Journal of Chromatography B, 879 (2011) 2642–2652

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Online extraction LC–MS/MS method for the simultaneous quantitative confirmation of urine drugs of abuse and metabolites: Amphetamines, opiates, cocaine, cannabis, benzodiazepines and methadone

Andrew D. de Jager*, Neville L. Bailey

Drugs of Abuse Laboratory, Clinical Chemistry, Level 6, Mater Adults Hospital, Raymond Terrace, South Brisbane, QLD 4101, Australia

ARTICLE INFO

Article history:
Received 23 May 2011
Accepted 24 July 2011
Available online 30 July 2011

Keywords:
LC–MS/MS
Drugs of abuse
Online extraction

ABSTRACT

A rapid LC–MS/MS method for confirmatory testing of five major categories of drugs of abuse (amphetamine-type substances, opiates, cocaine, cannabis metabolites and benzodiazepines) in urine has been developed. All drugs of abuse mandated by the Australian/New Zealand Standard AS/NZS 4308:2008 are quantified in a single chromatographic run. Urine samples are diluted with a mixture of isotope labelled internal standards. An on-line trap-and-flush approach, followed by LC–ESI-MS/MS has been successfully used to process samples in a functioning drugs of abuse laboratory. Following injection of diluted urine samples, compounds retained on the trap cartridge are flushed onto a reverse-phase C18 HPLC column (5-μm particle size) with embedded hydrophylic functionality. A total chromatographic run-time of 15 min is required for adequate resolution. Automated quantitation software algorithms have been developed in-house using XML scripting to partially automate the identification of positive samples, taking into account ion ratio (IR) and retention times (Rt). The sensitivity of the assay was found to be adequate for the quantitation of drugs in urine at and below the confirmation cut-off concentrations prescribed by AS/NZS 4308:2008.

1. Introduction

Increasingly, drug of abuse screening is becoming routine in the workplace. Testing has enjoyed the greatest acceptance in industries where employees are involved in activities that involve safety risks, such as mining and civil aviation. Rapid generation of analytical results is critical to a successful workplace screening programme, as delays in providing a requesting authority with results has adverse economic implications. This is mainly owing to the fact that individuals are not permitted to return to the workplace until their samples are declared free of illegal substances.

In Australia and New Zealand, screening and confirmation of drugs in the five major urine drug categories is regulated by the Australian/New Zealand Standard AS/NZS 4308:2008 [1]. Initial drug screening procedures are most commonly performed by immunoassay (IA), and while the limitations of IA with respect to specificity have been well documented, the generation of results is rapid relative to confirmation assays.

Traditionally, confirmation assays performed on samples that have returned a positive screening result have been done using GC–MS. While GC–MS has proven to be a robust and reliable technique for several decades, fairly rigorous sample preparation requirements, often involving derivatization of certain compounds and relatively long chromatographic runs make this a fairly time intensive approach. More recently, LC–MS/MS has been increasingly used for confirmation of drugs of abuse in various matrices. There are a number of inherent advantages when using LC–MS/MS as opposed to GC–MS. Most notably, derivatization is not required prior to instrumental analysis, which coupled with shorter chromatographic run times puts LC–MS/MS at an advantage with respect to rapid sample analysis. However, these advantages may be offset by the relative cost of an LC–MS/MS system, which is considerably more than for a GC–MS system. Regrettably, some of the time saving advantages of using LC–MS/MS are often lost because sample preparation protocols are often compartmentalised into drug groups. For example, laboratories develop a benzodiazepine assay, an opiate assay, etc., each with an associated set of chromatographic and instrumental conditions.

A second categorisation observed centres around the chemistry of the abused drugs, with laboratories developing an assay for acidic, basic and neutral compounds. While this is a fundamentally sound approach, the net effect is that total analysis times are not significantly different for GC–MS and LC–MS/MS confirmations. It was for this reason that we aimed

* Corresponding author. Tel.: +61 7 3163 8500; fax: +61 7 3163 7821.
E-mail addresses: theandy.dejager@gmail.com, andy.dejager@mater.org.au (A.D. de Jager).

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doi:10.1016/j.jchromb.2011.07.029

to develop an assay method that firstly involved the same sample preparation procedure for all drug classes, and secondly involved a single LC–MS/MS chromatographic run. In doing so, we hoped to overcome some of the factors that impede harnessing the advantages of using LC–MS/MS for drugs of abuse confirmation.

Combining multiple classes of drugs of abuse in a single assay is relatively commonplace. De Leenheer et al. [2] use a single solid phase extraction (SPE) procedure to determine opiates, amphetamines and cocaine related substances in oral fluid, followed by time-of-flight mass spectrometry. More recently, Thörngren et al. [3] reported a muticomponent screening method for 130 drugs, following a simple dilution of urine with a selection of isotope labelled internal standards. Simple dilution of urine prior to analysis is becoming increasingly prevalent in analytical literature, particularly as the sensitivity and robustness of LC–MS/MS instrumentation continues to improve. Beck et al. [4] validated a quantitative LC–MS/MS assay for opiates, following a simple dilution of urine. Importantly they avoid the need for hydrolysis by incorporating all the major metabolites of a particular opiate in the assay. For example, in the assay, morphine, morphine-3β-glucuronide (M3G) and morphine-6β-glucuronide (M6G) are quantitatively combined to produce a total morphine concentration. There may be an inherent advantage in this approach as Ilett et al. [5] report that inter-laboratory variation in reported codeine and morphine levels can often be attributed to differences in the efficiency of the hydrolysis process. Similarly, Beck et al. [6] point out that an incorrect determination of the proportion of total morphine and codeine in urine may result if hydrolysis is incorrectly performed.

Beck et al. report good agreement between this approach, and a reference GC–MS method involving hydrolysis of conjugated opiates. Owing to the absence of any rigorous sample clean-up, Beck et al. report that to varying degrees, compounds are affected by matrix effects, and that this is most acute for compounds that elute early from the analytical column.

While the most common approach to sample clean-up of urine samples prior to drugs of abuse confirmation analysis is SPE, on-line SPE (trap-and-flush) is a variation of the traditional SPE approach which is well worth considering. On-line extraction procedures have appeared in the literature and have demonstrated their suitability for routine analysis [7,8]. Weinmann et al. [9] used an on-line extraction method to determine a range of basic drugs of abuse in human serum following precipitation of proteins. They use a system of two alternating trap columns, allowing for adequate cleaning and equilibration of the trap column between injections.

In this paper, we describe an online extraction (trap and flush) LC–MS/MS method for the quantitation of 29 drugs of abuse in urine, following dilution with a mixture of isotope labelled internal standards. The online SPE methodology allowed for sufficient sample clean-up, bringing matrix effects to within acceptable limits. This method has proved itself suitable for a routine drugs of abuse laboratory.

2. Material and methods

2.1. Materials and reagents

All certified drug and isotope labelled internal standard solutions were purchased from Cerilliant (Round Rock, Texas, USA). Ammonium Acetate (Sigma Ultra grade) and methanol (Fluka, purity >99.9%) were purchased from Sigma–Aldrich (St. Louis, USA). Methanol (purity >99.9%) was purchased from Merck (Darmstadt, Germany). Deionised water (18.1 MΩ) was obtained from a Millipore-Q water system (Bedford, MA, USA).

