3.77	-0.54	2.01	2.27	2.14	-4.92	-6.13
13	3.13	3.77	-1.22	2.01	2.23	1.27	-5.78	-7.54
14	3.13	3.77	-1.75	2.01	1.95	0.37	-7.74	-9.32

2.5. Preparation of standards, calibration curves and quality control samples

Due to the adsorption of morphine, its glucuronide metabolites, fentanyl and norfentanyl [1] to the glassware, all stock and working solutions were prepared in plastic tubes or silanized glassware. Stock solutions of midazolam, 1-OH-MDZ and 4-OH-MDZ, 1-OH-MDZ-d5, fentanyl, and norfentanyl were prepared in methanol at 1 mg/mL. Morphine, M3G, and M6G were dissolved in 70% methanol in water at the same concentration. They were stored frozen at −20 °C for no more than 6 months.

Working solutions were made in methanol and aliquots of the working solutions were evaporated under nitrogen and then reconstituted in plasma so that a calibration range from 0.5 to 100 ng/mL for fentanyl, norfentanyl and midazolam; 1 to 200 ng/mL for 4-OH-MDZ, 2.5 to 500 ng/mL for 1-OH-MDZ and 3.5 to 700 ng/mL for morphine, M3G, and M6G were achieved. QCs contained only 2% methanol. Morphine-d3 (200 ng/mL) and 1-OH-MDZ-d5 (50 ng/mL) were chosen as internal standards for the polar (M3G, M6G and morphine) and less polar (midazolam and relevant metabolites, fentanyl and norfentanyl) analytes, respectively. All QCs, working standards, and stock solutions were stored frozen at −20 °C prior to use.

2.6. Assay performance

Aliquots of blank human plasma (150 μL) were spiked with 7 concentrations of all eight analytes of interest to produce the calibration standards, and for the QCs at 3 concentrations. The range of concentrations for each analyte is presented in Table 3. Three QCs for each analyte were prepared as follows: high (80% of the highest concentration of the calibration range), medium (50% of the highest calibration concentration) and low (3 times larger than the lowest calibration concentration).

2.6.1. Linearity

Linearity was assessed on six separate occasions using six different sources of plasma spiked with the mixture of all analytes at 7 different concentrations as shown in Table 3. A linear least squares regression model was applied to all calibration curves. The back calculated concentrations of the calibration standards, and also the CV% of the six replicates had to be within ±15% of the nominal concentrations, except for the LLOQ for which ±20% is acceptable.

2.6.2. Lower limit of quantification (LLOQ)

The LLOQ was assessed using the criteria that the analyte response at the LLOQ must be 5 times the baseline noise and it

Table 3
Linearity of the morphine, M3G, M6G, midazolam, 1-OHMid, 4-OHMid, fentanyl and norfentanyl standards spiked in 7 concentrations (ng/mL) in human plasma ($n = 6$).

Analyte name	Conc 1LLOQ	Conc 2Low	Conc 3	Conc 4	Conc 5Med	Conc 6High	Conc 7ULOQ	Accuracy range (%)	Precision range (%)	Mean r^2
Fentanyl	0.5	1.5	5	10	50	80	100	−0.3 to 9.4	2.1–11.7	0.9985
Midazolam	0.5	1.5	5	10	50	80	100	−7.9 to 2.9	4.0–17.5	0.9958
1-OH-midazolam	2.5	7.5	25	50	250	400	500	−2.8 to 7.3	1.8–12.8	0.9983
4-OH-midazolam	1	3	10	20	100	160	200	−0.3 to 15.4	2.6–14.8	0.9977
Norfentanyl	0.5	1.5	5	10	50	80	100	−6.9 to 1.3	2.3–13.6	0.9967
M3G	3.5	10.5	35	70	350	560	700	−2.5 to 5.9	4.6–17.2	0.9961
M6G	3.5	10.5	35	70	350	560	700	−4.3 to 2.1	5.6–15.7	0.9929
Morphine	3.5	10.5	35	70	350	560	700	−1.8 to 1.1	2.9–7.2	0.9982

Table 4
Recovery of eight analytes in three different concentrations ($n = 3$) from samples of human plasma.

