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High-throughput bioanalytical method for analysis of synthetic cannabinoid metabolites in urine using salting-out sample preparation and LC–MS/MS

Enrique G. Yanes*, Dennis P. Lovett

Air Force Drug Testing Laboratory, Lackland Air Force Base, TX 78236-5310, United States

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ABSTRACT

Herbal smoking mixtures which are sold as incense or potpourri and often referred to as 'Spice' are actually inactive plant matter adulterated with alkylamino indole based synthetic cannabinoids such as JWH-018 and JWH-073. Due to the inclusion of five synthetic cannabinoids, including JWH-018 and JWH-073, as Schedule I drugs by the Drug Enforcement Agency (DEA) in March 2011, it has become necessary for forensic laboratories to develop analytical methods to test for the presence of metabolites of synthetic cannabinoids. When a new analyte of interest emerges, most laboratories strive to develop a sample preparation procedure and validate an analytical method as quickly as possible and therefore, rely on effective but time consuming traditional protocols such as solid phase and liquid–liquid extraction. This research focuses on the examination of all aspects of sample preparation and analytical method development to streamline the analysis of four urinary metabolites of JWH-018 and JWH-073. A detailed evaluation of the β-glucuronide hydrolysis step lead to the reduction of time required for hydrolysis from 1 h at 50 °C to only 10 min at room temperature. By utilizing a salting-out assisted liquid–liquid extraction (SALLE) in place of traditional liquid–liquid extraction with a volatile solvent, processing time was saved and waste was reduced. The analysis run time was also shortened to one-third of a typical published run time by utilizing UPLC with isocratic conditions in place of conventional HPLC running a gradient method.

1. Introduction

Since their introduction into the market place in 2004 as "legal highs", synthetic cannabinoids, including JWH-018 and JWH-073, have rapidly gained popularity in the United States and Europe [1]. Originally sold under the brand name 'Spice', the term Spice has become a generic term to include the entire class of "legal high" smoking blends. Spice's popularity is due to several factors: it was originally touted as a "natural high", it was not legally controlled and it was not detected in standard drug screens. THC Pharma (Germany) and AGES PharmMed (Austria) reported independently in December 2008 the presence of JWH-018 in many herbal smoking blends [2]. A direct link was established in 2010, between herbal preparations containing JWH-018 and the psychotropic effects [3]. In early 2011, the DEA placed several synthetic cannabinoids (including JWH-018 and JWH-073) on their Schedule I list making the possession, manufacture and consumption of these compounds illegal.

The popularity of Spice extended to the United States Armed Forces leading to the US Department of Defense to ban military personnel from possession or use of synthetic cannabinoids under DoD Directive 1010.4 (Drug and Alcohol Abuse by DoD Personnel) [4]. A directive from the Air Force was issued to rapidly develop a screening and confirmation analysis for synthetic cannabinoid metabolites in urine.

A survey of sample preparation and analysis of synthetic cannabinoid metabolites revealed that application of current methods [5–11] would not provide the high throughput required (2500+ samples per month) to meet the needs of the Air Force Drug Testing Lab (AFDTL). A rapid, room temperature glucuronide hydrolysis and salting-out assisted liquid–liquid extraction (SALLE) was developed as a major improvement over the conventional protocols thus allowing for high-throughput screening and confirmation of synthetic cannabinoid metabolites. This paper also describes the method validation of an UPLC–MS/MS method to quantitate four urinary metabolites of JWH-018 and JWH-073.

2. Experimental

2.1. Chemicals, reagents and standards

All solvents were LC–MS grade. Acetonitrile, methanol, and water were obtained from Fisher Scientific (Pittsburgh, PA). Formic acid and ammonium acetate (both Optima LC–MS grade) were

* Corresponding author.
E-mail addresses: enrigy@yahoo.com, enrique.yanes_santos.ctr@us.af.mil (E.G. Yanes).

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also obtained from Fisher Scientific. β-Glucuronidase (*Escherichia coli*, Type IX-A, lyophilized powder, 1,000,000–5,000,000 units/g protein) was purchased from Sigma–Aldrich (St. Louis, MO). Reference standards of metabolites and internal standards: JWH-018 *N*-(4-hydroxypentyl), JWH-018 *N*-pentanoic acid, JWH-073 *N*-(3-hydroxybutyl), JWH-073 *N*-butanoic acid, JWH-073 *N*-(3-hydroxybutyl)-*d*5 and JWH-018 *N*-(4-hydroxypentyl)-*d*5 were obtained from Cerilliant Corporation (Round Rock, TX) in ampoules as 1 mL methanol solutions at 100 μg/mL. The remaining internal standards, JWH-073 *N*-butanoic acid-*d*5 and JWH-018 *N*-pentanoic acid-*d*4, were obtained from Cayman Chemicals (Ann Arbor, MI) in screw-cap vials as either 100 μL or 500 μL methanol solutions at 1000 μg/mL. The glucuronidated metabolite, JWH-018 *N*-(5-hydroxypentyl β-D-glucuronide), JWH-073 *N*-(4-hydroxybutyl), and JWH-018 *N*-(5-hydroxypentyl) were obtained from Cayman Chemicals (Ann Arbor, MI) as solids in screw-cap vials.

2.2. Chromatographic instrumentation

The chromatographic system was a Waters Acquity UPLC (Milford, MA) consisting of sample, solvent and column managers. The solvent manager included a binary pump and the column manager was comprised of a thermostatted column compartment. Chromatographic separation was performed on a Waters Acquity UPLC HSS T3 (2.1 mm × 100 mm, 1.8 μm) column which was connected to an Acquity UPLC HSS T3 VanGuard (2.1 mm × 5 mm, 1.8 μm) pre-column. The column temperature was maintained at 40 °C. Isocratic conditions were used for the chromatographic separation of the different analytes of interest. The optimal flow rate was set at 0.6 mL/min with a sample injection volume of 10 μL. Mobile phases consisted of LC/MS grade water containing 0.1% formic acid (mobile phase A) and LC/MS grade acetonitrile also containing 0.1% formic acid (mobile phase B). In order to eliminate carryover, the following washing solutions were used during the isocratic separation: a weak wash consisting of 50% acetonitrile and 50% water; a strong wash consisting of 90% acetonitrile and 10% water; and a seal wash consisting of 10% acetonitrile and 90% water.