2.2. Instrumentation

The HPLC system used was a Shimadzu Prominence UFLC system (Kyoto, Japan). Two binary high pressure pumps (LC-20AB), each fitted with a 100 μL high pressure mixer were used for solvent delivery. The autosampler was a SIL-20AHT. The column oven was a CTO-20A, which incorporated a 6-port switching valve. An in-line degasser (DGU-20A_3) was placed prior to the solvent delivery system.

An additional Rheodyne 10-port switching valve (Rheodyne, CA, USA) was purchased and was operated by contact closure. The LC–MS/MS system used was an ABSciex 5500QTRAP, with a TurboIonSpray® source (ABSciex, Concord, Ont., Canada). All data were collected using ABSciex Analyst software (version 1.5). Quantitation was performed using MultiQuant ver. 2 software (ABSciex).

2.3. Preparation of calibration standard solutions

Owing to the fact that 11-nor-Δ^9-tetrahydrocannibinol-9-carboxylic acid (THC-COOH) and 11-nor-Δ^9-tetrahydrocannibinol-9-carboxylic acid glucuronide (THC-COOH-GLU) degrade and adsorb to surfaces more readily than compounds in the remaining drug groups, it was decided to separate calibration solutions into cannabinoid calibrators and the remaining drug groups. This was done to minimise the impact of analyte losses, as it was deemed inefficient to re-prepare an entire calibration series for the sake of two compounds.

2.3.1. Non-THC calibration standard solutions

Five primary stock solutions (opiates, benzodiazepines, cocaine related substances, amphetamine-like substances and methadone) were prepared from certified reference material solutions in drug groups. Dilutions were made in methanol:water (1:1, v/v). 1 mL of each of these five stock solutions was placed in a 10 mL volumetric flask, which was made up to the mark with methanol:water (1:1, v/v). This tenfold dilution resulted in the calibration standard solution (S1). This calibration standard (S1) was then serially diluted (gravimetrically) producing four further calibration standard solutions S2, S3, S4 and SLLOQ (Table 1).

2.3.2. THC related calibration standard solutions

Similarly, a single primary stock solution containing THC-COOH and THC-COOH-GLU was prepared (each at 250 ng/mL) in methanol:water, 1:1 (v/v). This stock solution was then serially diluted (gravimetrically) to produce five calibration standard solutions (Table 2).

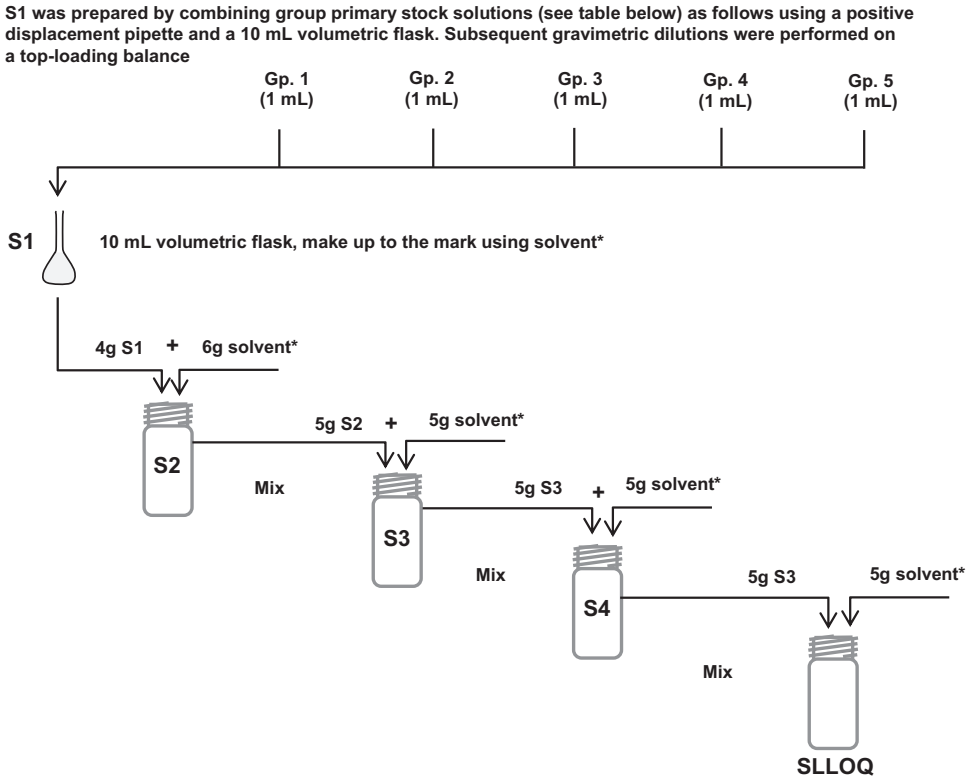
2.4. Preparation of urine quality controls

All urine used in the preparation of quality controls was obtained from staff volunteers within the hospital facility, and was screened prior to preparation of quality controls to ensure that it was drug free, using the assay procedure.

2.4.1. Non-THC urine quality controls

Five primary stock solutions were prepared from certified reference material solutions (in drug groups) in the same manner described in Section 2.3.1. In a 10 mL volumetric flask, spikes of the individual primary stocks were made according to the figure below using positive displacement pipettes.

Table 1
Calibration standard solution concentrations of non-THC solutions (ng/mL).



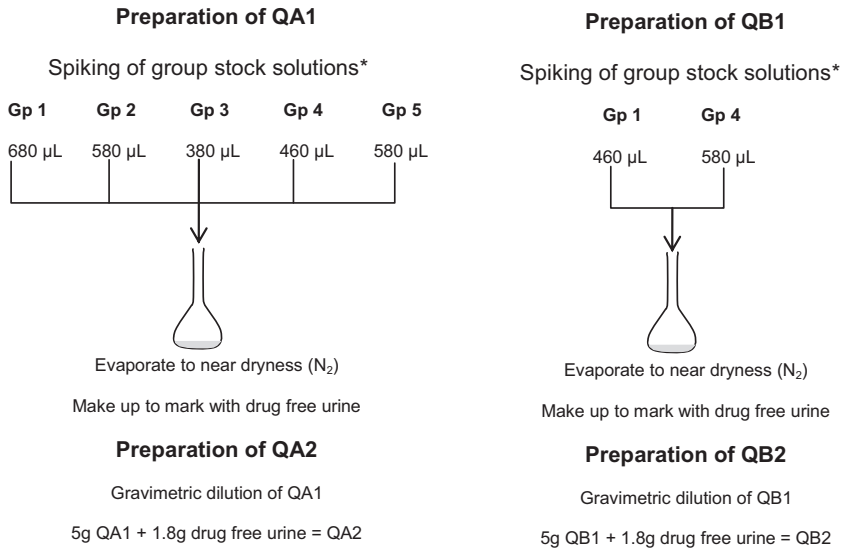
Compound name	Conc. in Cerilliant stock (ng/mL)	Group	Conc. in primary stock (ng/mL)	Calibration standard solution (ng/mL)				
				S1	S2	S3	S4	SLLOQ
Codeine	100,000	Gp. 1	5000	500	200	100	50	25
Codeine-6-β-D-glucuronide	100,000		5000	500	200	100	50	25
Morphine	100,000		5000	500	200	100	50	25
Morphine-3-β-D-glucuronide	100,000		5000	500	200	100	50	25
Morphine-6-β-D-glucuronide	100,000		5000	500	200	100	50	25
6-Acetylmorphine	100,000		1000	100	40	20	10	5
(±) Amphetamine	100,000	Gp. 2	3000	300	120	60	30	15
(±) Methylamphetamine	100,000		3000	300	120	60	30	15
(±) MDMA	1000,000		3000	300	120	60	30	15
(±) MDA	1000,000		3000	300	120	60	30	15
Benzylpiperazine	1000,000		10,000	1000	400	200	100	50
Phentermine	1,000,000		10,000	1000	400	200	100	50
Pseudoephedrine	1,000,000		10,000	1000	400	200	100	50
Methadone	100,000	Gp. 3	3000	300	120	60	30	15
EDDP	100,000		3000	300	120	60	30	15
Diazepam	1,000,000		5000	500	200	100	50	25
Nordiazepam	1,000,000		5000	500	200	100	50	25
Oxazepam	1,000,000		5000	500	200	100	50	25
Temazepam	1,000,000		5000	500	200	100	50	25
α-Hydroxyalprazolam	100,000	Gp. 4	2000	200	80	40	20	10
7-Aminoclonazepam	100,000		2000	200	80	40	20	10
7-Aminoflunitrazepam	100,000		2000	200	80	40	20	10
7-Aminonitrazepam	100,000		2000	200	80	40	20	10
Benzoylecgonine	100,000	Gp. 5	3000	300	120	60	30	15
Ecgonine methyl ester	100,000		3000	300	120	60	30	15

