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Determination of Urinary Opioids by Solid-phase Extraction LC-MS/MS for Clinical Research: Comparison of Automated and Manual Sample Preparation

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APPLICATION BENEFITS

- Efficient, automated sample preparation to reduce manual labor and errors in a busy laboratory environment
- Automated, error-free sample list generation using the Tecan® MassLynx® File Converter with sample traceability
- Robust SPE LC-MS/MS methodology for the determination of 21 urinary opioids
- Equivalent responses between manual and automated sample preparation

WATERS SOLUTIONS

ACQUITY UPLC® System

Waters® BEH Technology™

Oasis® MCX µElution Plate

Xevo® TQD Mass Spectrometer

MassLynx Software

TargetLynx™ Application Manager

Tecan MassLynx File Converter Software

KEYWORDS

Automation, Tecan, file converter, opiates, opioids, SPE, sample preparation, urine, clinical research

INTRODUCTION

Automated sample preparation improves laboratory operations by a) reducing errors in sample tracking and preparation, b) producing more consistent results free of analyst-to-analyst variation, c) allowing analysts to work more efficiently, and d) minimizing laboratory hazards in regard to solvent exposure and repetitive motions associated with manual pipetting. For labs considering automation, the aim of this study was to compare the performance and benefits of automated sample preparation using a Tecan Freedom EVO® 100 liquid handler to manual sample preparation in the context of a routine clinical research application. For the determination of a panel of 21 opioids in human urine by solid-phase extraction (SPE) LC-MS/MS, manual and automated sample preparation runs were performed on each of three days to compare linearity, precision, accuracy, carryover, and sample preparation time.

Tecan Freedom EVO 100 liquid handler.

EXPERIMENTAL

Methods

All analytes and internal standards were purchased from Cerilliant® (Round Rock, TX). Surine™ XTD was purchased from Dyna-Tek Industries (Shawnee Mission, KS).

A combined analyte stock solution was prepared in blank human urine (1000 ng/mL, 200 ng/mL fentanyl–norfentanyl). A combined internal standard stock solution was prepared in methanol and an internal standard working solution was prepared in Surine. Corresponding deuterated internal standards were used for all analytes except hydromorphone-3-β-D-glucuronide, which used morphine-3-β-D-glucuronide-D3 as an internal standard. Calibrators and QCs were prepared in human urine. Calibrators were prepared at six levels from 20–1000 ng/mL (4–200 ng/mL for fentanyl–norfentanyl); QCs were prepared at 30, 150, and 750 ng/mL (6, 30, and 150 ng/mL for fentanyl–norfentanyl). Calibrators and QCs were split for the automated and manual sample preparations.

Sample preparation

A robust solid-phase extraction (SPE) sample preparation method was developed for 21 opiate/opioid drugs and metabolites (see Table 1). An enzymatic hydrolysis step was not included in the method; rather, glucuronides were included as analytes. The following procedure was used for both automated and manual sample preparation.

Urine samples (150 µL) were combined with 50 µL of internal standard and 200 µL of 4% phosphoric acid in a 2 mL mixing plate. For extraction, samples were transferred to an Oasis MCX µElution 96-well plate and eluted into a 1 mL collection plate. The SPE procedure was as follows:

Condition:	200 µL MeOH
Equilibrate:	200 µL H_2O
Sample load:	375 µL
Wash 1:	200 µL H_2O
Wash 2:	200 µL MeOH
Elution (2x):	50 µL of 5% NH_4OH in 60:40 MeOH–ACN