Analyte name	Low	Med	High
Fentanyl	60	72.3	68.2
Midazolam	38	40.6	39.4
1-OH-midazolam	52	50.8	53.1
4-OH-midazolam	54	58.6	62.9
Norfentanyl	102	103.6	110.4
M3G	101.0	105.2	107.0
M6G	91.2	94.7	86.5
Morphine	111	107.8	112.5

should have a precision ≤20% coefficient of variation (CV%) and an accuracy of 80–120% of the nominal concentrations.

2.6.3. Accuracy and precision

The accuracy and precision of the assay were assessed on 3 different days (between-run), and using 5 replicates of high, medium, low and LLOQ in one day (within-run). The assessment was conducted using QCs prepared in advance. Measured concentrations were calculated by comparison with a freshly prepared standard curve. Accuracy (mean percentage of deviation from nominal concentrations in the replicate set), and precision (the CV% of the measured concentrations) ≤±15% were considered acceptable, except at the LLOQ which should not deviate by more than ±20%.

2.6.4. Recovery and matrix effect

Recovery was estimated by assaying three QCs compared with the same concentrations of analytes dissolved in aqueous solutions (ammonium acetate 50 mM, pH 9.25). The recovery should be consistent, precise and reproducible across the QC concentration range (Table 4).

The matrix effect was assessed based on the variability of the response from lot to lot of human plasma by analysing 6 different lots of plasma spiked at low and high concentrations. The CV for six replicates had to be ≤15%.

2.6.5. Selectivity

Human plasma samples from 6 different sources that were collected using Li-heparin as the anti-coagulant were extracted and analysed with the method described in Section 2.4 and were checked for peaks that might interfere with the detection of all analytes of interest or the internal standards. The background response was also checked to ensure that it was less than 20% of the LLOQ response and <5% of the internal standard response for each analyte.

2.6.6. Dilution integrity

A QC at 10 times the highest standard concentration of each analyte (ULOQ) was prepared. Five replicates were diluted 1 in 10 with drug free blank plasma and assayed alongside a freshly prepared standard curve. The concentrations were inversely predicted from the standard curve followed by application of the dilution factor (×10). The accuracy (deviation from nominal concentrations) and precision of these QCs must be within ±15%.

2.6.7. Carry-over

Carry-over was assessed following injection of a blank plasma sample immediately after 3 repeats of the ULOQ and the response was checked.

2.6.8. Stability

Three freeze and thaw cycles from −20 °C to room temperature were applied to three replicates of each QC and the concentrations were compared with freshly prepared standard curves.

The stability of QCs in the autosampler (48 h) and also after storage at room temperature for 2 and 5 h were tested against freshly prepared calibration curves.

3. Results and discussion

3.1. Method development for LC and on-line SPE

Various strategies have been described for sample preparation for LC–MS/MS quantification of the eight analytes of interest herein. For example, protein precipitation is a fast method that has been utilized previously for the quantification of fentanyl and its two metabolites [16], midazolam, 1-OH-MDZ and 4-OH-MDZ [20], as well as morphine and M3G in plasma samples [10]. However, this approach compromises the cleanliness of the samples, and it is not economical due to damage to the column and potential contamination of the detector. Although liquid–liquid extraction produces cleaner plasma sample extracts, and it has been used successfully for quantification of midazolam and its two metabolites [21,24,25], as well as fentanyl and norfentanyl in plasma samples [19], it is an expensive and labour intensive method using very toxic solvents such as toluene for fentanyl, norfentanyl [19] and midazolam [25], ethyl acetate for morphine and its glucuronide metabolites [26], or hexane for midazolam [21,24].

For weakly basic compounds, solid-phase extraction using cation exchange [7,9,14] or hydrophobic cartridges e.g. C18 [12] or HLB [13,17], with an acidic mobile phase for analyte elution is optimal and the latter option was utilized herein. Use of 96-well SPE trays was the first major improvement step in the automation of SPE for bioanalytical sample preparation and this approach has been used successfully for extraction of midazolam [23], morphine [12] and fentanyl [14] from plasma samples. Using this column switching on-line extraction, sample preparation time was reduced to 2 h for 96 samples by a single analyst for only one analyte; fentanyl [18]. Using on-line SPE and column-switching described here, a single analyst can extract all analytes of interest in less than 15 min.