* Solvent = methanol:water (1:1, v/v).
Note: Masses of stock solution and solvent were added drop-wise using glass Pasteur pipettes, and were added to the nearest ±0.01 g.

Table 2
Calibration standard solution concentrations of THC solutions (ng/mL).

Compound name	Conc. in primary stock (ng/mL)	Calibration standard solution (ng/mL)				
		$S1_{(THC)}$	$S2_{(THC)}$	$S3_{(THC)}$	$S4_{(THC)}$	$SLLOQ_{(THC)}$
THC-COOH	250	100	50	20	10	5
THC-COOH-GLU		100	50	20	10	5

Table 3
Preparation of quality controls for non-THC compounds.



Compound	Reporting level	QA1	QA2	QB1	QB2
Nominal concentration of compounds in quality controls					
Codeine	300	340	250		
Codeine-6-β-glucuronide	300	340	250		
Morphine	300	340	250		
Morphine-3-β-glucuronide	300	340	250		
Morphine-3-β-glucuronide	300	340	250		
6-Acetylmorphine	10			11.5	8.5
(±) Amphetamine	150	174	128		
(±) Methamphetamine	150	174	128		
(±) MDMA	150	174	128		
(±) MDA	150	174	128		
Phentermine	500	580	427		
Pseudoephedrine	500	580	427		
Methadone	100	114	83.8		
EDDP	100	114	83.8		
Diazepam	200	230	169		
Nordiazepam	200	230	169		
Oxazepam	200	230	169		
Temazepam	200	230	169		
α-Hydroxyalprazolam	100			116	85.3
7-Aminoclonazepam	100			116	85.3
7-Aminoflunitrazepam	100			116	85.3
7-Aminonitrazepam	100			116	85.3
Benzoylecgonine	150	174	128		
Ecgonine methyl ester	150	174	128		

* Group stock solutions prepared in the same manner described in Table 1 for calibration standards.

2.4.2. THC urine quality controls

When preparing urine THC quality controls (containing THC-COOH and THC-COOH-GLU), it was not possible to use the approach described in Section 2.4.1. Specifically, it was not possible to prepare quality controls at calculated nominal concentrations. Instead, it was necessary to prepare controls in urine, and then back-calculate these from a calibration curve in order to assign a mean measured concentration. The mean measured concentrations were then used throughout the validation studies. The reason for this is that both THC-COOH and THC-COOH-GLU were found to adsorb to surfaces (both glass and plastic) in aqueous medium, and in all cases, mean measured concentrations were found to be lower than expected concentrations.

A stock solution containing THC-COOH and THC-COOH-GLU was prepared in methanol:water (1:1, v/v) at 1 μg/mL for both compounds.

2.4.3. Aliquots of urine quality controls

Once quality controls were prepared, 100 μL (the assay volume) was transferred to a 1.8 mL snap-cap tube (the tube used for the assay dilution) and then kept at −20 °C. When preparing sample batches, tubes containing quality controls were thawed and used

Table 4
Preparation of quality controls – THC related compounds.

Compound	Spike vol (μL)	Urine mass (g)	THC-COOH (mean measured conc., ng/mL)	THC-COOH-GLU (mean measured conc., ng/mL)
QC HIGH	210	10	17.5	17.9
QC LOW	140	10	11.6	11.7

Table 5
Mass spectrometer parameters.

Parameter	Value
Polarity	Positive
Ionisation voltage (IS)	5200 V
Nebulising gas (GS1)	60
Desolvation gas (GS2)	60
Source temperature (TEM)	550 °C
Collision gas (CAD)	Medium
Q1 resolution	Unit
Q3 resolution	Unit

directly in the original tube, with no re-aliquotting done. While this facilitated speedy batch preparation, the more fundamental reason for this was to ensure that urine quality controls (particularly urine THC quality controls) were not exposed to additional plastic surfaces prior to dilution. A study of adsorptive losses of THC-COOH and THC-COOH-GLU is described in Section 3.7, illustrating why this was important.

2.5. Sample preparation

2.5.1. Preparation of daily urine calibration standards

100 μL of each of the two calibration standard solutions (described in Sections 2.4.1 and 2.4.2) was placed in a 1.8 mL eppendorf snap cap tube. 100 μL blank urine, 20 μL internal standard solution and 400 μL dilution solvent (methanol:water, 40:60 (v/v) containing 10 mM ammonium acetate) were added. Tubes were briefly vortexed and the diluted sample placed in autosampler vials.

2.5.2. Samples and quality controls (samples not requiring enzymatic cleavage)

100 μL urine, 200 μL methanol:water (1:1, v/v), 20 μL internal standard solution and 400 μL dilution solvent (described in Section 2.5.1) were placed in a 1.8 mL eppendorf snap cap tube. Tubes were briefly vortexed and the diluted sample placed in autosampler vials.

2.5.3. Samples requiring enzymatic cleavage (benzodiazepines)

100 μL urine, 20 μL internal standard solution and 50 μL β-glucuronidase solution (from *E. coli* K12, diluted 10-fold with water) were placed in a 1.8 mL eppendorf snap cap tube. The tubes were sealed and placed in a water bath (40 °C) for 3 h. Following this, tubes were cooled using tap water, and to each tube was added 100 μL methanol:water (1:1, v/v), and 400 μL dilution solvent (described in Section 2.5.1). The sample was mixed by vortexing and 500 μL was passed through a Millipore Micron centrifugal filter (10 Da) in order to remove the β-glucuronidase. The filtrate was placed in autosampler vials.