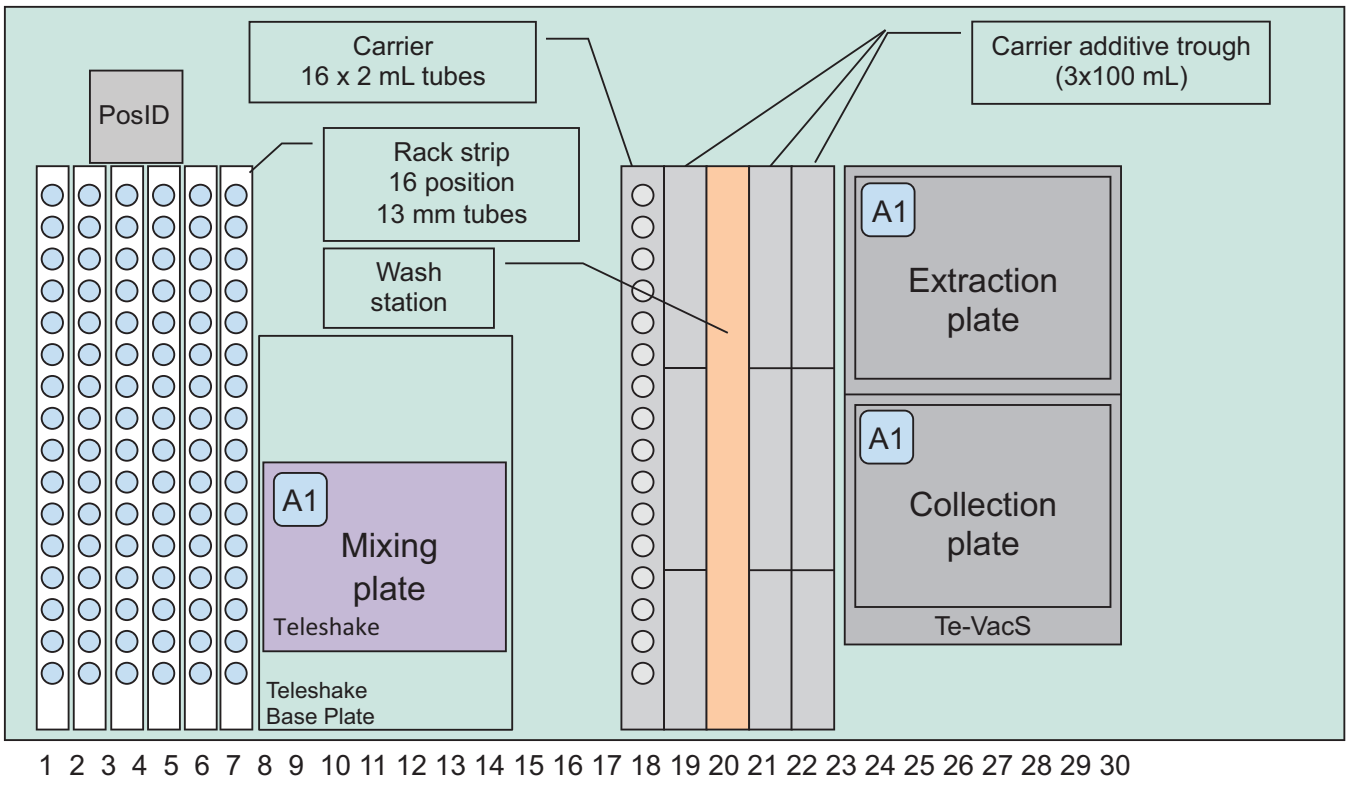
The eluted samples were blown down to dryness using a nitrogen evaporator and reconstituted in 50 µL of 2% formic acid in 98:2 water–acetonitrile before shaking for ten minutes.

The manual sample preparations were performed by an experienced analyst. A calibrated multichannel pipette was used throughout the extraction.