Due to the wide range in polarity of the analytes of interest in this study including the highly polar morphine metabolites, M3G and M6G, reversed phase chromatography was used with the hydrophobicity of the system being modified using an efficient mobile phase gradient. The gradient commenced with a high ratio

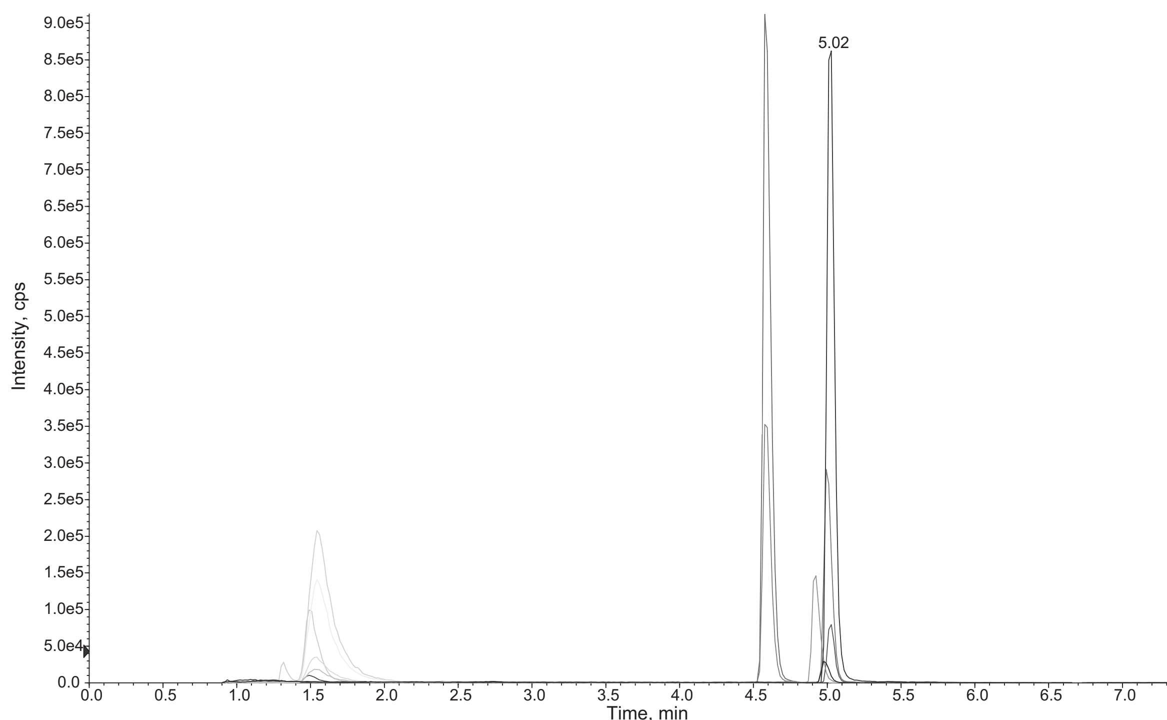

Fig. 2. Elution of the eight analytes and 2 internal standards.

of mobile phase A (96%) with a gradual increase in the organic component (mobile phase B) until M3G and M6G were eluted (Fig. 1). At 5 min, the proportion of mobile phase B was increased sharply to 80% until 6.5 min after which it was reduced to 4% until 7.5 min. M3G and M6G were eluted at distinct retention times. At the end of the run (7.5 min), mobile phase B was increased to 60% for 1 min to clean the column and vented to waste via a divert valve. A 4-min equilibration time was necessary before processing of the next sample.

For M3G and M6G, the pK_a of the glucuronic acid moiety is 4–4.5 and so a pH of 3.5 (0.1% formic acid in aqueous mobile phases) was used to ensure single peaks for each of these morphine metabolites. Plasma samples were mixed with ammonium acetate buffer (pH 9.25, 50 mM) during SPE cartridge loading thereby maximizing hydrophobicity and retention of all analytes. As 4-OH-MDZ is unstable at acidic pH [21], basic pH optimized the stability of this analyte in the autosampler.