2.3. MS instrumentation

The mass spectrometer used in this work was a Waters tandem quadrupole detector (TQD) which was operated in the positive electrospray ionization and multiple reaction monitoring (MRM) mode for selective and sensitive detection. The applied capillary voltage was 3.3 kV. The source temperature was set at 150 °C and desolvation temperature at 450 °C. Nitrogen was used as the nebulizing gas and argon as the collision gas. The desolvation gas flow was set at 600 L/h, cone gas at 50 L/h and collision gas at 0.2 mL/min. The collision cell pressure was approximately 5.28×10^{-3} mbar. The individual cone voltages and collision energies for the different metabolite reference standards are described in Table 1 and were determined by using the automated Waters IntelliStart® application. Two MRM transitions were monitored per metabolite and one MRM transition was monitored for their respective internal standard. Dwell times were 0.032 s for all transitions. All data was processed using TargetLynx/MassLynx® software from Waters Corporation.

2.4. Reagents and standards preparation

The salting out reagent (10 M ammonium acetate solution) was prepared by dissolving 38.55 g of ammonium acetate in a 50 mL volumetric flask with LC/MS grade water (~15 mL) to give exactly 50 mL. Solution was sonicated to assist with dissolution.

The β-glucuronidase solution was prepared as 12.3 units/μL in 0.1 M ammonium acetate by mixing 615,000 modified Fishman units of β-glucuronidase Type IX-A (370 mg of 1,661,000 units/g) and 500 μL of 10 M ammonium acetate solution into a 50 mL volumetric flask. The mixture was diluted to the mark with LC/MS grade water, shaken and sonicated for a few seconds to complete the mixing. The mixture was transferred into 1.7 mL microcentrifuge tubes and stored at −30 °C until use.

Metabolite reference standards (including internal standards) were stored at −30 °C according to the manufacture's recommendations until used. All standard solutions were further prepared in LC/MS grade methanol and kept in the refrigerator until needed.

Four different working internal standard solutions (A, B, C and D) were prepared. Internal standard solution A contained JWH-073 *N*-butanoic acid-*d*5 and JWH-018 *N*-pentanoic acid-*d*4 both at 5000 ng/mL. Internal standard solution B contained JWH-073 *N*-(3-hydroxybutyl)-*d*5 and JWH-018 *N*-(4-hydroxypentyl)-*d*5 both at 5000 ng/mL. Internal standard solution C (for quality control solution preparation) contained only JWH-073 *N*-butanoic acid-*d*5 at 1000 μg/mL. Internal standard solution D (500 ng/mL for all internal standards, to be spiked into urine specimens) was prepared by combining 1.0 mL of internal standard solution A and 1.0 mL internal standard solution B, then diluting to 10.0 mL with methanol. An additional stock solution without internal standards containing only a mixture of four metabolites at 2000 ng/mL was also prepared.

Five calibrator solutions (containing the four metabolites and four internal standards) were prepared to be spiked into negative urine and then extracted in the same manner as sample specimens. The three highest spiking calibrator solutions (2000, 1000 and 500 ng/mL) were prepared by mixing appropriate volumes (200, 100 and 50 μL, respectively) of individual metabolite reference standards with 1.0 mL of internal standard solutions A and B in a 10 mL volumetric flask. Methanol was added to make up the final volume. The two lowest spiking calibrator solutions (200 and 20 ng/mL) were prepared by mixing either 1.0 mL or 0.1 mL of the 2000 ng/mL stock solution with 1.0 mL of internal standard solutions A and B in a 10 mL volumetric flask and diluting to a final volume with methanol.

The QC solution (500 ng/mL) was prepared by transferring 50 μL of JWH-073 *N*-butanoic acid (100 μg/mL) to a 10 mL volumetric flask, then adding 1.0 mL of internal standard solution C and diluting to final volume with methanol.

2.5. Sample treatment

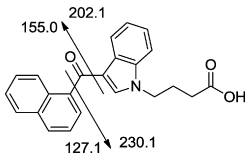
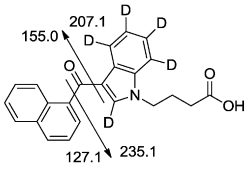
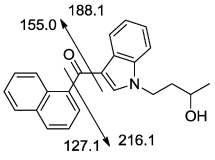
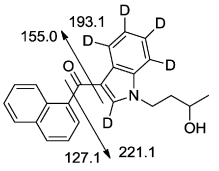
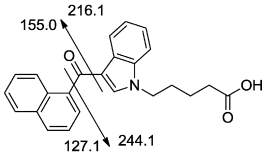
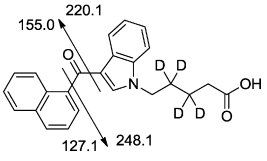
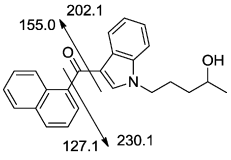
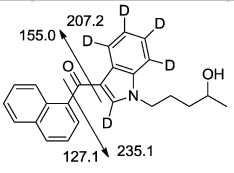
2.5.1. Hydrolysis

For initial studies, hydrolysis of a commercially available glucuronidated metabolite that was spiked into negative urine was chemically or enzymatically hydrolyzed by using hydrochloric acid and β-D-glucuronidase, respectively. A 1.7 mL microcentrifuge tube containing 100 μL of 200 ng/mL JWH-018 *N*-(5-hydroxypentyl β-D-glucuronide) prepared in unprocessed (unfiltered, undiluted, not pH adjusted) negative urine was treated with either 25 μL of 12 N HCl or 25 μL of β-D-glucuronidase solution (12.3 units/μL) and then mixed by vortexing gently for 5 s. The incubation was carried out at room temperature or 58 °C for a period of 1 h. Other parameters investigated for process optimization were the concentration of enzyme, substrate concentration and incubation time.

2.5.2. Extraction

After hydrolysis of the sample, 200 μL of LC/MS grade acetonitrile was added and the mixture was mixed by vortexing for 5 s. An aliquot of appropriate salting out reagent was then added and vortexed for 5 s. Microcentrifuge tubes were centrifuged at 10,000 rpm for 3 min. From the upper organic layer, 100 μL were removed and transferred to a clean HPLC vial and then further diluted by adding

Table 1
Summary of ionization parameters.