2.6. Chromatographic conditions

The online trap column used was a Thermo AQUASIL C18 5 μm 10 mm × 2 mm drop-in guard cartridge, and the analytical column was a Thermo AQUASIL C18, 100 mm × 2.1 mm, 5 μm particle size (San Jose, CA, USA). The analytical column was fitted with a guard cartridge (the same cartridge used for trapping). The aqueous mobile phase (phase A) consisted of 10 mM ammonium acetate in water, while the organic mobile phase (phase B) consisted of 10 mM ammonium acetate in methanol:acetonitrile (1:1, v/v).

Three chromatographic modes (Fig. 1) were programmed using the switching valves, namely (A) trap mode, (B) elute mode and (C) precolumn flush mode. In all instances, both pump 1 and pump 2 delivered solvent at a constant 270 μL/min, except for during the precolumn flush mode, where pump 2 delivered solvent at 400 μL/min to improve the cleaning of the precolumn.

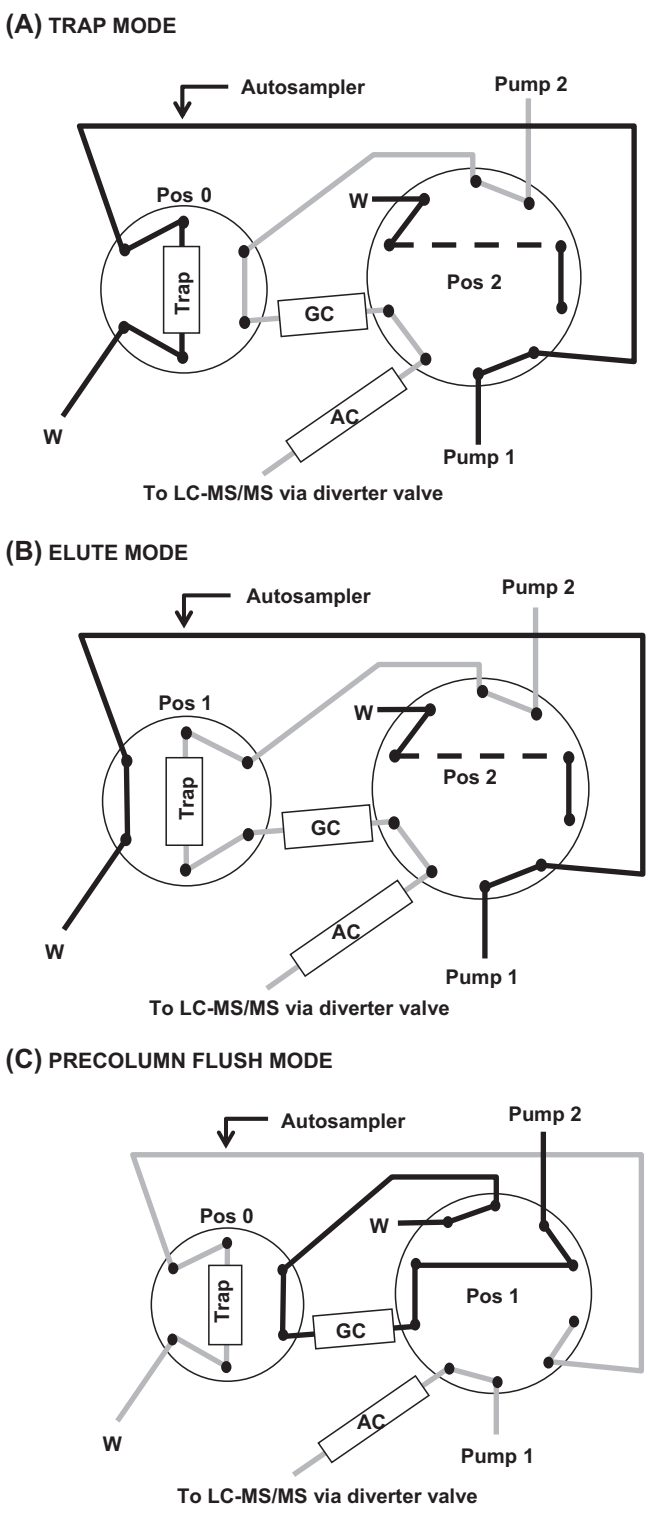


Fig. 1. HPLC trap/flush configuration (W = waste, GC = guard cartridge, AC = analytical column).

2.6.1. Trap mode

In trap mode, pump 1 delivered aqueous phase (99% phase A) to the trap column, while pump 2 kept the analytical column equilibrated at (99% phase A). Diluted urine samples were injected directly onto the trap cartridge (15 μL).

Table 6
Compound parameters, showing transitions for reporter and qualifier ions.

Compound	Q1 (m/z)	Q3 (m/z)	R time (min)	DP (V)	EP (V)	CE (eV)	CXP (V)
6-Monoacetylmorphine	328.0	165.1	8.5	150	10	51	14
	328.0	210.9	8.5	150	10	35	18
7-Aminoclonazepam	286.0	121.1	7.2	116	10	39	12
	286.0	222.0	7.2	116	10	35	12
7-Aminflunitrazepam	284.0	135.0	7.6	166	10	37	14
	284.0	226.9	7.6	166	10	35	18
7-Aminonitrazepam	251.9	121.0	7.2	61	10	35	12
	251.9	94.0	7.2	61	10	57	10
Amphetamine	136.0	90.9	7.9	46	10	25	10
	136.0	118.9	7.9	46	10	13	12
α-Hydroxyalprazolam	324.9	297.0	8.6	81	10	37	16
	324.9	215.9	8.6	81	10	55	18
Benzoylecgonine	290.0	167.9	7.2	86	10	27	22
	290.0	104.9	7.2	86	10	41	18
Morphine-3β-D-glucuronide	462.0	286.0	4.7	151	10	41	22
	462.0	151.9	4.7	151	10	129	14
Morphine-6β-D-glucuronide	462.1	286.0	5.3	151	10	41	22
	462.1	151.9	5.3	151	10	129	14
Benzylpiperazine	177.0	91.0	9.6	76	10	23	12
	177.0	64.9	9.6	76	10	57	10
Codeine-6β-D-glucuronide	476.0	300.0	6.2	106	10	43	22
	476.0	152.0	6.2	106	10	127	12
Codeine	300.0	152.0	9.0	151	10	87	14
	300.0	165.0	9.0	151	10	59	14
Diazepam	284.9	193.0	9.2	101	10	41	18
	284.9	154.0	9.2	101	10	37	12
Ecgonine methylester	200.0	182.0	6.3	61	10	23	16
	200.0	82.0	6.3	61	10	33	14
Ephedrine/pseudoephedrine	166.0	90.9	7.7	41	10	45	14
	166.0	115.0	7.7	41	10	35	16
MDA	180.0	104.9	7.8	41	10	31	10
	180.0	134.9	7.8	41	10	25	12
MDMA	194.0	162.8	8.4	36	10	17	16
	194.0	105.0	8.3	36	10	33	12
Methamphetamine	150.0	91.0	8.5	51	10	27	10
	150.0	64.9	8.5	51	10	53	12
Morphine	286.0	151.9	7.7	91	10	77	16
	286.0	165.0	7.7	91	10	57	16
Nordiazepam	270.9	139.9	8.9	81	10	39	14
	270.9	208.0	8.9	81	10	39	18
Oxazepam	286.9	240.9	8.5	81	10	31	20
	286.9	104.0	8.5	81	10	45	14
Phentermine	150.0	90.9	8.3	46	10	31	14
	150.0	133.1	8.3	46	10	13	12
Temazepam	300.9	255.0	8.8	76	10	31	16
	300.9	177.1	8.8	76	10	53	10
Methadone	310.1	265.0	10.9	111	10	21	22
	310.1	105.1	10.9	111	10	35	10
EDDP	279.1	235.2	10.3	181	10	43	12
	279.1	250.1	10.3	181	10	33	12
THC-COOH	344.9	299.2	9.4	151	10	29	16
	344.9	193.0	9.4	151	10	37	18
THC-COOH-GLU	344.9	299.2	8.5	200	27	29	16
	344.9	193.1	8.5	200	27	37	18