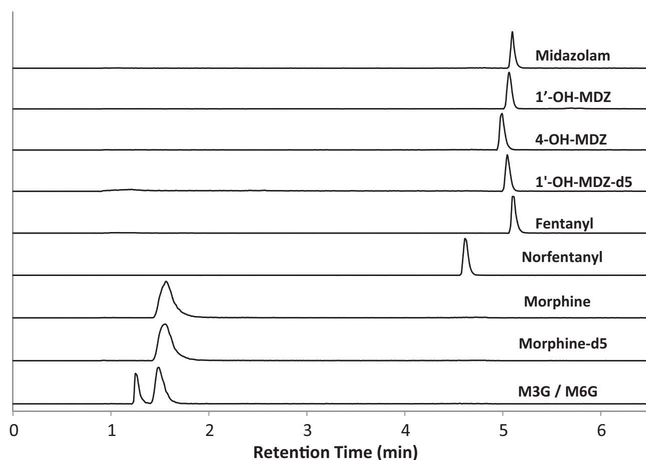

Fig. 3. On-line SPE extracted plasma sample chromatograms for each analyte separately. The chromatograms are normalized to show similar intensity.

The cartridges were conditioned with 100% methanol, 1 mL (5 mL/min), followed by ammonium acetate buffer 50 mM, pH 9.25, 2 mL (1 mL/min). Ammonium acetate buffer (50 mM, pH 9.25) was used to transfer plasma samples from the autosampler to the SPE cartridges. Each sample was washed with 10% methanol in ammonium acetate buffer (50 mM, pH 9.25; 1 mL, 2 mL/min). Analyte elution was performed using the first 5 min of the mobile phase gradient.

The resulting peaks are presented in Figs. 2 and 3.

3.2. MS conditions

Table 1 summarizes the MS parameters used for detection of all analytes. No signal suppression/enhancement were observed due to the internal standards.

3.3. Assay performance

3.3.1. Linearity

The simultaneous assay and quantification of 8 analytes was found to be linear on 6 separate occasions using a linear regression model. $1/x$ weighting provided the simplest fit for all 8 analytes. The precision and accuracy of the assays of all analytes were within the required ranges and the mean regression coefficients (r^2) were all greater than 0.9929 (Table 3).

3.3.2. Lower limit of quantification

The signal to noise ratios for all analytes were >5 (Table 7). The precision and accuracy results for 5 replicates at the LLOQ for each analyte, are presented in Tables 5 and 6, and all are in the acceptable range. It is clear from the signal to noise ratios that the lower limit of quantification for most of the analytes especially fentanyl, midazolam and norfentanyl, could potentially be improved 10-fold, if required. However, the proposed application of this assay to the quantification of the analytes of interest in plasma samples collected from patients on ECMO, does not require such a high level of assay sensitivity.

Table 5
Within-run assay precision and accuracy (% mean deviation from the nominal concentration) ($n = 5$).

Analyte name	Precision LLOQ	Accuracy LLOQ	Precision Low	Accuracy Low	Precision Med	Accuracy Med	Precision High	Accuracy High
Fentanyl	9.7	−0.9	8.3	1.1	5.1	10.0	6.2	10.9
Midazolam	13.6	1.2	7.7	−8.8	7.2	9.4	4.7	8.4
1-OH-midazolam	8.8	−2.8	3.7	11.4	7.4	1.6	5.7	−1.8
4-OH-midazolam	16.8	16.6	11.7	4.0	6.1	−5.2	5.9	5.0
Norfentanyl	19.2	−4.7	9.4	−0.5	2.3	4.6	2.4	10.6
M3G	4.9	−8.5	11.6	−4.8	20.0	11.9	18.3	15.3
M6G	8.1	−1.6	5.8	5.7	4.7	2.7	16.2	−0.6
Morphine	7.1	3.8	7.7	1.9	6.3	−1.6	6.0	1.0

Table 6
Between-run assay precision and accuracy ($n = 3$).