Metabolite			Internal standard		
MRM transitions	Cone voltage (V)	Collision energy (eV)	MRM transitions	Cone voltage (V)	Collision energy (eV)
	 **JWH-073-N-Butanoic acid** Chemical Formula: $C_{23}H_{19}NO_3$ Exact Mass: 357.1			 **JWH-073-N-Butanoic acid-d5** Chemical Formula: $C_{23}H_{14}D_5NO_3$ Exact Mass: 362.2	
358.1 → 155.07	38	28	363.2 → 155.07	44	22
358.1 → 127.02	38	48	363.2 → 127.09	44	46
	 **JWH-073-N-(3-Hydroxybutyl)** Chemical Formula: $C_{23}H_{21}NO_2$ Exact Mass: 343.2			 **JWH-073-N-(3-Hydroxybutyl)-d5** Chemical Formula: $C_{23}H_{16}D_5NO_2$ Exact Mass: 348.2	
344.2 → 155.00	38	28	349.2 → 155.07	42	24
344.2 → 127.02	38	52	349.2 → 127.08	42	50
	 **JWH-018-N-Pentanoic acid** Chemical Formula: $C_{24}H_{21}NO_3$ Exact Mass: 371.2			 **JWH-018-N-Pentanoic acid-d4** Chemical Formula: $C_{24}H_{17}D_4NO_3$ Exact Mass: 375.2	
372.1 → 155.01	40	26	376.2 → 155.01	40	28
372.1 → 127.08	40	46	376.2 → 127.02	40	50
	 **JWH-018-N-(4-Hydroxypentyl)** Chemical Formula: $C_{24}H_{23}NO_2$ Exact Mass: 357.2			 **JWH-018-N-(4-Hydroxypentyl)-d5** Chemical Formula: $C_{24}H_{18}D_5NO_2$ Exact Mass: 362.2	
358.2 → 155.01	34	26	363.2 → 155.07	38	26
358.2 → 127.05	34	56	363.2 → 127.09	38	46

100 μL of water/0.2% formic acid. The extracted solution was mixed well by vortexing for 5 s.

2.6. Method validation studies

The analytical method validation included the following parameters: extraction recovery, matrix effect studies, precision, accuracy, carryover, selectivity, cross talk and linearity.

2.6.1. Extraction recovery, matrix effects

For extraction recovery and matrix effect studies the protocols described by Matuszewski et al. [12], Hughes [13] and Van Eeckhaut and co-workers [14] were used. For the determination of extraction recoveries, MS signal responses of pre-extraction spiked samples were compared to the responses of post-extraction spiked samples at low and high concentration levels. For the recovery and matrix effect studies, the matrix variability from three different lots of negative urine was taken into consideration. Twelve 100 μL aliquots per lot of unprocessed negative urine were removed, placed into individual 1.7 mL micro-centrifuge tubes and respectively labeled. A total of 36 sample tubes were thus prepared.

Pre-extraction experiments were carried out by spiking the first 18 microcentrifuge tubes with 20 μL of the appropriate spiking calibrator solution (each urine lot spiked with low or high spiking calibrator in triplicate). Therefore, nine microcentrifuge tubes were spiked with low spiking calibrator and nine were spiked with high spiking calibrator solution. The remaining 18 microcentrifuge tubes were set up as the post-extraction samples and were spiked with 20 μL of pure methanol to normalize the percentage of methanol in all samples. All 36 microcentrifuge tubes were then hydrolyzed following the optimized hydrolysis protocol. After the hydrolysis and salting-out organic-aqueous phase separation, 100 μL aliquots were removed from the upper organic layer and placed into labeled HPLC vials. The solvent was evaporated under the fume hood overnight. Following solvent evaporation, the pre-extraction sample extracts were reconstituted with 190 μL of diluent solution and 10 μL of methanol.

The post-extraction sample extracts were further prepared by reconstituting the matrix residues with 20 μL of the proper spiking calibrator solution. In a similar manner as the pre-extraction experiments, nine vials were spiked with the low spiking calibrator solution and the other nine were spiked with the high spiking calibrator solution. The vials were further diluted by addition of 380 μL of the diluent solution (50% water/acetonitrile). All 36 vials thereby contained theoretically equivalent metabolite and internal standard concentrations and the same percentage of methanol.

For the determination of matrix effects, signal responses of post-extraction samples were compared to the responses of neat samples prepared by adding the appropriate spiking calibrator into diluent solution. The post-extraction samples were prepared following all the steps described previously. The neat samples, which did not contain the sample matrix, were prepared as the post-extraction solutions. Six clean HPLC vials were spiked with 20 μL of the appropriate spiking calibrator solution. For statistical purposes, three vials were spiked with low calibration and three with high calibration solution. Vials were labeled and further diluted by addition of 380 μL of the diluent solution. Again all 36 vials contained theoretically equivalent metabolite and internal standard concentrations and the same percentage of methanol.

2.6.2. Precision, accuracy and carryover

Precision and carryover studies were conducted simultaneously during the extraction recovery experiments. To allow for statistical analysis, intra-day precision experiments were executed in triplicate. For inter-day precision and percent accuracy determinations, negative urine spiked with positive QC solution containing only JWH-073 N-(butanoic acid) was processed and analyzed on 16 different days. The evaluation of carryover was assessed by injecting a solvent blank immediately after the injection or analysis of the highest calibration standard solution.

2.6.3. Selectivity

For the evaluation of method selectivity, interferences from different sources possibly encountered in authentic urine specimens were simulated. To test for endogenous interferants, unprocessed negative urine from three different sources were hydrolyzed, extracted and analyzed. These same lots of urine were spiked with two different drug metabolite cocktails. One of the solutions was a QC solution typically used in the Air Force Drug Testing Lab at Lackland AFB for the quantitation of common drugs of abuse. It is a mixture of THC-acid glucuronide, benzoylecgonine, morphine glucuronide, phencyclidine (PCP), amphetamine, oxycodone, MDMA and 6-monoacetylmorphine at different concentration levels. The second solution contained a mixture of synthetic cannabinoid metabolites at concentration of 200 ng/mL each: JWH-200 4-hydroxyindole, JWH-200 5-hydroxyindole, JWH-200 6-hydroxyindole, JWH-210 5-hydroxyindole, JWH-210 *N*-pentanoic acid, JWH-250 *N*-(5-hydroxypentyl) and JWH-250 *N*-pentanoic acid.