2.6.2. Elute mode

In elute mode, the analytical column was placed in line using the 6-port switching valve. Pump 2 delivered a gradient (see Fig. 2) and with increasing organic content, flushed the retained compounds from the trap column into the analytical column, which were then separated on the analytical column (Fig. 3).

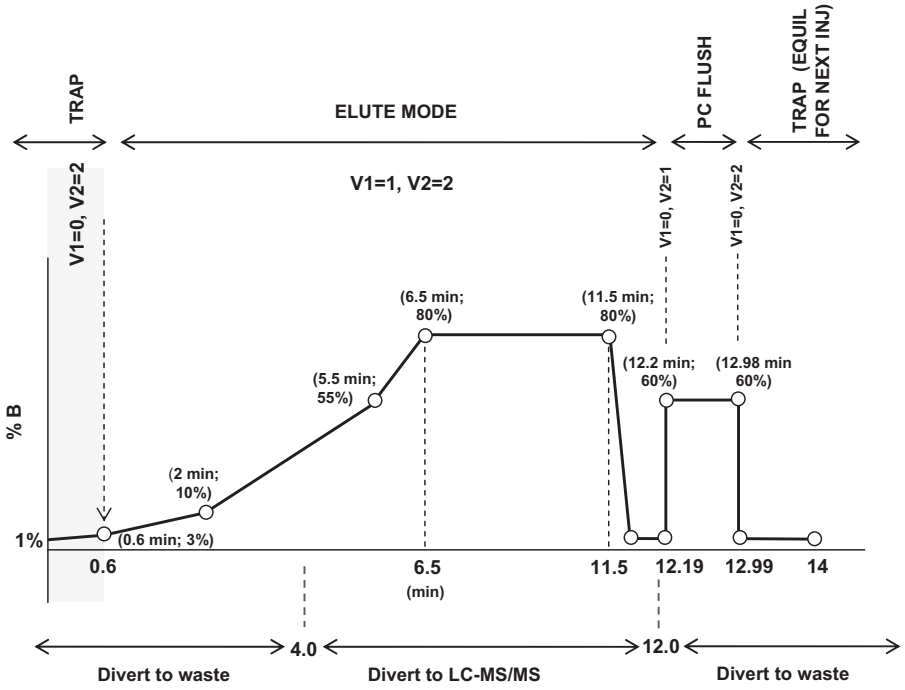
2.6.3. Precolumn flush mode

By switching the 10-port switching valve, the precolumn was isolated and flushed in the reverse direction with 60% phase B, to waste, while pump 1 equilibrated the analytical column for the next injection. This greatly lengthened the lifetime of the analytical column.

2.7. Mass spectrometer conditions

The mass spectrometer used was an ABSciex 5500QTRAP (Concord, Ontario) fitted with an ESI (TurboIonSpray™) source. Using the diverter valve integrated on the mass spectrometer, the first 4 min of chromatographic eluent was diverted to waste. All analytes were ionised in positive mode. Scheduled multiple reaction monitoring (sMRM) was used in order to optimise the instrument cycle time.

It is worth noting that for THC-COOH and THC-COOH-GLU, the same Q1 to Q3 transitions were used. However, for the THC-COOH-GLU, higher source voltages (DP and EP) were used, which effectively reverted a large fraction of the glucuronidated species back to THC-COOH in the mass spectrometer source. This was



Mobile phase A : 10 mM ammonium acetate in water (18.1 MΩ)

Mobile phase B : 10 mM ammonium acetate in methanol : acetonitrile (1:1; v/v)

Fig. 2. Gradient elution profile for pump 2.

possible owing to the fact that the two compounds were fully resolved from one another chromatographically (see Fig. 3).

2.8. Data collection and quantitation

All data were collected using Analyst version 1.5 software (ABSciex, Concord, Ontaria, Canada). Quantitation of data was done using MultiQuant version 2.0 (ABSciex). Following integration, XML scripting (integral to MultiQuant) was used to interrogate data, and automatically produce a list of samples that satisfied the requirements of AS/NZS 4308:2008 [1] with respect to being above the reporting concentration, displaying the correct ion ratio and having an acceptable retention time (within ±2% of the retention time of the calibration standards).

3. Results and discussion

3.1. Linearity

The linearity of the assay was established between the highest and the lowest calibration standard as shown in Tables 1 and 2 for all analytes, which reflects not only the concentration of the

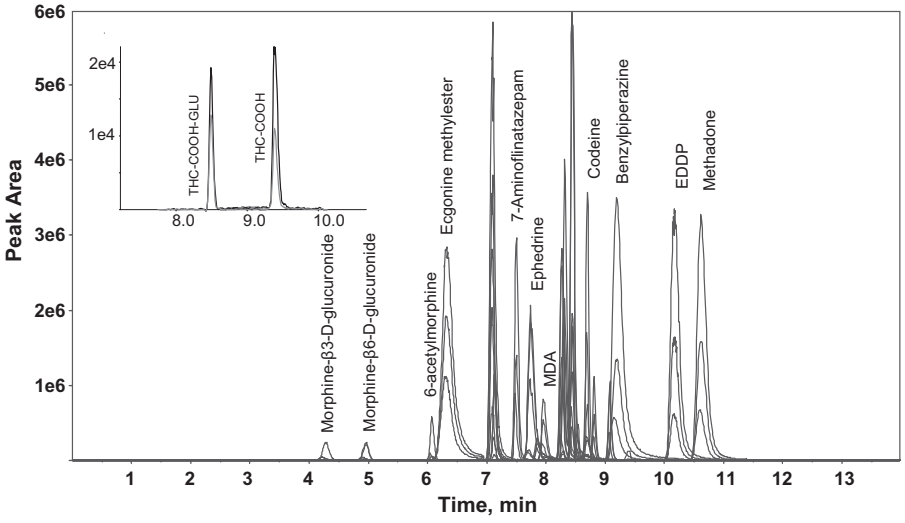
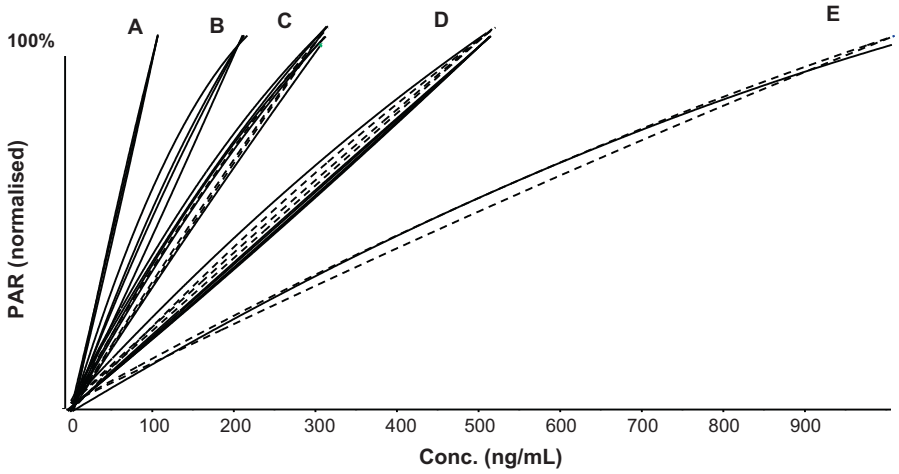