Analyte name	Precision LLOQ	Accuracy LLOQ	Precision Low	Accuracy Low	Precision Med	Accuracy Med	Precision High	Accuracy High
Fentanyl	2.6	11.6	7.7	−7.6	5.5	3.5	6.4	6.1
Midazolam	13.6	−0.6	2.5	−0.4	12.0	2.1	13.9	−2.1
1-OH-midazolam	6.4	6.5	4.3	11.5	4.6	5.3	7.4	7.4
4-OH-midazolam	6.8	14.3	9.8	−5.2	4.6	−5.8	14.8	−0.2
Norfentanyl	0.95	13.8	5.9	−5.8	1.0	−0.7	7.9	1.7
M3G	20.0	9.0	13	3.2	5.4	3.0	20.0	12.7
M6G	7.4	−7.7	11.2	4.2	11.7	−0.5	8.1	1.8
Morphine	13.2	1.1	1.0	−1.9	1.4	−0.1	5.3	−0.4

Table 7
Signal to noise at LLOQ ($n = 5$).

Compound's name	S/N
Fentanyl	132
Midazolam	98
1-OH-midazolam	20
4-OH-midazolam	9
Norfentanyl	80
M3G	11
M6G	13
Morphine	25

It is well understood that the required level of assay sensitivity is determined by the intended application of each bioanalytical method of interest. For example, although midazolam is a widely used sedative hypnotic drug, it is also used as a probe compound for measuring CYP3A enzyme activity in in vivo phenotyping studies in humans. For phenotyping studies, an LLOQ of 0.1 ng/mL is required that is achieved by using a relatively large plasma volume and reconstitution in a much smaller volume of mobile phase prior to injection into the mass spectrometer [21,24,25]. Likewise, measurement of plasma fentanyl concentrations following administration by the transdermal route requires assays of much higher sensitivity than for applications involving the parenteral dosing route [17,18]. Highly sensitive assays are also required where there is a plasma sample volume limitation such as for the conduct of PK studies of fentanyl in children [16]. The range of concentrations utilized in the present bioanalytical method for simultaneous quantification of eight analytes herein, is based on the anticipated concentration ranges expected following use of sedative and analgesic agents in patients on ECMO in the intensive care unit.

3.3.3. Accuracy and precision

The within-run and between-run accuracy and precision were assessed using low, medium and high QCs (Tables 5 and 6). The precision and accuracy were also assessed at the LLOQ level. The precision and accuracy of six replicates for each of the QCs for each analyte were within the ±15% range (20% for LLOQ), except for 4 within-run and one between-run occasions for M3G/M6G where these values were within ±20%. The most likely explanation is detector difficulty in differentiating between these two structural analogues that also have close retention times.

3.3.4. Recovery and matrix effects

The recovery of all analytes is reported in Table 4. The recovery values are reproducible for the low, med and high QC's.

The CV for the high and low QC's in 6 different plasma samples was within ±15%.

3.3.5. Selectivity

The response of the assay was checked in 6 different lots of human plasma and no significant responses for any of the 8 analytes (<20% of the LLOQ, and 5% of IS) were detected.

3.3.6. Dilution integrity

The accuracy and precision of the diluted QCs after including the dilution factor were within the 15% limit.

3.3.7. Carry-over

None of the analytes showed any significant peak (≥20% of the LLOQ and 5% of the IS) in blank samples injected after the ULOQ samples. Adding 4 extra minutes to the end of the run using the ballistic gradient (Fig. 1) effectively washed the system between samples thereby eliminating carry-over.

3.3.8. Stability

All 8 analytes were stable after three cycles of freeze and thaw, 2 and 5 h at room temperature, and 48 h in the autosampler at 4 °C. The only exception was for 4-OH-MDZ where the nominal concentration was reduced by 22% after 24 h of storage in the autosampler. The good stability of the analytes used in this study is well established. Morphine (and metabolites) and fentanyl have been previously shown by others to be stable for at least 15 months and 6 months at −20 °C, respectively [11,18]. Midazolam and 1-OH-MDZ were stable at −20 °C for at least 10 months [24].