Automation

The Tecan Freedom EVO 100 liquid handler has a user-configurable worktable and components to automate a variety of sample preparation operations. For this study, the liquid handler was equipped with sample and internal standard tube racks, reagent racks and troughs, 4-tip liquid handling arm for sample transfers and reagent additions, robotic manipulator arm for moving plates, bar code reader (posID™), plate shaker (Teleshake), wash station, and vacuum manifold (Te-VacS™). Pipetting tips were fixed (i.e., non-disposable) and were washed between transfers with the vendor-recommended solution of 5% isopropanol in water. The liquid handler executed the extraction as specified by the software script. Upon completion of the script, the Tecan MassLynx File Converter software automatically created a sample list with specimen IDs, plate locations, and pre-populated method information for import into MassLynx via .csv file.
The combined use of automated sample preparation with the file converter provides sample traceability from the sample tube through the completion of the LC-MS/MS analysis, thereby reducing the potential for sample mix-ups as well as errors associated with sample preparation and sample information transcription.

1A



1B

Queue Is Empty

Spectrum Chromatogram Map Edit Samples

	File Name	Sample ID	MS File	MS Tune File	Inlet File	Bottle	Inject Vol...	Sample Type	Conc A	Conc B
1	20150924_Plate1_001	2200001	Opioids MS	Opioids Tune	Opioids Inlet	1:1	5.000	Blank		
2	20150924_Plate1_002	1010020	Opioids MS	Opioids Tune	Opioids Inlet	1:2	5.000	Standard	20	4
3	20150924_Plate1_003	1010050	Opioids MS	Opioids Tune	Opioids Inlet	1:3	5.000	Standard	50	10
4	20150924_Plate1_004	1010100	Opioids MS	Opioids Tune	Opioids Inlet	1:4	5.000	Standard	100	20
5	20150924_Plate1_005	1010200	Opioids MS	Opioids Tune	Opioids Inlet	1:5	5.000	Standard	200	40
6	20150924_Plate1_006	1010500	Opioids MS	Opioids Tune	Opioids Inlet	1:6	5.000	Standard	500	100
7	20150924_Plate1_007	1011000	Opioids MS	Opioids Tune	Opioids Inlet	1:7	5.000	Standard	1000	200
8	20150924_Plate1_008	2200002	Opioids MS	Opioids Tune	Opioids Inlet	1:8	5.000	Blank		
9	20150924_Plate1_009	1110030	Opioids MS	Opioids Tune	Opioids Inlet	1:9	5.000	QC	30	6
10	20150924_Plate1_010	1120030	Opioids MS	Opioids Tune	Opioids Inlet	1:10	5.000	QC	30	6
11	20150924_Plate1_011	1130030	Opioids MS	Opioids Tune	Opioids Inlet	1:11	5.000	QC	30	6
12	20150924_Plate1_012	1140030	Opioids MS	Opioids Tune	Opioids Inlet	1:12	5.000	QC	30	6
13	20150924_Plate1_013	1150030	Opioids MS	Opioids Tune	Opioids Inlet	1:13	5.000	QC	30	6
14	20150924_Plate1_014	1160030	Opioids MS	Opioids Tune	Opioids Inlet	1:14	5.000	QC	30	6

Figure 1. 1A) Tecan worktable layout. 1B) Waters proprietary Tecan MassLynx File Converter software automatically generates importable MassLynx-compatible sample lists pre-populated with Batch ID (defined by user), Sample ID (barcode), sample location, and method information (from user-customizable template).

Analyte	RT (min)	MRM transitions	Cone voltage (V)	Coll. energy (eV)
1 Morphine-3β-D-glucuronide	0.81	462>286 462>201	58	30 46
2 Oxymorphone-3β-D-glucuronide	0.81	478>284 478>227	52	30 44
3 Hydromorphone-3β-D-glucuronide	0.96	462>286 462>185	58	30 50
4 Morphine-6β-D-glucuronide	1.08	462>286 462>201	66	32 44
5 Morphine	1.11	286>201 286>165	60	26 38
6 Oxymorphone	1.24	302>227 302>198	44	29 44
7 Hydromorphone	1.4	286>185 286>157	60	30 42
8 Codeine-6β-D-glucuronide	1.76	476>300 476>215	66	30 40
9 Codeine	1.91	300>215 300>165	60	26 42
10 Noroxycodone	2.12	302>187 302>227	38	25 29
11 Oxycodone	2.18	316>241 316>256	44	30 26
12 Norhydrocodone	2.27	286>199 286>128	54	28 52
13 O-desmethyltramadol	2.33	250>58 250>42	26	16 60
14 Hydrocodone	2.35	300>199 300>171	56	30 40
15 Norfentanyl	2.97	233>84 233>150	34	20 18
16 Tramadol	3.34	264>58 264>42	28	35* 60
17 Norbuprenorphine	3.87	414>101 414>187	68	38 38
19 Buprenorphine	4.23	468>101 468>396	76	42 40
20 EDDP	4.32	278>249 278>186	60	24 35
21 Methadone	4.47	310>105 310>223	34	45* 22

