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LC–MS/MS method for the quantitation of metabolites of eight commonly-used synthetic cannabinoids in human urine – An Australian perspective

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ABSTRACT

An LC–MS/MS method for the quantitation of urinary metabolites of eight JWH-type synthetic cannabinoids (SCs) has been developed and validated. Urine samples are subjected to deconjugation using β-glucuronidase, followed by a solvent extraction procedure. Compounds are separated on a reverse-phase HPLC column within a 14 min cycle. Low assay limits are required in order to demonstrate prior exposure to SCs. Matrix effects were studied and proved to be significant for selected analytes, and were challenging to circumvent as isotope-labeled internal standards are not available. An elimination profile from a naïve user following a single smoke of “Kronic” was constructed, showing urinary excretion over 2–3 days with peak concentrations of different metabolites 3–16.5 h after smoking. This method has been developed to process several hundred samples within a high-throughput drugs of abuse laboratory, with growing evidence that the use of synthetic cannabinoid blends is common within the Australian workforce.

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1. Introduction

The Chinese emperor Shen-Nung was first to mention in his writings (2737 BC) the benefits of cannabis, noting its properties against malaria and rheumatism [1]. Throughout history, its use as a narcotic that produces tranquility has been well known. There are several active constituents in cannabis, but the effects are primarily attributed to Δ^9-tetrahydrocannabinol (THC), a known agonist of cannabinoid receptors types 1 (CB_1) and 2 (CB_2). CB_1 is highly expressed in the CNS. Interaction with this receptor is understood to be responsible for the euphoria, appetite stimulation and pain-relieving properties associated with cannabis use [2–4].

In modern times, cannabis has remained one of the most frequently used illicit drugs in the Western world [1], and as such, analytical techniques relating to the detection of cannabis use have enjoyed much attention and as a result are well understood.

Following the isolation of THC in 1964 [5] and discovery of CB_1 and CB_2 in the 1980s [6], a large number of cannabinoid receptor agonists have been synthesised in an effort to exploit their antiemetic, analgesic, appetite modulating and anti-glaucoma effects for therapeutic purposes. In the 1990s, Huffman et al. synthesised a large number of naphthoylindoles, naphthoylpyrroles and related compounds with cannabinoid receptor agonist activity, which have become known as the ‘JWH compounds’ [7].

Regrettably, the potential market for products that produced cannabis-type euphoria (with the added benefit of not being prohibited or readily detectable) was swiftly appreciated. In Europe, mixtures of dried herbs laced with two of the compounds first synthesized by Huffman (JWH-018 and JWH-073) appeared for sale on the Internet and in head shops, marketed as herbal incense. At the same time, discussions surrounding the highs that could be experienced through smoking these herbal mixtures became increasingly visible on the Internet, particularly on ‘drug forums’, giving rapid momentum to their popularity.

Cannabinoid receptor agonists added to herbal blends are not limited to JWH-type compounds (naphthoylindoles and phenylacetylindoles). A number of other synthetic cannabinoid sub-classes have also been detected, such as classical cannabinoids (e.g. HU-210 and nabilone) and cyclohexylphenols (e.g. CP47,497 and CP47,497 C8).

Demonstration of an individual’s exposure to herbal blends containing SCs (such as ‘SPICE’, ‘K2’ and in the case of Australia ‘KRONIC’) poses a number of challenges from an analytical perspective. Having observed the phenomenon in Europe and America, it is clear that what needs to be measured in biological matrices will always be a moving target.

Auwärter et al. analysed so-called herbal incense blends available in Germany at regular intervals, and report that not only are the active constituents varied within the blends, but that even within individual products marketed under a single brand,

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changes in active constituents were observed. This was attributed to a response by manufacturers to circumvent legal control in Germany. It was reported that not only were blends fortified with SCs, but in some cases with other compounds including *O*-desmethyltramadol, caffeine and oleamide. Conversely, in some of the blends studied, no measurable levels of SCMs could be detected [8].

In Australia, the use of synthetic cannabinoids has become a significant workplace health and safety issue, particularly in the mining industry [9]. This has resulted in a need for SC testing to be developed for use in random occupational drugs-of-abuse screening. A similar legislative reaction to that which took place in Europe and the United States took place in Australia in 2011, with different Australian states prohibiting small numbers of specific SC compounds in an ad hoc fashion [10].

Within our own Australian laboratory, a number of herbal mixes ("Kronic" blends) were purchased online in June 2011 and analysed in-house for synthetic cannabinoids. JWH-018 and/or JWH-073 were found in all five blends tested, with one blend found to also contain JWH-250. Following an interval of three months, during which legislation to ban some JWH compounds other SCs was enacted in various states, a further blend was purchased locally and found to contain two synthetic cannabinoids unknown to us. Consultation with Cayman Chemicals (Ann Arbor, Michigan, USA [11]) revealed that the mass spectra captured for these two compounds were highly likely to be those of JWH-022 and AM2201, which are known 2nd generation synthetic cannabinoids being identified by crime laboratories in the US [Cayman Chemicals, personal communication].