2.6.4. Cross-talk

"Cross-talk" was assessed by processing and analyzing two spiked urine aliquots. In case 1, an aliquot of negative urine was spiked with 20 μL of 2000 ng/mL spiking solution without internal standard. In case 2, a second aliquot of negative urine was spiked with 20 μL of internal standard solution D (containing only a mixture of internal standards at 500 ng/mL). Both aliquots were processed using the optimized hydrolysis/extraction protocol. For case 1, analysis of sample extracts was carried out by monitoring the internal standards' MRM transitions response. For case 2, analysis of sample extracts was accomplished by monitoring the metabolites MRM transitions response.

2.6.5. Linearity

For determination of linearity, five aliquots of negative urine were spiked with 20 μL of the appropriate spiking calibrator solution. The spiked urine was processed using the hydrolysis and extraction protocol to obtain the extracted five non-zero calibrator solutions. Preparation of the zero-matrix sample was carried out by spiking negative urine with internal standard solution D only prior to hydrolysis and extraction.

3. Results and discussion

3.1. UPLC method development

Initial method development studies focused on the chromatographic separation of six metabolites from the parent drugs JWH-018 and JWH-073 (three metabolites for each). Based on scientific literature reports [5–11], reversed phase chromatographic conditions were selected for the resolution of the six different metabolites of interest. Four different Acquity UPLC separation columns (BEH C18: 50 and 100 mm and HSS T3: 50 and 100 mm) were evaluated using both gradient and isocratic conditions. As previously reported [11,22], one of the main chromatographic challenges was the isomeric separation of JWH-018 hydroxylated metabolites (4-hydroxypentyl and 5-hydroxypentyl). These compounds cannot be differentiated solely by MS/MS because both share the same MRM transitions. Fig. 1a shows the total ion chromatogram (TIC) of the successful separation of the six different metabolites using the UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm). The retention time window for all metabolite reference standards and internal standards, under the optimized conditions,

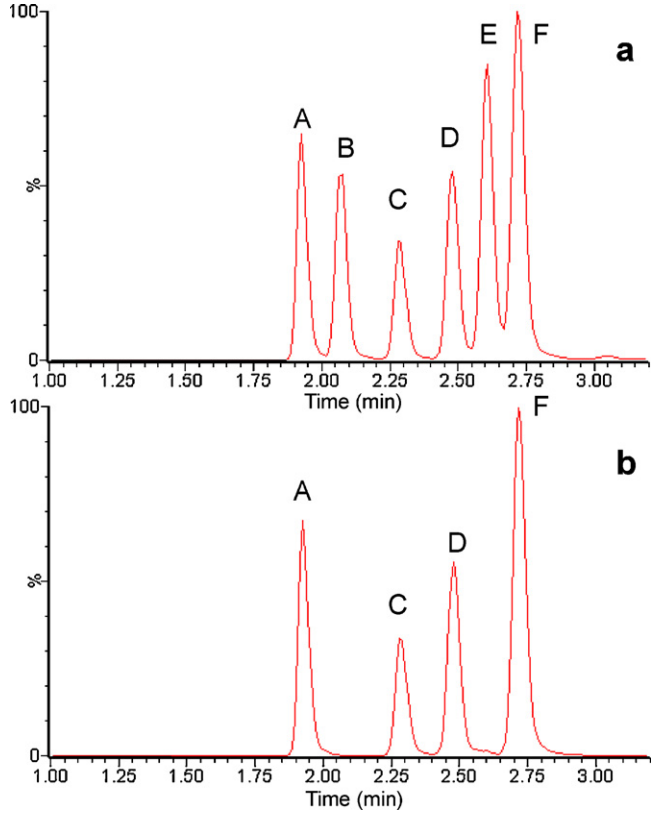


Fig. 1. (a) TIC for a mixture of six metabolite reference standards, each at 40 ng/mL, (b) TIC for a mixture of four metabolite reference standards each at 40 ng/mL [A – JWH-073 *N*-butanoic acid, B – JWH-073 *N*-(4-hydroxybutyl), C – JWH-018 *N*-pentanoic acid, D – JWH-073 *N*-(3-hydroxybutyl), E – JWH-018 *N*-(5-hydroxypentyl), F – JWH-018 *N*-(4-hydroxypentyl)].

is between 1.7 and 3.2 min. Optimum experimental conditions included isocratic conditions of 50% A and 50% B, a flow rate of 600 μL/min and a column temperature of 40 °C which resulted in a column pressure of approximately 8900 psi.

After several internal discussions and reviews of the literature, it was decided to focus on the quantitation of the more prevalent (ω−1) hydroxylated metabolites of JWH-018 and JWH-073 as well as the carboxylic acid metabolites [8,10]. Fig. 1b shows a chromatogram of the four metabolites currently being monitored.

3.2. Sample hydrolysis and extraction protocol development

According to several research groups [7,8,10,11], synthetic cannabinoid metabolites are primarily excreted as glucuronic acid conjugates and therefore, quantitative analysis of these metabolites requires the release from their corresponding glucuronide conjugates. Even though hydrolysis of glucuronides is generally performed by chemical or enzymatic treatment, the enzymatic hydrolysis approach using β-D-glucuronidase from various sources is the most prevalent means for the liberation of metabolites.

Since the main goal was the development of a high throughput analytical method, the initial goal was to devise a hydrolysis protocol with mild conditions to provide a quick release of metabolites of interest but at the same time provide cleaner extracts that would further eliminate or minimize issues during extraction and UPLC–MS/MS analysis. It was envisioned that chemical hydrolysis would provide those benefits. Experiments were designed to compare the efficiency of chemical versus enzymatic hydrolysis. Other hydrolytic conditions investigated included temperature, incubation time, substrate concentration and amounts of enzyme. Due to the initial unavailability of authentic positive urine specimens, initial assay development was performed by hydrolyzing and then extracting negative urine spiked with commercially available JWH-018 *N*-5-hydroxypentyl β-D-glucuronide conjugate. Hydrolysis efficiency was measured by UPLC–MS/MS by monitoring the decrease of the glucuronide conjugate response (peak area counts) and the increase of metabolite response. MRM transitions for both the glucuronide conjugate and metabolite were monitored, m/z 534 → 155 and 358 → 155, respectively. The percent conversion was calculated by subtracting the remaining conjugate area count from the area count from a sample without enzyme and dividing by the area count of the sample without enzyme. Fig. 2 shows representative chromatograms obtained from urine spiked with glucuronide conjugate incubated in absence (a) or presence (b) of β-glucuronidase. The JWH-018 *N*-(5-hydroxy) glucuronide conjugate (shown in Fig. 2a) elutes at 1.03 min and the free JWH-018 *N*-(5-hydroxy) metabolite elutes at 2.87 min. The peak at 0.62 min is an unknown peak also seen in un-spiked urine.