Fig. 3. Chromatogram resulting from second highest calibration standard (with selected peaks labelled) showing the relative intensities. As expected, hydrophylic compounds (such as morphine and heroin metabolites) elute early during the highly aqueous part of the gradient, while hydrophobic compounds (such as THC-COOH and methadone) elute in the latter part of the chromatogram. The inset shows THC-COOH and THC-COOH-GLU (not observable in the full scale chromatogram), which are the two compounds for which the sensitivity is poorest in +ESI mode.



Overlay of calibration curves for all analytes (5 non-zero calibration points for each curve), run in a single batch. Calibration curves are normalised to 100% based on the peak area ratio (analyte/ISTD) for each analyte.

A = THC-COOH and THC-COOH-GLU, 6-monoacetylmorphine
B = α-OH alprazolam, 7-aminoclonazepam, 7-aminonitrazepam, 7-aminoflunitrazepam
C = methadone, EDDP, amphetamine, methamphetamine, MDMA, MDA, benzoylecgonine, ecgonine methylester
D = codeine, codeine-6β-D-glucuronide, morphine, morphine-3β-D-glucuronide, morphine-6β-D-glucuronide, diazepam, nordiazepam, oxazepam, temazepam
E = benzylpiperazine, phentermine, pseudoephedrine

Fig. 4. Overlay of calibration curves ($n = 27$ analytes) from a sample batch, based on peak area ratio (PAR). Overlay of calibration curves for all analytes (5 non-zero calibration points for each curve), run in a single batch. Calibration curves are normalised to 100% based on the peak area ratio (analyte/ISTD) for each analyte. A = THC-COOH and THC-COOH-GLU, 6-monoacetylmorphine. B = α-OH alprazolam, 7-aminoclonazepam, 7-aminonitrazepam, 7-aminoflunitrazepam. C = methadone, EDDP, amphetamine, methamphetamine, MDMA, MDA, benzoylecgonine, ecgonine methylester. D = codeine, codeine-6β-D-glucuronide, morphine, morphine-3β-D-glucuronide, morphine-6β-D-glucuronide, diazepam, nordiazepam, oxazepam, temazepam. E = benzylpiperazine, phentermine, pseudoephedrine.

compounds in solution, but also the effective urine concentration. The regression line of best fit for each analyte was assessed by comparing the summed absolute percentage of the relative error (Σ%RE) for each calibration curve [10,11]. The data showed that a quadratic curve fit (no weighting) was the simplest and most rugged fit for all compounds. Any samples above the calibration range were diluted to within the calibration range using drug free urine. Fig. 4 shows all calibration curves (constructed from a total of just 5 injections, one for each calibration level) overlaid on a single system of axes. The peak area ratio (analyte peak area/ISTD peak area) has been normalised on the *y*-axes.

Table 7
Between run accuracy and precision ($n = 6$ runs).

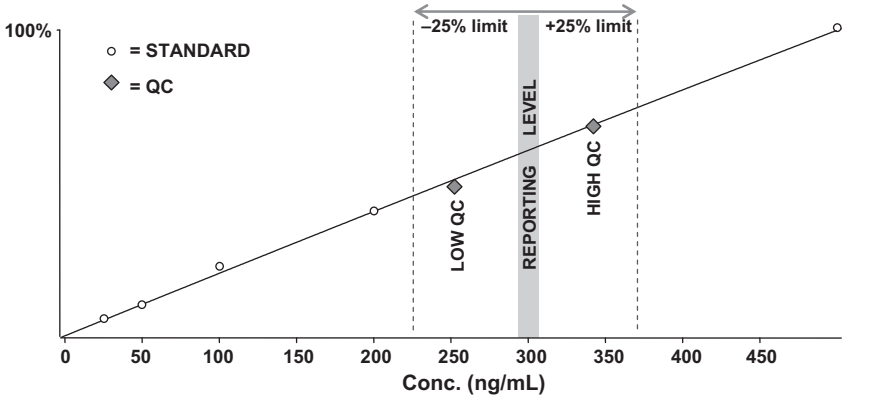
Compound	+25% QC		−25% QC	
	CV%	%dev	CV%	%dev
6-Monoacetylmorphine	4.58	−1.7	4.62	1.3
7-Aminoclonazepam	9.71	−9.7	5.85	−7.1
7-Aminoflunitrazepam	5.62	−7.2	6.13	−6.6
7-Aminonitrazepam	7.72	−3.2	5.96	−0.7
Amphetamine	7.12	−3.9	6.45	−2.0
α-Hydroxyalprazolam	5.01	−4.2	6.67	−4.7
Benzoylecgonine	7.42	−0.5	3.80	0.1
Benzylpiperazine	5.82	−2.0	2.88	1.1
Codeine	7.13	−0.8	4.34	−2.3
Codeine-6β-D-glucuronide	4.28	−1.2	2.80	−1.3
Diazepam	2.59	−2.8	5.33	−1.0
Ecgonine methylester	2.86	−6.1	4.47	−6.3
EDDP	3.17	−1.5	5.09	−1.0
Pseudoephedrine	3.01	−4.7	2.60	−2.3
MDA	3.87	−2.5	5.93	−1.8
MDMA	6.10	−2.9	2.60	1.4
Methadone	3.43	−4.7	4.67	−2.9
Methamphetamine	3.20	−0.7	4.84	−2.5
Morphine	6.89	−3.9	4.16	5.3
Morphine-3β-D-glucuronide	6.28	−2.6	4.19	−0.2
Morphine-6β-D-glucuronide	5.45	−2.6	3.17	−2.1
Nordiazepam	5.63	−1.2	6.19	−0.4
Oxazepam	4.27	−3.7	3.83	−1.5
Phentermine	2.32	−3.6	3.91	−3.2
Temazpam	4.37	−2.8	4.38	−1.6
THC-COOH	2.29	−3.4	5.59	−4.7
THC-COOH-GLU	4.30	−1.6	3.89	−3.8

3.2. Between-run accuracy and precision

Between run accuracy and precision was calculated using a single replicate of each of the urine quality controls levels prepared over six occasions (each run on a different day). For each analyte, two quality controls were used, the first no more than 25% above the reporting concentration, and the second no more than 25% below the reporting concentration for the particular analyte (see Fig. 5). The data are shown in Table 7. Precision is expressed as CV%, and accuracy is expressed as mean % deviation from the nominal concentration of the QC (nominal concentrations of QCs are shown in Tables 3 and 4).