The choice of biological matrix is an important analytical consideration. In a paper dealing with the measurement of selected SCs in serum, Dresen et al. demonstrate that parent SCs (non-metabolised) can be readily measured in serum following exposure. This work was done within hospitals, psychiatric and detoxification centres, and suggests that SCs are being widely used by subjects undergoing withdrawal treatment. Once again, this paper elegantly illustrates how manufacturers are tailoring products to the appropriate legislation: the study was undertaken after JWH-073 and JWH-018 had been banned in Germany and neither was detected in any samples tested ($n = 100$), while JWH-081 and JWH-250 (uncontrolled at the time of the study) were the two most prevalent compounds detected [12].

As a matrix of measurement, serum has an inherent advantage. Dresen et al. show that it is generally appropriate to measure the JWH-type SCs directly (not as metabolites). This is beneficial because what is measured in the herbal blends can in turn be measured in the serum. This is significant as it is generally possible to obtain certified drug standards for these compounds, which means that it is possible produce analytical methodology that conforms to the accepted international norms. Similarly, Kacinko et al. [13] measured JWH-073, JWH-018, JWH-019 and JWH-250 in whole blood, but owing to significant matrix effects, JWH-019 was not reported quantitatively. However, in workplace drugs-of-abuse screening, serum is not a viable matrix of measurement for routine screening, and it is necessary to turn to urine (and in some cases oral fluid) as the preferred matrix.

In early work on SCs, using high resolution mass spectrometry and collision induced-dissociation (CID) Thevis et al. [14] demonstrated that, as opposed to serum, no intact JWH-018 can be measured in urine following exposure. Sobolevsky et al. [15] reported on metabolites of JWH-018 and confirmed this finding. Similarly Auwärter et al. [16] reported on metabolites of several SCs found in herbal blends and show that hydroxylation and carboxylation are common Phase I biotransformations prior to conjugation. While screening for SC metabolites in urine is likely to provide a longer detection window than would serum or oral fluid screening for parent drug, the availability of certified standards of known urinary SC metabolites is currently limited. Several urinary metabolites of JWH-073 and JWH-018 have been published and reference standards manufactured [17]. Certified standards for putative metabolites of newer SCs are made available as they are developed (Cayman Chemicals) and one of the challenges of keeping a screening test up to date will be the incorporation of new metabolite standards as the SCs in herbal blends change.

In this paper, we focussed on the simultaneous determination of a series of metabolites of JWH-type substances. While not rapid, the solvent extraction procedure developed was deliberately 'generic' and was aimed at future extension to additional analytes, as new cannabinoid receptor agonists begin to appear in herbal blends.

2. Materials and methods

2.1. Materials and reagents

All certified drug and isotope-labeled internal standard solutions were purchased from Cayman Chemicals (Ann Arbor, Michigan, 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). β-Glucuronidase (*H. pomatia*/arylsulfatase) was purchased from Roche (Penzberg, Germany). Deionised water (18.1 MΩ) was produced by a Millipore-Q water system (Bedford, MA, USA).

2.2. Instrumentation

The HPLC system used was a Shimadzu Prominence UFLC system (Kyoto, Japan). A Shimadzu LC-20AB high-pressure pump was used for solvent delivery. The autosampler was a SIL-20AHT. The column oven was a CTO-20A. An in-line degasser (DGU-20A_3) was placed prior to the solvent delivery system.

The LC–MS/MS system used was an ABSciex 5500QTRAP, with a TurboIonSpray® source (ABSciex, Concord, Ontaria, Canada). All data were collected using ABSciex Analyst software (version 1.5). Quantitation was performed using MultiQuant version 2.1 software (ABSciex).

Creatinine was measured on an Abbott Architect c8000 using a modified Jaffe method (Abbott Laboratories, Illinois, USA).

2.3. Preparation of calibration standard solutions

Certified reference materials (Cayman Chemical) were reconstituted in organic solvent as per the manufacturer's instructions. These stock solutions were then used to prepare a range of methanolic calibration standards, using a combination of volumetric and serial dilution (see Table 1).

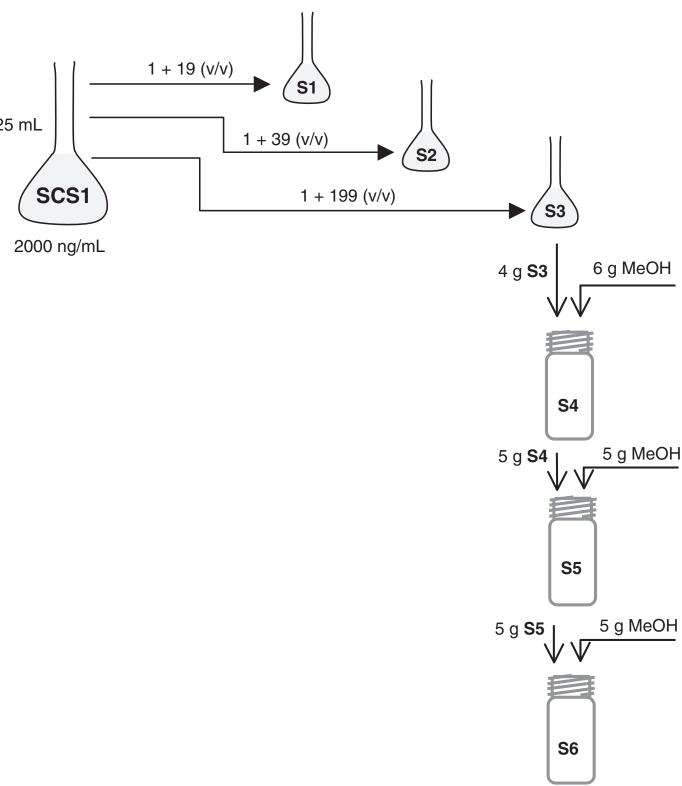
2.4. Preparation of internal standard working solution