To assess the chemical and enzymatic hydrolysis, the JWH-018 glucuronide was dissolved in negative urine to achieve a concentration of 200 ng/mL. Aliquots of 100 μL were subjected to hydrolysis with either 25 μL of 12 N HCl or 25 μL β-glucuronidase (12.3 units/μL) for 1 h at room temperature or 58 °C. Under investigated conditions, even at high temperature, chemical hydrolysis was very inefficient and only provided a maximum conversion of 17.7%. Results also confirmed what other groups [7,8,10,11] have observed and that enzymatic hydrolysis is much more convenient and gentle than acidic conditions [6]. However, it was interesting to observe that close to a 100% conversion was achieved with enzyme treatment and incubation at either 58 °C or room temperature. Therefore, subsequent incubation studies were performed using enzymatic hydrolysis at room temperature.

With respect to hydrolysis reaction time, when incubating the spiked urine at different time periods of 1, 2, 5, 10, 15, 30, 45 or 60 min, no significant benefits were achieved in terms of hydrolytic efficiency beyond 10 min. In fact, a 1 min reaction time averaged a

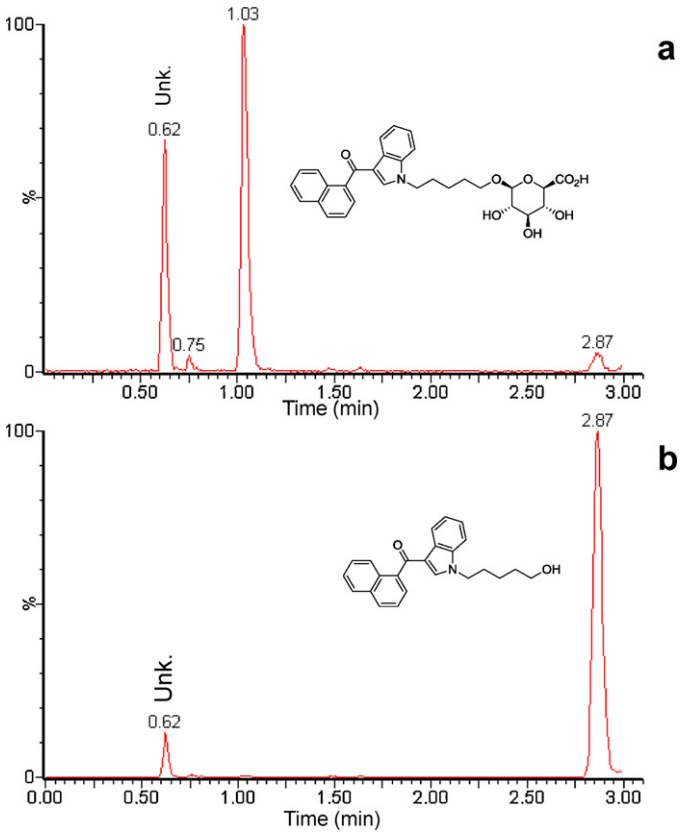


Fig. 2. TIC of extracted urine spiked with glucuronide conjugate (200 ng/mL) incubated in absence (a) or presence (b) of β-glucuronidase.

95% conversion, indicating that a very rapid reaction is achieved after enzyme addition. The experiments were performed in triplicate. To stop the hydrolysis reaction at specified times, 200 μL of acetonitrile was added (which is also a step included in the extraction protocol). The effective and immediate enzymatic hydrolysis of glucuronide conjugate under the study may be correlated to the slightly high unit levels of enzyme and the low volumes of urine and/or low substrate concentration levels.

Fig. 3 shows the hydrolysis results obtained by increasing the concentration of β-glucuronidase from 0 to 12.3 units/μL and an incubation time of 10 min at room temperature. The initial concentration of the glucuronide conjugate remained constant

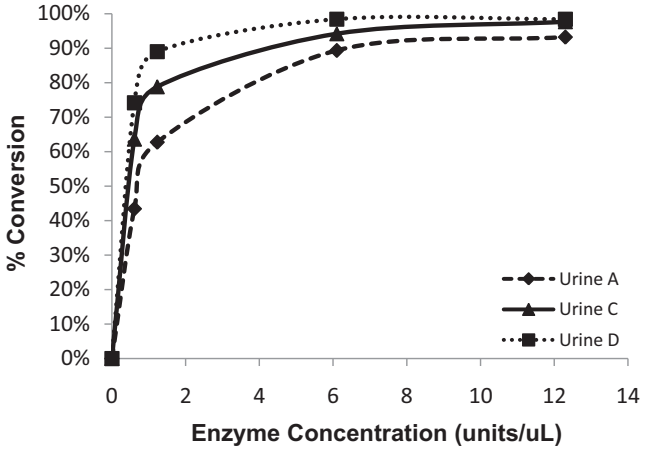


Fig. 3. Effect of β-glucuronidase concentration on the hydrolysis of JWH-018 *N*-(5-hydroxypentyl)-β-D-glucuronide conjugate in three urine lots.