3.3. Within-run accuracy and precision

Similarly, within-run accuracy and precision was calculated using urine quality controls ($n = 5$) from a single assay occasion. For each analyte, the same quality controls described above were used (Tables 5–7). The data are shown in Table 8. Precision is expressed as CV%, and accuracy is expressed as mean % deviation from the nominal concentration of the QC (nominal concentrations of QCs are shown in Tables 3 and 4).



For the example curve shown above (morphine-3β-D-glucuronide, one of 27 analytes), the nominal concentration of the low and high QCs were 250 and 340 ng/mL respectively, which were within the limits prescribed by AS/NZS 4308:2008. The accuracy of these two QCs (expressed as a %deviation from nominal concentration) was **-3.2%** and **-0.7%** respectively. The figure above shows the appropriate placement of QCs as prescribed by the standard (AS/NZS 4308:2008) and this was done for all analytes. For certain analytes (notably THC-COOH, THC-COOH-GLU and 6-monoacetylmorphine) the cut-off concentrations are nearer the low end of the respective calibration curve, and placement of the low and high QCs was adjusted appropriately.

Fig. 5. Example calibration curve (morphine-3β-D-glucuronide) from a sample batch For the example curve shown above (morphine-3β-D-glucuronide, one of 27 analytes), the nominal concentration of the low and high QCs were 250 and 340 ng/mL respectively, which were within the limits prescribed by AS/NZS 4308:2008. The accuracy of these two QCs (expressed as a %deviation from nominal concentration) was −3.2% and −0.7% respectively. The figure above shows the appropriate placement of QCs as prescribed by the standard (AS/NZS 4308:2008) and this was done for all analytes. For certain analytes (notably THC-COOH, THC-COOH-GLU and 6-monoacetylmorphine) the cut-off concentrations are nearer the low end of the respective calibration curve, and placement of the low and high QCs was adjusted appropriately.

3.4. Carry-over

Carry-over was assessed by injecting the highest calibration standard repeatedly, followed immediately by a blank urine sample. The blank sample was then examined, and carry-over calculated and expressed as a percentage (based on peak area) of the lowest calibration standard. For all analytes, the area of any peaks detected in the blank was less than 7.8% of the LLOQ peak, except for benzypipierazine, which had a peak representing 18.2% of the LLOQ peak. This study showed that the maximum carry-over effect did not affect the assay at the reporting level of any analyte.

Table 8
Within run accuracy and precision.

Compound	+25% QC		−25% QC	
	CV%	% Dev	CV%	% Dev
6-Monoacetylmorphine	6.41	−2.6	3.78	−1.4
7-Aminoclonazepam	6.70	−6.0	6.53	−4.4
7-Aminoflunitrazepam	5.44	−2.8	6.21	−2.9
7-Aminonitrazepam	4.40	5.0	5.84	5.2
Amphetamine	4.49	−1.4	5.47	−0.3
α-Hydroxyalprazolam	1.76	−5.9	3.62	−3.6
Benzoylecgonine	5.80	1.6	0.84	2.2
Benzylpiperazine	6.25	−0.7	1.49	2.6
Codeine	3.80	−1.9	0.82	1.0
Codeine-6β-D-glucuronide	2.81	−2.4	2.56	2.2
Diazepam	4.57	−3.0	1.91	2.0
Ecgonine methylester	3.76	−6.7	0.93	−4.4
EDDP	5.24	0.7	0.51	2.7
Pseudoephedrine	4.79	−3.5	1.60	−4.1
MDA	3.63	−2.5	4.35	−1.3
MDMA	3.95	−2.2	1.70	−0.2
Methadone	5.40	−1.6	0.42	2.0
Methamphetamine	3.41	−4.9	2.28	−1.3
Morphine	4.17	−2.2	4.93	−0.2
Morphine-3β-D-glucuronide	6.60	4.3	5.62	5.6
Morphine-6β-D-glucuronide	3.65	−4.3	0.95	1.0
Nordiazepam	7.91	3.0	3.00	6.9
Oxazepam	5.73	−0.9	0.75	3.4
Phentermine	4.67	−5.0	1.88	0.2
Temazpam	5.88	2.0	1.76	1.9
THC-COOH	4.40	−7.3	4.84	−1.8
THC-COOH-GLU	2.87	−3.9	6.21	4.0

3.5. Matrix effects

A study was done in various sources of urine (not pooled) in order to assess whether or not normal variations in human urine affected the quantitative integrity of the assay. The study was done by preparing calibration standards at high (ST2), medium (ST3) and low (ST4) concentration. These calibrators encompassed the reporting concentration for all analytes (see Tables 1 and 2). The study was done by preparing the said three calibrators in each of the five discrete sources of drug free human urine (a total of 15 samples). In analytical literature, a high degree of variation in inter-matrix analyte peak area is accepted as an indicator of the presence of matrix effects [12]. In order to assess imprecision, the CV% was calculated for each of the three calibration levels, and across the five sources of human urine, based on the peak area ratio (analyte peak area/ISTD peak area). The data for this study are shown (Table 9). From the data it is apparent that there are, to varying degrees, matrix effects that affect production of ions in the electrospray source (the point at which matrix effects manifest), and 6-acetylmorphine is the analyte most affected by this phenomenon. However, from the peak area ratio (PAR) data, it is clear that the use of isotope labelled internal standards compensates acceptably for these effects, and in particular at the reporting concentration for each analyte.

3.6. Calculation of concentrations for unknown samples

In order to revert phase II metabolites (predominantly glucuronides and sulphates) back to unconjugated species prior to measurement, laboratories generally subject opiate and cannabis samples to either enzymatic cleavage, or acid/base hydrolysis prior to preparation. Owing to the fact that it was possible to obtain certified reference materials for all the significant metabolites of these compounds, urinary levels are calculated as follows:

- Total morphine = morphine + morphine-3β-D-glucuronide + morphine-6β-D-glucuronide
- Total codeine = codeine + codeine-6β-D-glucuronide
- Total THC = THC-COOH + THC-COOH-GLU

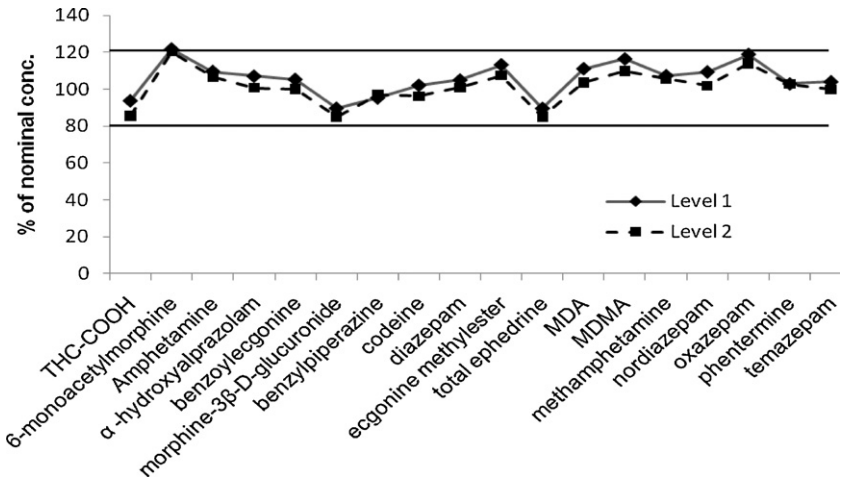


Fig. 6. Accuracy of UTAK external quality controls measured in-house.