Only two isotope-labeled internal standards were commercially available at the time of method development. It is not unexpected that this had negative quantitative implications for selected analytes. This is discussed in Section 4.6.

JWH-018-N-pentanoic acid metabolite-d_4 and JWH-018-5-hydroxyindole metabolite-d_4 were reconstituted in methanol as per the manufacturer's instructions. Aliquots of these solutions

Table 1
Preparation of methanol calibration standard solutions.

- Solvate Cayman materials with methanol (all compounds* at 500 μg/mL).
- Spike 100 μL of each of the above into a single 25 mL volumetric flask, and make up to the mark with methanol. This results in SCS1, effectively containing 11 compounds (see Table 3) at 2000 ng/mL.
- S1, S2 and S3 are produced volumetrically, while S4, S5 and S6 are produced gravimetrically, according to the procedure below:



Name	Conc. in solution (ng/mL)	Effective urine conc. (ng/mL)
ST1	100	10
ST2	50	5
ST3	10	1
ST4	4	0.4
ST5	2	0.2
ST6	1	0.1

were then combined and diluted in methanol to a working concentration of 20 ng/mL.

2.5. Preparation of ex vivo urine quality controls

A methanol stock solution, containing all analytes at 2 μg/mL was prepared. 20 μL of this stock solution was then made up to 10 mL using blank urine. This resulted in the level 1 QC, with a nominal concentration of 4 ng/mL. A portion of the level 1 QC was then diluted 1 + 3 with blank urine, resulting in the level 2 QC (1 ng/mL). When these freshly prepared QCs were assayed, it was not possible in all cases to achieve the expected nominal concentration, and some losses were observed. Possible reasons for this are discussed in Section 4.2.

2.6. Use of an incurred sample as a quality control

While ex vivo quality controls are useful in assessing day-to-day performance of most aspects of the assay, one notable exception is the inability to monitor the enzymatic cleavage of glycones of the SCM metabolites. To the authors' knowledge, no certified reference materials for conjugated forms of SCMs are commercially available. For this reason a urine sample from an individual who had smoked a blend containing JWH-018, JWH-073 and JWH-250 on a single

occasion was used as an additional quality control during validation, as well as for subsequent sample runs. This incurred sample was collected approximately 7.5 h after use of the blend.

2.7. Preparation of B-glucuronidase/arylsulfatase solution

An aqueous acetate buffer was prepared by adjusting a 200 mM ammonium acetate solution to pH 5.5 using glacial acetic acid. A β-glucuronidase/aryl sulfatase solution was then prepared by combining 0.5 mL enzyme with 9.5 mL acetate buffer. The purpose of this solution was not only to ensure optimum enzyme (β-glucuronidase) activity, but also to control urine pH prior to solvent extraction. This solution was prepared freshly with each batch and not stored. It should be noted that to the authors' knowledge, there are not significant levels of SC sulfate metabolites present in urine, and the developed protocol was aimed at maximal β-glucuronidase activity, without any consideration for the arylsulfatase.

3. Sample preparation and data acquisition

Sample preparation was by way of solvent extraction. Details of the procedure are shown in Table 2.

3.1. Chromatographic conditions

The analytical column was a Thermo AQUASIL C18, 100 mm × 2.1 mm, 5 μm (San Jose, CA, USA). This was fitted with a Thermo AQUASIL C18 5 μm 10 mm × 2 mm drop-in guard cartridge. The aqueous mobile phase (phase A) consisted of 5 mM ammonium acetate in water, while the organic mobile phase (phase B) consisted of 5 mM ammonium acetate in methanol:acetonitrile (1:1; v/v). The analytical column was maintained at 55 °C, and the flow rate was 270 μL/min. The gradient settings, as well as the resulting chromatogram are shown in Fig. 1.

3.2. Mass spectrometer conditions

The mass spectrometer used was an ABSciex 5500QTRAP (Concord, Ontario) fitted with an ESI (TurboIonSpray^{TM}) source. Using the diverter valve integrated on the mass spectrometer, the first 2 min of chromatographic eluent was diverted to waste. All analytes were ionised in positive mode. It is important to note that owing to structural similarities of many SC metabolites, there are a number of instances in which even two selected reaction monitoring (SRM) experiments (qualifier and reporter ion) are not sufficient to unambiguously identify the SC metabolite (see Table 3), and retention time plays an important role in the identification of the particular SCM.