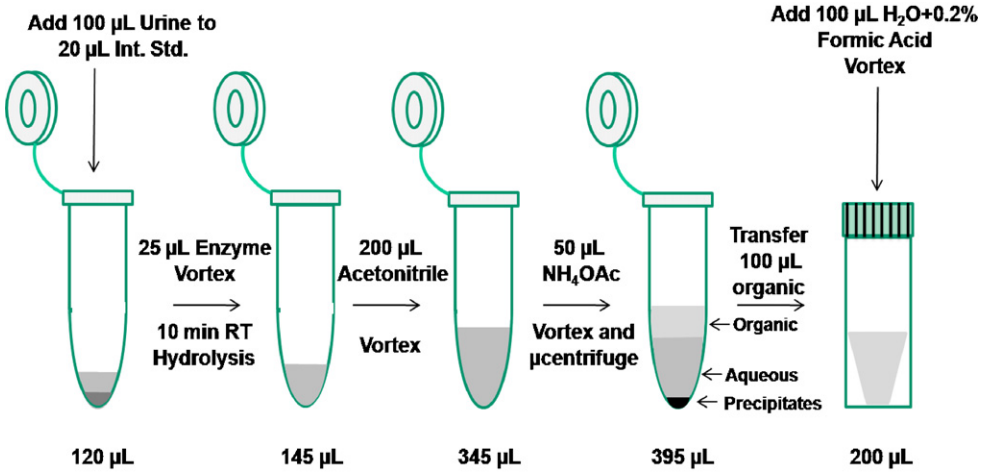


Fig. 4. Summary of the optimized protocol used for hydrolysis and extraction of the metabolites of interest from urine.

(200 ng/mL). As expected, higher rates of hydrolysis were observed when using high enzyme concentrations. It was also found out that even at concentration levels of 600 ng/mL of substrate, 12.3 units/µL of enzyme incubated at room temperature for 10 min was more than enough time to achieve almost complete hydrolysis. For further studies, an incubation time of at least 10 min was utilized to assure complete hydrolysis.

Due to the utilization of salting-out assisted liquid–liquid extraction, it was discovered early in the project that the enzymatic hydrolysis is quenched by the addition of acetonitrile and not by the addition of the salting-out reagent ammonium acetate. This allowed for the stopping of the hydrolysis reaction at precise times for the hydrolysis kinetics experiments as well as assisting in the cleanup of the samples by precipitation of solutes.

Even though the hydrolytic protocol was developed using only one glucuronide conjugate it was anticipated that the protocol will be equally efficient for different glucuronides expected to be in synthetic cannabinoid positive urine. The optimized hydrolytic conditions (25 µL of 12.3 units/µL enzyme and at least 10 min of room temperature incubation) were further applied to 12 authentic positive urine specimens containing different glucuronide conjugates. The different metabolites were quantified and compared to values determined by a certified drug testing lab using standard hydrolysis methods. Results were in a close agreement with the results reported by the certified lab (results not shown).

Based on a limited survey of current literature, both classical liquid–liquid extraction (LLE) and solid phase extraction (SPE) are among the most frequently used sample preparation techniques for the extraction of target synthetic cannabinoid metabolites from urine and plasma samples [6–11]. LLE has been performed with chlorobutane, chloroform or methyl *t*-butyl ether (MTBE) as the extraction solvent. Several different SPE cartridges have been utilized; including polymeric strong cationic exchange (Phenomenex), Accubond ODS-C_{18} (Agilent Technologies) and C_{18} high-load end-capped Resprep (Restek). Both LLE and SPE methods suffer from the same drawbacks, namely lengthy sample preparation, extensive production of waste and high per sample cost.

In this report, a simple and cost effective salting out assisted liquid/liquid extraction (SALLE) was evaluated and utilized for extraction of metabolites from urine specimens. This method of sample preparation was successfully applied and validated for a high-throughput LC–MS analysis by Zhang et al. [15,16] for the extraction of drugs of therapeutic interest from human plasma samples. To date there has been no published work applying this technique to extraction of urine samples for LC–MS/MS analysis. The salting out protocol involves the addition of a high molarity inorganic salt solution to a mixture of water and a water miscible organic solvent causing phase separation into two distinct layers but more importantly, the extraction of the analytes of interest into the organic layer [17]. As one would expect, the degree of extraction and phase separation will depend upon the analyte's physical and chemical properties as well as the nature and concentration of the salting out reagent. It is feasible to achieve nearly spontaneous phase separation at high salt concentrations. Although several inorganic salts have been reported as reagents for SALLE [18–21], this research focused on ammonium acetate because it is considered to be a "mass spectrometry friendly salt" [15]. In addition, acetonitrile was utilized as the water miscible solvent and extractant of targeted metabolites.

The sample preparation method described by Zhang et al. [15] for human plasma was applied with some minor modifications for the extraction of metabolites from urine. Fig. 4 summarizes the protocol used for both the hydrolysis and extraction of the metabolites of interest from urine. Because the extracting solvent was acetonitrile, the extract only needed to be further diluted with an equal amount of water/formic acid (thus matching the isocratic

Table 2
Recovery.

Sample	JWH-018 4-OH		JWH-018 acid		JWH-073 3-OH		JWH-073 acid	
	Abs. Rec. (%)	Norm. Rec. (%)	Abs. Rec. (%)	Norm. Rec. (%)	Abs. Rec. (%)	Norm. Rec. (%)	Abs. Rec. (%)	Norm. Rec. (%)
Urine A low	101.6	100.9	94.2	107.3	94.9	95.0	83.2	99.2
Urine B low	105.3	103.3	90.5	100.9	92.6	91.4	80.9	94.8
Urine C low	104.6	103.3	85.2	98.9	95.4	97.6	88.2	105.0
Average	103.8	102.5	90.0	102.4	94.3	94.7	84.1	99.7
Urine A high	100.6	99.7	88.8	100.4	95.4	96.0	81.0	99.7
Urine B high	102.7	99.5	89.8	97.9	98.3	96.3	87.4	102.5
Urine C high	102.4	103.5	84.6	98.9	91.9	98.0	78.0	99.4
Average	101.9	100.9	87.7	99.1	95.2	96.8	82.1	100.5

mobile phase conditions) and injected into the LC–MS/MS system. This bypasses the need for evaporation of a solvent and reconstitution of the residue, thus shortening the sample processing time. The results of the extraction recovery studies are discussed in Section 3.2.

One potential drawback of using SALLE may be the extraction of many other untargeted analytes from the urine. This possibility would be offset by the intrinsic selectivity of tandem mass spectrometry. To further offset this shortcoming, the first 1.5 min of effluent from the UPLC was directed to waste, thus avoiding the introduction of salts and weakly retained substances into the MS system. This would have the added benefit of minimizing the fouling of the sample cones and thus increasing the time between required cleaning of the sample cones.