3.3.9. Application of the assay

The assay method described herein has been used successfully in a pharmacokinetic study for measuring the concentrations of morphine, midazolam, fentanyl and their major metabolites in samples of human plasma. The study was approved by the Human

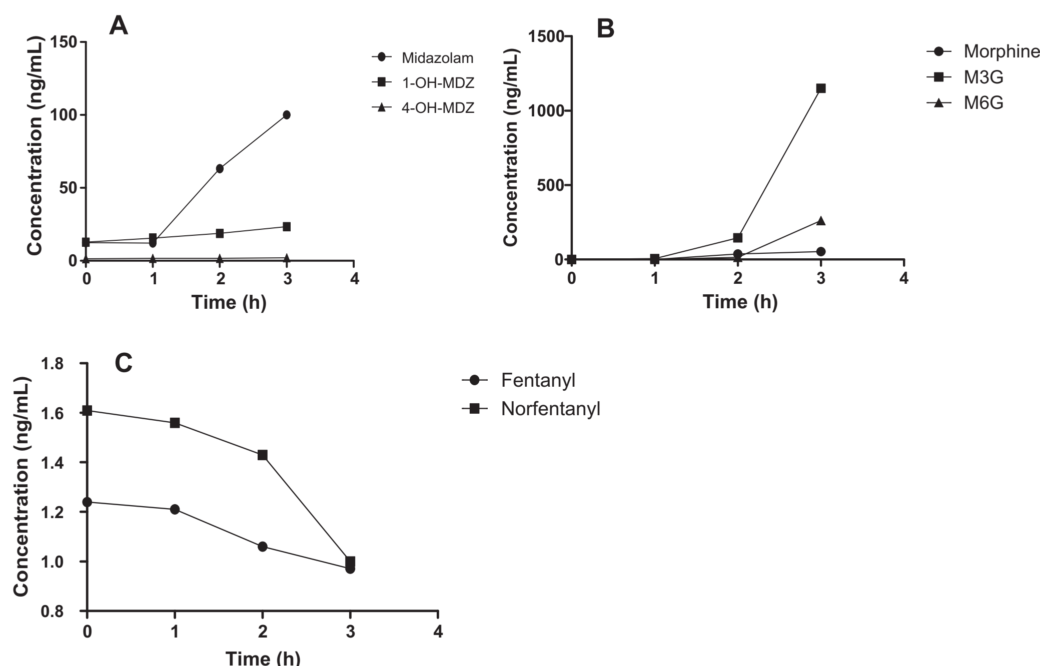

Fig. 4. Plasma concentrations vs time curves for (A) Midazolam, 1-OH-MDZ and 4-OH-MDZ; (B) Morphine, M3G and M6G and (C) Fentanyl and norfentanyl in one patient on ECMO.

Research Ethics committee of The Prince Charles Hospital (TPCH HREC/11/QPCH/121), Brisbane Australia. A case study has been presented here as an application of the assay in the clinic. The plasma concentration versus time curve data for morphine, fentanyl and midazolam and their major metabolites are shown in Fig. 4 for one patient on ECMO who failed to wake up after being free of sedative drugs for more than 48 h.

This patient was on venoarterial ECMO for seven days and had a significantly altered level of consciousness despite stopping sedative drugs for more than 48 h. Prior to cessation of sedative drugs, the patient was receiving midazolam and fentanyl at 0.5 mg/h and 30 μg/h, respectively. The patient had a normal CT (computed Tomography) head and there was no seizure activity on the electroencephalogram (EEG). In the absence of any other obvious cause, there were clinical concerns regarding whether the ECMO circuit was acting as a reservoir releasing sequestered drugs over a period of time. This prompted clinicians to undertake PK sampling. The patient was eventually palliated, as there was no cardiac recovery and further mechanical cardiac support or heart transplantation was not possible. Morphine, midazolam and fentanyl were recommenced at 5 mg/h each for patient comfort. As PK sampling was done during this period of clinical decision making, the assay results initially showed reducing fentanyl and midazolam concentrations with time. The subsequent rise in plasma concentrations of morphine, midazolam and their metabolites of interest correspond with re-commencing administration of these morphine and midazolam.

4. Conclusion

The robotic on-line SPE LC–MS/MS assay method described herein for the simultaneous quantification of 8 analytes of interest in samples of human plasma has been successfully validated. This method replaces at least 3 separate assays thereby saving considerable analysis time, as well as eliminating manual solvent handling, evaporation and reconstitution steps used in previously reported separate methods.

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