3.3. 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 2.1 (ABSciex). Following integration, XML scripting (integral to MultiQuant) was used to interrogate data, and automatically produce a list of samples that were above the in-house reporting threshold. The in-house reporting threshold was set above the validated lower limit of quantification (LLOQ) (100 pg/mL) for all analytes, based on the uncertainty of measurement (UM) (100 pg/mL + UM, see Table 4). Uncertainty of measurement calculations deliberately incorporated data from the matrix effects study, because the intended application of the method was to analyse donor urine samples:

$$CV_{TOTAL} = SQRT[(CV_{M.E.})^2 + (CV_{V.f.})^2 + (CV_{Pipette})^2]$$

where $CV_{M.E.}$ is the matrix effects run CV%, based on LLOQ in multiple sources of urine; $CV_{V.f.}$ is the variation data calculated from volumetric flask performance certificate; $CV_{Pipette}$ is the allowable performance limits of positive displacements pipettes used.Following this, the 95% C.I. was calculated as follows and applied to samples:

$$1.96 \times STDEV = 95\%\,C.I. = \frac{CV\% \times \text{nominal conc.}}{100} \times 1.96$$

In addition to being above the applied limit, samples were required to be within ion ratio requirements (±30% of calibration standards) and have an acceptable retention time (±2% of calibration standards).

4. Results and discussion

4.1. Linearity

The linearity of the assay was established between the highest (10 ng/mL) and the lowest (0.1 ng/mL) calibration standard for all analytes. 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 [18,19]. The data showed that a quadratic curve fit (no weighting) was the simplest and most rugged fit for all compounds.

4.2. Between-run accuracy and precision based on QCs

Between run accuracy and precision was calculated from urine QC data over three occasions (each on a different day). On each of the three occasions, QCs at each level (level 1 = 1 ng/mL, level 2 = 4ng/mL) were included in triplicate. The summary data are shown in Table 5. Precision is expressed as CV%, and accuracy is expressed as mean % deviation from the nominal concentration of the QC.

Table 5 shows a negative bias (accuracy) for a significant proportion of analytes. Interestingly, the bias appears to affect the metabolites with hydroxyl moiety, but not those containing a carboxyl moiety. While the reason for this bias is not fully understood, it is speculated that it may be caused either by differences in aqueous solubility related to the two moieties (QCs were prepared in urine), or alternatively differences in adsorption to plastics under aqueous conditions. QCs were prepared on more than one occasion (data not shown), and this phenomenon (negative bias) was found to be reproducible.

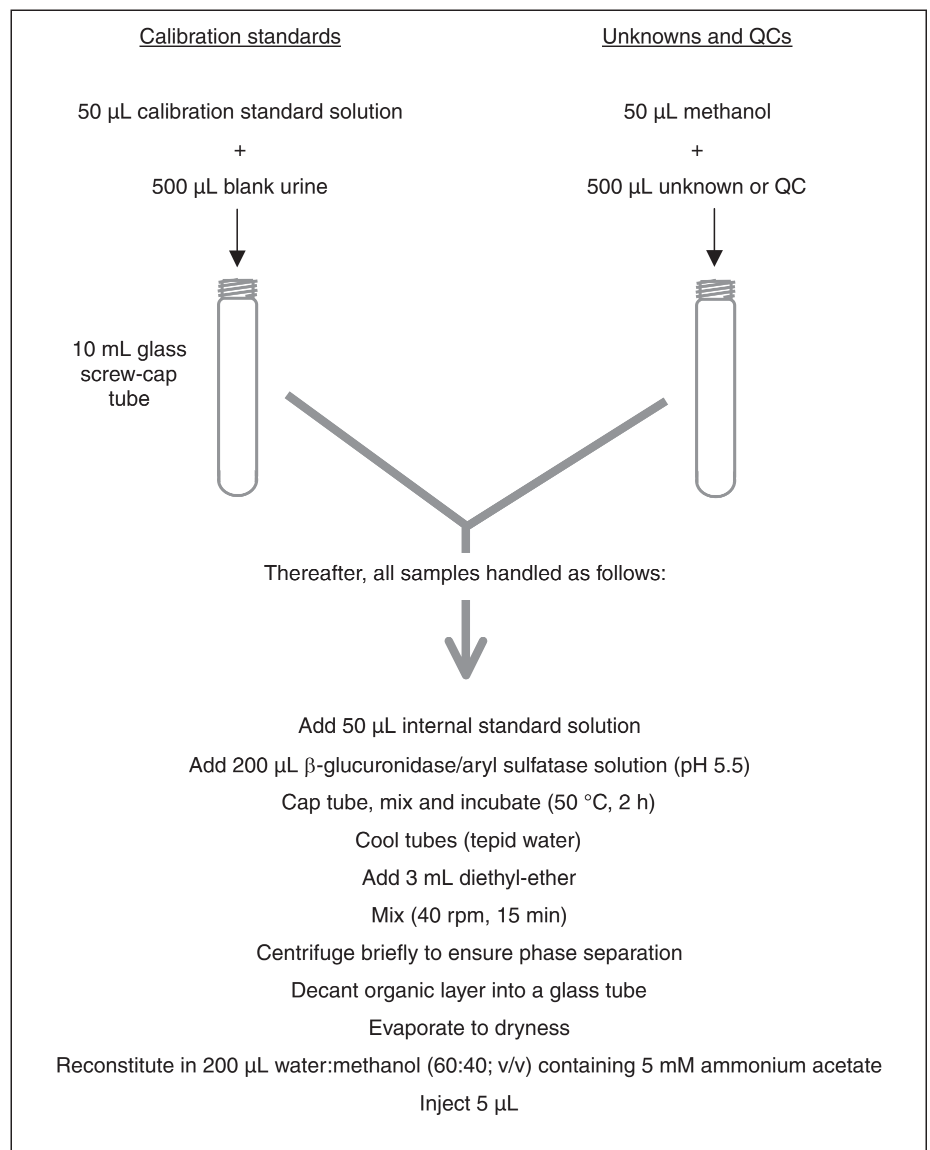
4.3. Within-run accuracy and precision based on QCs