By using this sample treatment protocol, the preparation time for 20 samples from aliquoting of the urine specimen through the capping of the extraction solutions in the HPLC vials was less than 70 min. To further reduce the instrument analysis cycle time, the next sample injection was preloaded into the injector loop 1 min prior to the completion of the prior analysis. The net cycle analysis time from injection to injection was less than 3.5 min per sample.

3.3. Method validation

3.3.1. Extraction recovery and matrix effects

As described in the experimental section, extraction recovery studies were validated by comparing the MS response (peak area counts) from pre-extraction spiked samples (spiked with appropriate standards prior to extraction) with the MS response from post-extraction spiked samples which is expressed as the absolute recovery (Abs. Rec.). The mean extraction recoveries, shown in Table 2, ranged from 82.1% to 104.6% for the four metabolites. By taking into account the internal standard responses, the normalized recovery (Norm. Rec.) was obtained. In summary, it can be concluded that excellent recoveries were achieved across all extractions for urine spiked at low and high concentrations.

The matrix effect was determined by comparing the analytical response (peak area counts) obtained from the post-extraction spiked samples versus the neat samples. Care was taken to ensure that the percentage of methanol was constant in both preparations.

From the data shown in Table 3, it can be concluded that the sample matrices from the three lots of urine did not have any significant matrix effects in the MS signal/response for three of the metabolites of interest and all internal standards. The exception is the JWH-073 *N*-(3-hydroxybutyl) metabolite, which the data suggests slight ion suppression for all three lots of urine when spiked at both low and high concentrations. However no ion suppression was observed for its respective deuterated internal standard. In theory, since both the metabolite and internal standard should behave chemically and physically in the same way, they should undergo fairly similar ionization processes, which was not the case. The inclusion of a deuterated internal standard is meant to compensate for matrix effects. If an actual matrix effect would have been present, these effects should have been compensated or minimized by the use of an individual deuterated internal standard per metabolite. Normalized data is also shown in Table 3.

3.3.2. Precision and accuracy

Both the intra-day and inter-day precisions were examined. The intra-day precision from triplicate extractions per lot of urine (spiked with low and high calibrator solution) using the salting out assisted liquid–liquid extraction protocol is shown in Table 4. All %RSDs were less than 10% for all metabolites. As expected, extractions from the high spiking calibrator solution had a smaller %RSD than solutions at the LOQ. The inter-day percent accuracy and

Table 3
Matrix effects.

Sample	JWH-018 4-OH			JWH-018 acid			JWH-073 3-OH			JWH-073 acid		
	Abs. matrix (%)	IS[a] ME (%)	Norm. matrix (%)	Abs. matrix (%)	IS[a] ME (%)	Norm. matrix (%)	Abs. matrix (%)	IS[a] ME (%)	Norm. matrix (%)	Abs. matrix (%)	IS[a] ME (%)	Norm. matrix (%)
Urine A low	103.2	98.4	104.9	100.8	102.8	98.1	89.7	99.1	90.5	108.0	99.3	108.8
Urine B low	102.4	100.2	102.2	101.2	104.0	97.3	91.0	99.4	91.5	102.5	100.5	102.0
Urine C low	101.7	98.8	102.9	99.9	102.8	97.2	89.0	99.2	89.7	104.0	102.2	101.8
Average	102.4	99.1	103.3	100.7	103.2	97.6	89.9	99.3	90.5	104.8	100.7	104.1
Urine A high	100.0	98.0	102.0	101.3	101.9	99.4	92.5	102.9	89.9	100.9	102.2	98.7
Urine B high	99.8	96.0	104.0	101.5	101.5	100.0	94.3	95.3	99.0	99.7	99.1	100.6
Urine C high	101.7	98.5	103.2	102.6	102.8	99.8	96.2	96.6	99.6	102.2	102.4	99.8
Average	100.5	97.5	103.1	101.8	102.0	99.8	94.3	98.3	95.9	100.9	101.2	99.7

[a] Absolute matrix effect of respective internal standard.

Table 4
Intra-day precision.

Sample	JWH-018 4OH (%)	JWH-018 acid (%)	JWH-073 3OH (%)	JWH-073 acid (%)
Urine A low	2.0	1.7	1.7	5.3
Urine B low	3.5	9.6	5.6	2.4
Urine C low	0.7	6.3	9.7	9.9
Urine A high	1.4	1.5	2.0	2.6
Urine B high	1.0	0.3	0.9	1.4
Urine C high	1.6	0.9	2.7	1.1

precision from 36 injections of the QC solution (nominal concentration of 100 ng/mL) run on 16 different days (2 or 3 per day) was determined to be 102.7 ± 3.4%.

3.3.3. Carryover

Analysis of a solvent blank injected immediately following an injection of the highest calibrator solution showed no measurable carryover of any metabolites of interest or internal standards within the optimized retention time window.

3.3.4. Selectivity

The evaluation of the selectivity of the method was based on the determination of potential interferants from three different sources. The most abundant source of possible interferants is the presence of typical endogenous urine components (e.g. salts, minerals, lipids, small molecules and proteins). These can be expected to be present in every urine sample tested. The other two sources of potential interferants are metabolites from typical drugs of abuse and other cannabinoids, due to the rapidly changing adulterants found in "Spice" smoking blends that might be expected to be seen in addition to or instead of the four metabolites of interest. As a result of the selective and specific monitoring of MRM transitions, it was anticipated that the presence of endogenous components, common drugs of abuse or other synthetic cannabinoids would have zero or minimal interference with UPLC–MS/MS analysis of metabolites of interest.

For all three sources investigated, no interfering peaks were detected in the retention time window (from 1.7 to 3.2 min) corresponding to the metabolites' elution time. From these results it can be concluded that the presence of endogenous components, metabolites from eight drugs of abuse or certain other synthetic cannabinoids potentially found in unknown/authentic urine specimens will not have any effect or interference on the UPLC–MS/MS analysis of the metabolites of interest.

3.3.5. Cross-talk

For "cross-talk" studies, the MS/MS channels for the different metabolites and their respective internal standards were monitored in the following manner: when extracts from urine spiked with high concentration metabolite standards (case 1) were injected and monitored for their respective internal standard MRMs, responses for the internal standards were not detected. In similar fashion, when extracts from urine spiked with internal standards (case 2) were injected and monitored their respective metabolite MRMs, responses for the different metabolites were not detected. In summary, no "cross-talk" was observed in any of the two circumstances.