Table 1. Analyte-specific parameters for all analytes, and internal standards.
**non-optimized setting to extend linear range*

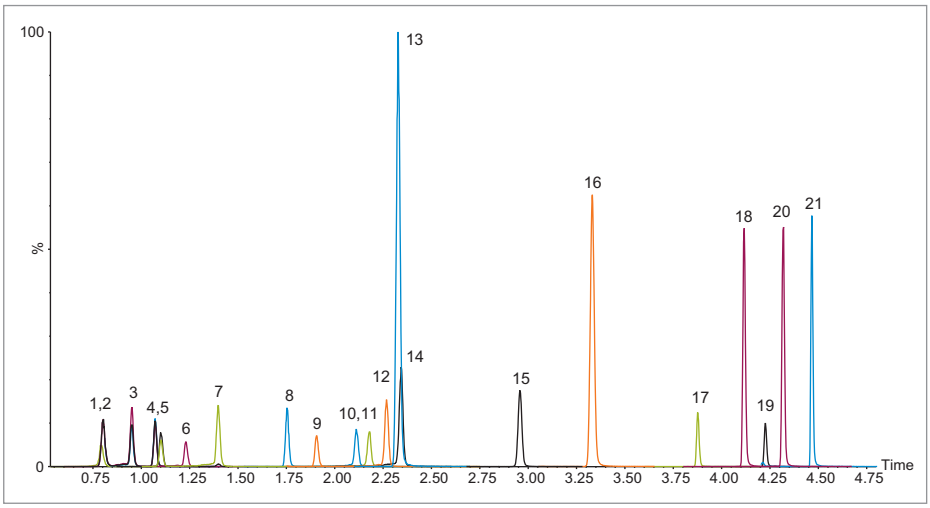


Figure 2. Representative chromatogram of a 20 ng/mL (4 ng/mL fentanyl–norfentanyl) standard; peak assignments are provided in Table 1.

LC conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 mm x 100 mm (P/N 186002352)
Column temp.:	40 °C
Sample temp.:	10 °C
Mobile phase A:	H_2O with 0.1% formic acid
Mobile phase B:	ACN with 0.1% formic acid
Weak needle wash:	2% ACN in H_2O
Strong needle wash:	ACN

Gradient:

Time (min)	Flow (mL/min)	%A	%B
0.00	0.6	98	2
3.00	0.6	80	20
4.00	0.6	55	45
4.10	0.6	90	10
4.60	0.6	90	10
4.70	0.6	98	2
6.20	0.6	98	2

Injection volume: 5 µL

MS conditions

MS system:	Xevo TQD Mass Spectrometer
Ionization mode:	ESI+
Acquisition mode:	MRM (see Table 1 for transitions)
Capillary voltage:	0.5 kV
Cone voltage (V):	Optimized for each analyte
Collision energy (eV):	Optimized for each analyte

Data management

Data were acquired and processed using MassLynx v4.1 Software. Quantification was performed using TargetLynx Application Manager.