Similarly, within run accuracy and precision were calculated from urine QCs on a single occasion (5 replicates at each QC level). The data are shown in Table 5. Precision is expressed as CV%, and accuracy is expressed as mean % deviation from the nominal concentration of the QC.

4.4. Within-run precision based on an incurred sample

As discussed in Section 2.6, ex vivo QCs do not fully represent incurred samples. Most notably, precision and accuracy calculations do not encompass the de-conjugation procedure. During method development, it was found that the addition of β-glucuronide to clinically incurred samples (see Section 4.7) prior to extraction significantly increased the instrument response for SCMs. From this it was concluded that the three SCMs contained in these samples (see Fig. 2A and B) were significantly conjugated in urine. Based on the structural similarities of JWH-type SCs, the authors speculate that the other SCMs described in this

Table 2
Sample preparation protocol.



paper also undergo conjugation. In keeping with this, Auwärter et al. [12] subjected all urine samples to de-conjugation prior to solvent extraction.

Because there are no certified materials available for glycones of these metabolites, it is not possible to accurately determine the absolute efficiency of the deconjugation procedure. However, it is prudent to have an understanding of the reproducibility of the assay, when used on real samples.

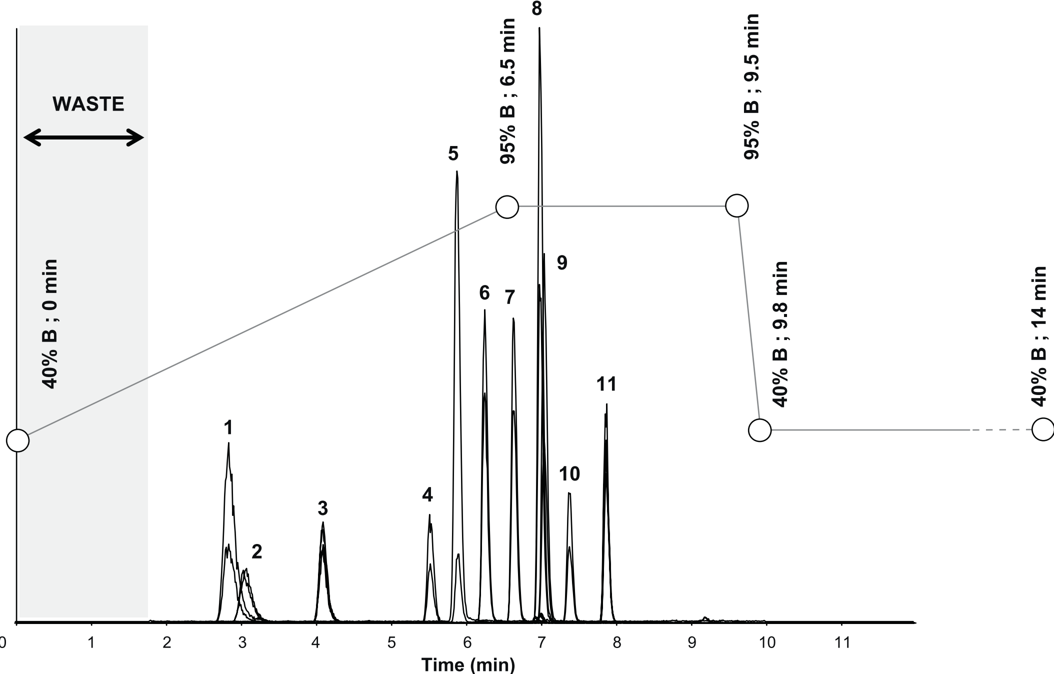
An incurred sample, known to contain metabolites of JWH-018, JWH-073 and JWH-250, was assayed in triplicate, on three separate days. The results of this study are shown in Table 6. The reproducibility (CV%) of the back-calculated concentration is consistent with the between-run precision (Section 4.2) based on QCs, and it was concluded from the results that the deconjugation procedure does not contribute significantly to the imprecision of the assay, based on the 5 compounds detected in the incurred sample.