3.3.6. Linearity

For the evaluation of the UPLC–MS/MS method linearity, five non-zero calibration points (containing IS) were assessed. All calibration solutions, prepared in methanol, were spiked into negative urine to mimic the unknown sample matrix and processed using the previously described hydrolysis and extraction protocol. Due to the lack of historical data at the drug testing lab, the range of the calibration standards included concentration levels at the limit of quantitation (LOQ) and a high calibration point based on recent scientific literature (233 ng/mL for the most abundant metabolite)[10].

The method linearity was demonstrated over a range of 4–400 ng/mL for each metabolite, which corresponds to an extracted metabolite solution concentration of 1–100 ng/mL; assuming that 100 percent recovery is obtained. A standard calibration curve was constructed using the known concentrations of metabolite versus the ratio of metabolite response to the internal standard response. The calibration curves generated for each metabolite of interest using five calibration points showed linearity with coefficients of determination (R^2) ranging from 0.997 to 0.999. Based on the metabolite with the lowest response, JWH-073 *N*-butanoic acid, it was administratively determined that the lowest standard solution (4 ng/mL) on the calibration curve would be accepted as the LOQ. This criterion was applied for all four metabolites even though it was already known that a lower LOQ could be defined for both hydroxylated metabolites, JWH-073 *N*-(3-hydroxybutyl) and JWH-018 *N*-(4-hydroxypentyl).

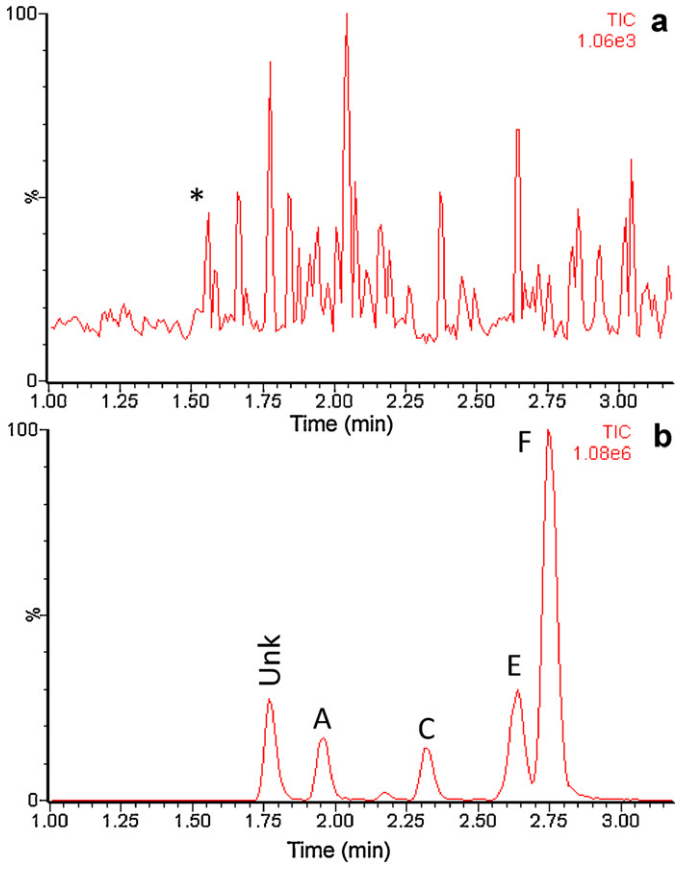


Fig. 5. Representative chromatograms of a solvent blank (a) and an authentic positive sample (b). * – The first 1.5 min of effluent flows to waste [A – JWH-073 *N*-butanoic acid, C – JWH-018 *N*-pentanoic acid, E – JWH-018 *N*-(5-hydroxypentyl), F – JWH-018 *N*-(4-hydroxypentyl)].

3.4. Analysis of authentic urine specimens

The validated method was used for both a screening and confirmation analysis for the quantitation of four metabolites of synthetic cannabinoids JWH-018 and JWH-073 in authentic urine samples. To date over 500 urine specimens have been analyzed in-house resulting in a few positive specimens which contain metabolites ranging from 5.4 to 37.8 ng/mL. Fig. 5 shows representative chromatograms of a solvent blank (a) and an authentic positive sample (b). To some extent, the confirmation method is more extensive and consequently includes many of the same elements as the screening (semi-quantitative) method. In both methods, all four metabolites of interest and their respective internal standards are monitored. Both also use the same two quality control solutions; a negative control only containing blank sample matrix and a positive control containing only one metabolite (100 ng/mL). The primary difference in the two methods is the number of calibrator solutions used. The screening method includes a single calibrator solution equivalent to the highest calibration (400 ng/mL) point of confirmation method. The confirmation method includes five non-zero calibration points already mentioned previously.

A result is considered positive based upon all of the following criteria being met: presence of at least one synthetic cannabinoid metabolite at a concentration of 4.0 ng/mL or greater; the retention time (t_R) of the synthetic cannabinoid metabolite in samples and controls are within ±3% of the average t_R of the cannabinoid metabolite in the calibrators; MRM transition ratios (response of the quantifier over the qualifier) of the cannabinoid metabolite in samples and controls are within ±20% of the average MRM transition ratios of the cannabinoid metabolite in the calibrators.

4. Conclusions

A high-throughput bioanalytical method consisting of enzymatic hydrolysis, salting-out assisted liquid–liquid extraction and UPLC–MS/MS has been developed and validated to quickly extract and quantify the metabolites of synthetic cannabinoids JWH-018 and JWH-073 present in urine specimens. It was demonstrated that use of enzymatic hydrolysis for releasing the metabolites of interest from the glucuronide conjugates can be achieved in only 10 min at room temperature. High extraction efficiencies of all four metabolites of interest were obtained by SALLE using acetonitrile as the organic extractant and ammonium acetate as the salting-out reagent. In addition to metabolites of JWH-018 and JWH-073, current work is directed toward the use of this protocol for the extraction of other “Spice” metabolites from urine. It is anticipated that the lipophilic nature of most synthetic cannabinoid metabolites would provide similar extraction efficiencies.

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