RESULTS AND DISCUSSION

Manual and automated sample preparation LC-MS/MS runs were performed on each of three days to compare linearity, inter-assay precision and accuracy, carryover, and sample preparation time. Plates from manual and automated sample preparation each included blank samples, duplicate bracketing calibrators at six levels from 20–1000 ng/mL (4–200 ng/mL fentanyl–norfentanyl), and three levels of QCs (n=6/level) at 30, 150, and 750 ng/mL (6, 30, and 150 ng/mL fentanyl–norfentanyl). Results are summarized in Tables 3–5.

Both types of sample preparation produced linearity, precision, and accuracy results that met industry-standard acceptance criteria; in many cases, inter-assay means and variance were not statistically different (t-test and F-test).[3] For both types of sample preparation, carryover – evaluated by comparing the mean analyte response from the blanks injected after the highest standard (n=2) to the mean response from the lowest standard (n=2) – was less than 4% for all 21 analytes.

Sample processing time for the manual and automated approaches did not differ significantly. However, the use of the Tecan MassLynx File Converter to generate MassLynx sample lists saved considerable amounts of time in the overall analysis, while minimizing transcription errors.

Analyte	Manual prep	Automated prep
	R^2	R^2
Morphine-3µ-D-glucuronide	1.00	0.999
Oxymorphone-3µ-D-glucuronide	0.999	0.998
Hydromorphone-3µ-D-glucuronide	0.999	0.995
Morphine-6-B-D-glucuronide	0.999	0.998
Morphine	0.998	0.998
Oxymorphone	0.999	0.999
Hydromorphone	0.999	0.999
Codeine-6µ-D-glucuronide	0.999	0.998
Codeine	0.991	0.993
Noroxycodone	0.998	0.997
Oxycodone	0.998	0.994
Norhydrocodone	0.998	0.997
O-desmethyltramadol	0.997	0.997
Hydrocodone	0.999	0.996
Norfentanyl	0.999	0.999
Tramadol	0.992	0.991
Norbuprenorphine	0.999	0.999
Fentanyl	0.999	0.999
Buprenorphine	0.998	0.998
EDDP	1.00	0.998
Methadone	0.999	0.998

Table 3. Linearity – comparison of calibration curve coefficient of determination (R^2), day 1.