4.5. 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. For all analytes, no peaks were observed in the blank sample. This study showed that the assay was not affected by carry-over, when samples were found to be within the calibration range. However, as a precaution, if a sample was found to contain SCMs at a level above the top calibration standard (10 ng/mL) within a batch, the sample immediately following the high sample was re-injected post-batch, following a blank urine extract, in order to eliminate any carry-over effects that could occur at this untested level.

4.6. Matrix effects

In analytical literature, a high degree of variation in inter-matrix analyte peak area is accepted to be an indicator of the presence of matrix effects [20]. It is generally accepted that the use of isotope-labeled internal standards is the most effective way to minimise matrix effects. For SCMs however, there is a very limited range of isotope-labeled internal standards available, and out of necessity, the assay was developed and validated using only two (see Table 3). The selection of which of the two internal standards to use for an individual analyte was based on the proximity of the



Mobile Phase A : 5 mM ammonium acetate in water

Mobile Phase B : 5 mM ammonium acetate in methanol : acetonitrile (1:1 ; v/v)

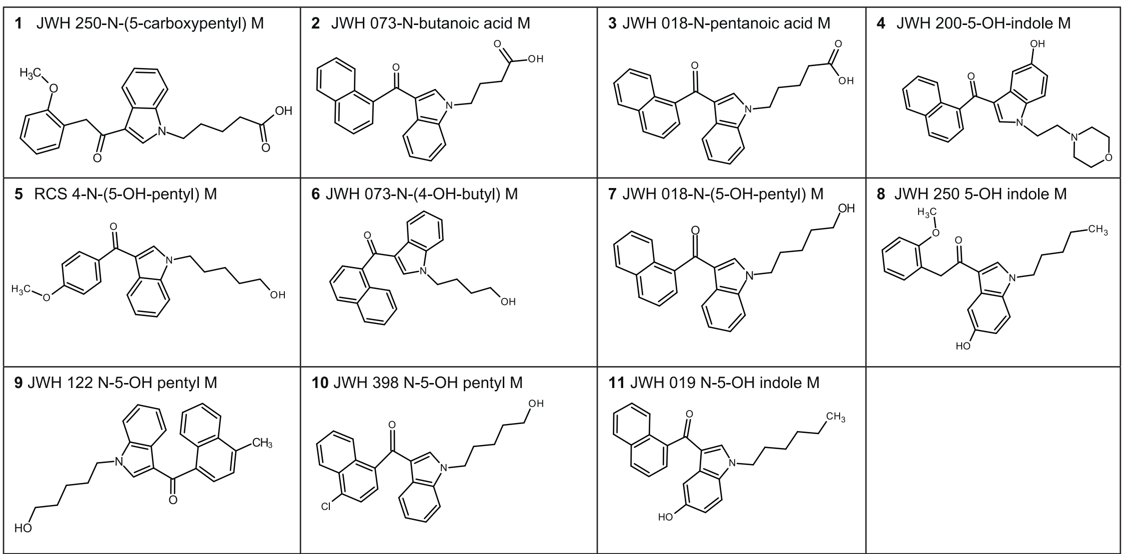


Fig. 1. HPLC gradient and resulting chromatogram for synthetic cannabinoid metabolites.

retention times. While this is not ideal, it was a necessary compromise. Effectively only one of the twelve analytes is quantitated using an isotope-labeled internal standard.

Therefore, in order to minimise matrix effect, it was decided at the outset of method development to optimise a solvent-extraction procedure. Although solvent extraction cannot be readily automated and requires the handling of volatile substances, it was chosen above solid-phase extraction because, in our experience, it produces cleaner extracts. In the absence of isotope-labeled internal standards, starting with the cleanest possible sample extract is an important factor in the amelioration of matrix-based variation in ionisation efficiency.

To assess the effect of the extracted sample matrix on assay results, a comparison was performed between 5 replicates of a single sample of spiked urine and spiked urine specimens from 5 different sources. The urines were spiked with low concentrations of metabolites of JWH-018, JWH-073, JWH-398, JWH-250, JWH-122, JWH-019, JWH-200 and RCS-4. Results are shown in Table 7. From the data it is apparent that there are, to varying degrees, matrix effects present, as in some cases there are marked differences in assay reproducibility between the single urine and the multiple urine data. Notably, the JWH-200-OH-indole metabolite was the poorest performer, with a CV% of 27.8% across multiple urines, but a CV% of only 2.5% in a single source of urine, signifying significant matrix effects for this analyte in particular. On order to compensate for this matrix-based imprecision, an adjustment was made to the LLOQ (see Section 3.3), based on the uncertainty of measurement (UM) calculation.

Table 3
Compound parameters, showing transitions for reporter and qualifier ions.