Table 9
Summary of matrix effects (CV%), based on peak area ratio (analyte/ISTD).

Compound	ST2	ST3	ST4
6-Monoacetylmorphine	20.8	10.5	12.7
7-Aminoclonazepam	8.4	6.4	2.8
7-Aminoflunitrazepam	4.0	2.5	3.2
7-Aminonitrazepam	7.3	5.2	5.2
amphetamine	6.6	5.8	3.7
α-Hydroxyalprazolam	5.2	11.7	7.7
Benzoylecgonine	6.3	5.4	5.1
Benzylpiperazine	1.9	4.0	2.0
Codeine	1.8	4.5	1.6
Codeine-6β-D-glucuronide	8.9	11.8	3.9
Diazepam	3.2	2.5	3.7
Ecgonine methylester	1.4	2.6	1.9
EDDP	1.3	2.3	1.4
Pseudoephedrine	2.1	6.0	5.1
MDA	3.0	6.3	5.9
MDMA	3.0	3.3	5.1
Methadone	5.8	2.7	1.6
Methamphetamine	6.5	2.6	1.6
Morphine	2.7	4.1	3.5
Morphine-3β-D-glucuronide	4.5	5.1	4.7
Morphine-6β-D-glucuronide	3.1	2.1	5.6
Nordiazepam	5.8	2.4	4.0
Oxazepam	2.2	3.0	4.4
Phentermine	5.0	4.7	2.0
Temazpam	1.9	3.7	2.9
THC-COOH	2.2	1.2	1.3
THC-COOH-GLU	3.0	3.1	3.1

For benzodiazepines that contain a hydroxyl moiety (oxazepam, temazepam and α-hydroxyalprazolam), a similar approach is appropriate owing to the fact that these compounds exist to a considerable degree as conjugated species in urine. However, it is currently difficult to source certified reference materials for oxazepam glucuronide, temazepam glucuronide and α-hydroxyalprazolam. For this reason, these samples are currently still subjected to enzymatic cleavage prior to preparation (see Section 2.5.3).

3.7. Study on adsorptive losses of cannabis related compounds

As described in Section 2.4.3, aliquots (100 μL) of urine quality controls (prepared in-house) were placed in 1.8 mL snap-cap tubes upon preparation, and these tubes were used *in situ* during the assay (no sub-aliquot taken, but tube used directly). During the development phase of the assay, a study was done which compared this approach to the traditional approach of taking a sub-aliquot of a thawed quality control on the day ($n = 3$). Importantly, this experiment was done on a single batch of prepared QCs. Following preparation, three additional aliquots were prepared in which 1 mL urine was placed in the 1.8 mL snap-cap tube, and a 100 μL sub-aliquot was taken on the day of assay and placed in a fresh sample tube. Results for the two approaches were compared and results are shown in Table 10.

The data show that exposing aqueous quality controls to a single additional surface resulted in adsorptive losses for both analytes. While THC-COOH-GLU losses are significant, they are somewhat less than for THC-COOH, and this seems appropriate, as GLU conjugation increases hydrophilicity, and thus a smaller fraction lost on the adsorptive surface. When a significant fraction of methanol is added directly to the QC tube (as described in the dilution procedure, Section 2.5.2), the compounds appear to desorb from the plastic surface of the tube. It is for this reason that the diluents are added directly to the QC tube. This way any THC-COOH and THC-COOH-GLU that has adsorbed from the 100 μL aliquot onto the surface of the QC tube over time is re-dissolved. The final diluted sample described in Section 2.5 contains around 38% methanol. Losses of THC related compounds to soft plastic materials has been previously described in the literature. The data above show the effect of exposure to a single additional surface only. While it is not possible to extrapolate this to different tube types, the data illustrates the complexities of measuring THC related compounds in aqueous medium. The implication on measurement of samples should be considered, as collection devices used in the field may vary by size and material type. Moreover, dividing of a sample into primary and a referee samples at the site of collection may result

Table 10
Study on adsorptive losses of THC-related compounds in urine.

Compound	Level	Mean ($n = 3$) of *in situ* aliquot (100 μL)	Mean ($n = 3$) of Sub-aliquot (100 μL taken from 1 mL)	Adsorptive loss (%)
THC-COOH	High	16.6	10.7	−38
	Low	11.4	6.8	−40
THC-COOH-GLU	High	18.2	13.0	−29
	Low	11.7	8.8	−25

in losses based on variable surface exposure in each of the two containers. Adding methanol directly to containers to circumvent adsorption (as is done for QCs) is not feasible for samples for myriad reasons. Sub-aliquots of samples taken in a laboratory for assay are necessarily taken from an entirely aqueous sample, where adsorptive losses are most acute. It is thus reasonable to suggest that levels of THC-related compounds in urine are generally underestimated due to adsorptive losses, but owing to the number of variables, it is not possible to make a meaningful estimation of the losses.

3.8. Cross-validation of methodology

Following the development and validation of the methodology, it was deemed prudent to compare the newly developed method with an external reference. For this reason, a set of two commercially prepared controls were purchased (UTAK, Valencia, CA), containing most of the target compounds near the reporting levels of AS/NZS 4308:2008. These external quality controls were re-suspended as per the manufacturer instructions, and assayed. The UTAK level 1 and level 2 quality control back-calculated results were then compared with the nominal concentrations supplied by the manufacturer. Accuracy was expressed as a percentage of the expected nominal concentration, and the results are summarised in Fig. 6. The data show that the methodology produces results consistent with the expected concentrations in the external quality controls. While it should be stated that certain key compounds are not present in the UTAK quality controls (notably the 7-amino benzodiazepines, and selected opiate metabolites), the compounds that are included make these external quality controls a useful external reference check.

4. Comments/conclusions

The objective of the authors was to develop a robust and rapid assay procedure for the quantitation of all drugs appropriate to the AS/NZS 4308:2008, and this was achieved. While the trap and flush approach used is not entirely novel to drugs of abuse, this is, to the authors' knowledge, the first paper dealing with all the drugs pertaining to the standard in a single assay and run. The approach described has enabled rapid response to client needs.

The trap and flush approach employed results in considerable ongoing cost benefit to the laboratory. While adding switching valves to the HPLC system did add some initial cost, a single trap cartridge (capable or processing at least 100 samples) was more cost effective than using the conventional single use SPE cartridge.

It should be said that key to the success of the assay is the availability of isotope-labelled internal standards for all but a few of the compounds.

Acknowledgements

We thank Wendy Ferguson for the generation of immunoassay data during method development, as well as Michael Henman for help with accuracy and proofreading. We also express our thanks to the Mater Adults Hospital for the resources and funding made available for this project.

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