Analyte	Nominal conc. (ng/mL)	Manual preparation (N=18) Mean	%Dev	%CV	Automated preparation (N=18) Mean	%Dev	%CV
1 Morphine-3β-D-glucuronide	30	29.4	-1.9	3.2	29.3	-2.4	3.0
	150	151	0.8	1.9	155	3.1	2.2
	750	756	0.8	1.3	802	6.9	1.6
2 Oxymorphone-3β-D-glucuronide	30	29.9	-0.3	3.2	28.5	-5.1	4.5
	150	152	1.3	3.0	153	1.6	2.7
	750	746	-0.6	3.7	777	3.6	2.7
3 Hydromorphone-3β-D-glucuronide	30	29.7	-1.0	3.4	29.9	-0.3	3.6
	150	152	1.1	2.5	159	5.8	4.5
	750	753	0.3	3.1	821	9.4	3.3
4 Morphine-6β-D-glucuronide	30	30	-0.2	4.0	29	-3.5	3.0
	150	153	2.0	2.0	154	2.6	3.0
	750	745	-0.7	3.0	782	4.3	2.8
5 Morphine	30	30.2	0.8	4.7	29.2	-2.8	7.6
	150	154	2.5	4.3	159	5.7	4.5
	750	723	-3.6	2.8	779	3.9	3.0
6 Oxymorphone	30	29.3	-2.4	3.3	28.1	-6.3	2.5
	150	151	0.4	2.7	155	3.1	3.0
	750	754	0.6	2.8	801	6.8	2.0
7 Hydromorphone	30	29.8	-0.6	3.6	29.5	-1.8	2.7
	150	149	-0.5	4.3	154	2.8	3.0
	750	767	2.3	3.5	825	10.0	3.5
8 Codeine-6β-D-glucuronide	30	30	-0.2	2.3	28.8	-4.1	3.3
	150	151	1.0	2.9	152	1.6	2.4
	750	745	-0.6	1.9	780	4.0	2.4
9 Codeine	30	30.9	3.0	2.3	29.5	-1.6	3.0
	150	161	7.2	2.1	163	8.6	2.9
	750	698	-7.0	1.6	735	-2.0	2.2
10 Noroxycodone	30	29.6	-1.5	3.1	28.8	-4.0	3.4
	150	151	0.7	2.5	153	1.7	4.6
	750	763	1.7	2.0	802	7.0	2.7
11 Oxycodone	30	30.2	0.8	2.4	28.3	-5.8	3.3
	150	153	2.1	2.4	158	5.0	2.7
	750	721	-3.9	2.3	765	2.1	2.7
12 Norhydrocodone	30	29.7	-0.9	2.7	28.9	-3.8	5.2
	150	153	2.2	2.9	158	5.6	3.2
	750	737	-1.7	2.6	777	3.6	2.4
13 O-desmethyltramadol	30	30.1	0.3	1.8	29.4	-1.9	2.9
	150	158	5.3	1.9	162	8.2	2.5
	750	722	-3.7	3.1	776	3.5	1.9
14 Hydrocodone	30	30.4	1.4	4.3	29.8	-0.8	3.2
	150	153	2.3	3.4	159	5.8	4.9
	750	760	1.3	4.5	827	10.3	3.2
15 Norfentanyl	6	5.94	-1.0	1.8	5.71	-4.8	2.7
	30	30.7	2.4	2.1	31.3	4.3	2.4
	150	148	-1.6	1.2	155	3.6	1.6
16 Tramadol	30	30.5	1.8	1.6	29.7	-1.0	1.6
	150	159	5.7	1.4	163	8.5	2.0
	750	692	-7.8	1.2	733	-2.3	1.4
17 Norbuprenorphine	30	29.6	-1.3	3.2	29.4	-1.9	1.7
	150	151	0.8	2.5	158	5.3	3.4
	750	752	0.2	1.7	796	6.2	1.8
18 Fentanyl	6	6	-0.4	2.3	5.92	-1.4	1.8
	30	30.3	0.9	2.0	31.5	5.0	3.9
	150	151	0.8	1.8	160	6.6	1.7
19 Buprenorphine	30	29.7	-1.2	2.0	29.4	-2.1	2.5
	150	151	0.5	2.7	158	5.1	4.8
	750	768	2.3	2.6	831	10.8	2.0
20 EDDP	30	29.7	-1.1	1.7	29.1	-3.1	2.0
	150	150	0.1	1.6	155	3.5	2.9
	750	761	1.5	1.5	795	6.0	2.3
21 Methadone	30	29.6	-1.5	1.8	29.4	-1.9	1.9
	150	149	-0.7	1.5	154	3.0	3.1
	750	761	1.5	1.5	804	7.2	1.6

Table 4. Inter-assay precision (%CV) and accuracy (% deviation).

Sample preparation	Pipette samples (min)	Extraction (min)	Dry-down (min)	Reconstitution and mixing (min)	Generation of MassLynx sample list (min)
Manual	45	30	5	11	5–20 min
Automated	21	55	5	11	Automatic

Table 5. Time required to process 96 samples using manual and automated approaches.

CONCLUSIONS

Automated sample preparation produced results similar, and in many cases statistically equivalent to, manual sample preparation. The time required for automated sample preparation was also similar to that required for manual preparation. However, automated sample preparation was overall faster when the Tecan MassLynx File Converter was used to automatically generate an importable MassLynx sample list. Automated sample preparation has the additional benefits of allowing analysts to spend more time on tasks requiring human intervention while also reducing the potential for variation and error at multiple points during sample preparation and analysis. The Oasis MCX µElution Plate provides identical results when used in either manual or automated sample preparation procedures. Finally, the combination of the sample-tracking capabilities of the Tecan liquid handler with the Tecan MassLynx File Converter software can reduce transcription errors.

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