Compound		Q1 (m/z)	Q3 (m/z)	R Time (min)	CE (eV)	CXP (V)
JWH 073 -N-butanioc acid metabolite	*	**358.1**	**155.0**	**2.8**	29	18
		358.1	**127.0**		67	20
JWH 018 –N-5-OH-pentyl metabolite	*	**358.1**	**155.0**	**6.5**	29	18
		358.1	**127.0**		67	20
JHW 073 N-4-OH butyl metabolite		344.1	155.1	6.2	29	12
		344.1	127.1		61	6
JWH 018 -N-pentanioc acid metabolite	*	**372.1**	**155.1**	**3.8**	29	12
		372.1	**127.1**		67	22
JWH 398 –N-OH-pentyl metabolite		392.2	189.1	7.3	35	11
		392.2	161.1		60	11
JWH 250-5-OH-indole metabolite		352.1	121.1	6.9	29	12
		352.1	91.0		65	14
JWH 122 N-5-OH -pentyl metabolite		372.3	169.1	7.0	30	11
		372.2	141.1		55	11
JWH 250 N-pentanoic acid metabolite		366.1	121.1	2.6	27	11
		366.1	91.1		55	11
JWH 019 OH-indole metabolite	*	**372.2**	**155.1**	**7.8**	36	11
		372.2	**127.1**		60	11
RCS-4 N-OH-pentyl metabolite		338.1	135.0	5.8	29	12
		338.1	91.9		89	14
JWH 200 5-hydroxyindole metabolite		401.1	155.0	5.4	29	18
		401.1	127.0		73	12

Source parameters (used for all compounds)

Parameter	Value
Polarity:	Positive
Ionisation voltage (IS):	4800 V
Nebulising gas (GS1):	50
Desolvation gas (GS2):	60
Desolvation gas temperature (TEM):	650℃
Curtain gas (CUR):	30
Declustering potential (DP):	160 V
Entrance potential (EP):	10 V
Collision gas (CAD):	Medium

[*] Compounds for which it is not possible to distinguish between, based on SRM alone (retention time required for differentiation).
Bold indicates analytes for which MRM transition alone is not sufficient to guarantee specificity.

4.7. Application of method to a series of samples

The developed method was used to analyse a series of urine samples produced by a drug-naïve individual (one of the authors) who had smoked 0.15 g of the herbal blend Kronic, known to contain JWH-073 and JWH-018, before synthetic cannabinoids were prohibited in Australia. The absolute amount of compound present in the herbal blend used is not known, and it is thus not possible to state the exact dose. Urine was collected prior to use (0 h), and then at 1.5, 3.0, 4.5, 7.5, 11, 16, 19, 22, 24, 26.5, 31, 40, 48 and 65 h. Creatinine levels for all urine samples were measured in-house.

The results for this study are shown in Fig. 2, which shows a detection profile. These data suggest that at a LLOQ of 100 pg/mL, a detection window of 2–3 days is achievable, following a single use of a blend containing JWH-018 and JWH-073. Peak concentrations of different metabolites were seen 3–16.5 h after smoking.

Less clear is how high levels may be following larger doses of these compounds and what detection window may be appropriate following chronic use of blends containing SCs. It is not known whether or not there is SCM 'accumulation' in fatty tissues (as is the case with THC-COOH). However, experience with other samples passing through the laboratory suggests that the metabolites shown in Fig. 2 remain detectable 2–3 weeks after cessation of chronic use.

Fig. 2B suggests a role for normalisation of SCM concentration to the urine creatinine level, which may prove useful in assessing whether or not an individual has re-used a herbal blend.

4.8. Comments/conclusions

The objective of the authors was to develop a generic assay procedure for the quantitation of a range of JWH-type synthetic cannabinoids, and this was achieved. While solvent extraction

Table 4
Applied cut-off values following application of U.M. calculation.

	JWH-018-N-5-OH-pentyl metabolite	JWH-073-N-OH-butyl metabolite	JWH-018-N-pentanoic acid metabolite	JWH-073-N-butanoic acid metabolite	JWH-398-N-OH pentyl metabolite	JWH-250-Indole-OH metabolite	JWH-122-OH-pentyl metabolite	JWH-250-COOH-pentyl metabolite	JWH-019-OH-indole metabolite	RCS-4 OH-pentyl metabolite	JWH-200-OH-indole metabolite
% $RSD_{M.E.}$	11.9	15.9	3.8	8.2	3.9	11.2	12	2.9	11.4	14.8	27.8
% $RSD_{V.f.}$	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054
% $RSD_{Pipette}$	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
% RSD_{TOTAL}	11.93	15.92	3.88	8.24	3.98	11.23	12.03	3.01	11.43	14.82	27.81
Limit adjustment (pg/mL)	100 ± 23.3	100 ± 31.2	100 ± 7.6	100 ± 16.1	100 ± 7.8	100 ± 22.0	100 ± 23.6	100 ± 5.9	100 ± 22.4	100 ± 29.1	100 ± 54.5
Applied cut-off (pg/mL)	123.4	131.2	107.6	116.1	107.8	122.0	123.6	105.9	122.4	129.1	154.5

Table 5
Within-run (WR) and between-run (BR) accuracy and precision, based on quality controls.

	JWH-018-N-5-OH-pentyl metabolite	JWH-073-N-OH-butyl metabolite	JWH-018-N-pentanoic acid metabolite	JWH-073-N-butanoic acid metabolite	JWH-398-N-OH pentyl metabolite	JWH-250-indole-OH metabolite	JWH-122-OH-pentyl metabolite	JWH-250-COOH-pentyl metabolite	JWH-019-OH-indole metabolite	RCS-4 OH-pentyl metabolite	JWH-200-OH-indole metabolite
Accuracy											
BR accuracy (% dev)											
L	−21.1	−24.4	−2.0	17.4	−15.5	−26.5	−21.3	1.2	−28.2	−27.1	−3.7
H	−22.0	−27.8	−2.5	13.6	−8.5	−24.6	−22.5	−2.2	−6.4	−31.3	−12.9
WR accuracy (% dev)											
L	−20.6	−26.0	−3.1	17.5	−13.6	−26.2	−22.4	−0.1	−27.3	−34.5	−6.9
H	−19.7	−24.9	−3.1	14.9	−7.4	−21.7	−20.9	−2.7	−9.8	−34.4	−7.6
Precision											
BR precision (% CV)											
H	10.0	13.1	1.8	3.6	3.5	8.9	9.6	4.0	5.7	16.7	9.0
L	6.1	7.2	1.5	4.6	1.9	9.0	7.5	6.3	8.0	10.3	6.2
WR precision (% CV)											
H	2.0	1.0	1.9	1.6	2.2	1.6	1.2	1.4	2.5	1.9	1.9
L	1.3	0.5	1.0	1.5	1.3	2.3	1.5	2.4	2.5	0.8	1.3

L = 1000 pg/mL; H = 4000 pg/mL.

A : Urinary levels of three SCMs (pg/mL) without correction for creatinine.

B : Urinary levels of three SCMs (ng/mgcreatinine) with correction

Fig. 2. Urinary levels of three detected SCM metabolites over a 65 h time course. (A) Urinary levels of three SCMs (pg/mL) without correction for creatinine. (B) Urinary levels of three SCMs (ng/mg creatinine) with correction.

can be considered fairly laborious, the resulting extracts allowed for acceptable assay performance. The absence of isotope-labeled internal standards means that reliable quantitation can be challenging. Matrix effects should be rigorously investigated by laboratories undertaking synthetic cannabinoid metabolite assay development.

Table 6
Assay precision based on back-calculated concentrations (pg/mL) of an incurred sample.

JWH-018-N-5-OH-pentyl metabolite	JWH-073-N-OH-butyl metabolite	JWH-018-N-pentanoic-acid metabolite	JWH-073-N-butanoic acid metabolite	JWH-250-COOH-pentyl metabolite
Occasion 1				
8042	637	892	508	261
8424	660	883	515	270
8198	607	894	472	257
Occasion 2				
7765	637	897	550	271
8475	633	953	547	264
11,046	849	898	563	266
Occasion 3				
9002	713	844	600	239
8791	651	875	605	219
8917	702	881	626	226
Mean				
8740	677	891	554	252
%CV				
10.9	10.8	3.2	9.1	7.8

Table 7
Comparison of assay reproducibility (at LLOQ) in a single urine versus multiple urines.

	JWH-018-N-5-OH-pentyl metabolite	JWH-073-N-OH-butyl metabolite	JWH-018-N-pentanoic acid metabolite	JWH-073-N-butanoic acid metabolite	JWH-398-N-OH pentyl metabolite	JWH-250-indole-OH metabolite	JWH-122-OH-pentyl metabolite	JWH-250-COOH-pentyl metabolite	JWH-019-OH-indole metabolite	RCS-4 OH-pentyl metabolite	JWH-200-OH-indole metabolite
ISTD used	a	a	a	a	a	a	a	b	a	a	a
Single urine ($n=5$ replicates)	98.5	96.8	104.3	101.7	104.0	101.2	99.9	102.2	107.0	97.4	91.6
	105.8	97.6	113.9	109.3	103.7	98.9	101.8	107.2	109.0	101.7	92.7
	100.7	99.7	103.0	104.3	103.6	102.5	99.2	102.9	107.5	99.4	89.4
	100.9	95.8	102.5	105.7	99.7	95.5	95.4	101.2	105.0	92.9	87.6
	101.9	95.3	108.4	108.9	98.2	101.2	99.2	104.9	109.0	98.5	87.8
CV%	2.6	1.8	4.5	3.0	2.6	2.8	2.3	2.3	1.5	3.3	2.5
Multiple urines ($n=5$)	115.6	117.4	104.3	140.2	112.8	121.6	121.7	106.8	119.2	121.4	153.6
	94.1	84.4	99.2	130.0	104.6	94.3	96.5	101.6	113.5	94.6	95.1
	125.0	114.0	98.4	149.4	103.6	115.6	129.1	100.3	127.7	123.1	79.2
	97.4	83.8	97.2	121.8	109.2	95.9	101.9	106.7	94.5	88.2	93.1
	106.1	100.3	105.7	144.5	111.9	108.0	113.3	105.7	105.3	115.6	130.0
CV%	**11.9**	**15.9**	3.8	8.2	3.9	**11.2**	**12.0**	2.9	**11.4**	**14.8**	**27.8**

Bold indicates analytes for which MRM transition alone is not sufficient to guarantee specificity.

[a] JWH-018 5-OH indole metabolite-d9.

[b] JWH-018-N-pentanoic acid metabolite-d4.

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