Anal Bioanal Chem (2014) 406:3599–3609
DOI 10.1007/s00216-013-7574-x

RESEARCH PAPER

LC-QTOF-MS as a superior strategy to immunoassay for the comprehensive analysis of synthetic cannabinoids in urine

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Received: 31 October 2013 / Revised: 2 December 2013 / Accepted: 10 December 2013 / Published online: 15 January 2014

Abstract The objective of this study was to compare the performance of an immunoassay screening for synthetic cannabinoids with a newly developed confirmation method using liquid chromatography quadrupole time-of-flight mass spectrometry. The screening included metabolites from JWH-018, JWH-073, and AM-2201. The confirmation included metabolites from AM-2201, JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-210, JWH-250, JWH-398, MAM-2201, RCS-4, and UR-144. The immunoassay was tested and found to have no cross-reactivity with UR-144 metabolites but considerable cross-reactivity with MAM-2201 and JWH-122 metabolites. Sensitivity and specificity for the immunoassay were evaluated with 87 authentic urine samples and found to be 87 % and 82 %, respectively. With a cutoff at 2 ng/ml, the confirmation showed 80 positive findings in 38 cases. The most common finding was JWH-122 5-OH-pentyl, followed by JWH-018 5-OH-pentyl. There were 9 findings of UR-144 metabolites and 3 of JWH-073 metabolites. In summary, the immunoassay performed well, presenting both high sensitivity and specificity for the synthetic cannabinoids present in the urine samples tested. The rapid exchange of one cannabinoid for another may pose problems for immunoassays as well as for confirmation methods. However, we consider time-of-flight mass spectrometry to be superior since new metabolites can be quickly included and identified.

Keywords LC-Q-TOF · Synthetic cannabinoids · Immunoassay · Metabolites · Forensic

Published in the topical collection *Forensic Toxicology* with guest editor Helena Teixeira.

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Introduction

A range of synthetic cannabinoids are currently used as recreational drugs. They belong to several groups based on their structures, and when current compounds are scheduled new similar compounds appear [1]. The most common groups are the naphthoylindoles (for example JWH-018), the tetramethylcyclopropylindoles (for example XLR-11), and very recently the adamantoylindoles (for example AKB-48), although there are many other classes too.

Like many drugs, cannabinoids seem to be eliminated via cytochrome P450 enzymes, and then conjugated with glucuronic acid by UDP-glucuroronsyltransferases. Common metabolic pathways (JWH-018, JWH-073, JWH-081, JWH-122, JWH-203, JWH-210, JWH-200, JWH-250, JWH-251, RCS-4) are mono- or dihydroxylation of the N-carbon side chain or the indole group [2–7] or hydroxylation of the aliphatic adamantoyl ring in AB-001 and AKB-48 [8, 9]. Another common pathway is stepwise carboxylation of the terminal carbon on the N-pentyl side chain, resulting in both N-pentyl- and N-butyl-carboxylated metabolites, as exemplified using AM-2201 by Hutter et al. [10].

JWH-018, JWH-122, JWH-210, UR-144, and AKB-48 all have fluorinated analogs that have been shown to metabolize through hydroxylation and carboxylation of the terminal carbon, resulting in de-fluorinated metabolites similar to those afforded by their unfluorinated analogs [8, 10–13]. The phase I metabolites are then further conjugated, and high levels of

glucuronide metabolites have been found in human urine, animal models, and during in vitro studies [4, 8, 14, 15].

In summary, studies have confirmed that monohydroxylation is the primary urinary metabolic pathway, followed by glucuronidation. The detection of synthetic cannabinoids in urine is complicated by their structural similarities resulting in common metabolites, which makes interpretation difficult. The omega-1 hydroxylated metabolites may be considered unique to one parent compound [10].

Despite this paucity of data, there has been a great demand for assays that determine synthetic cannabinoids, and many laboratories have begun analyzing metabolites from synthetic cannabinoids in case work. Most of the methods employed are based on liquid chromatography tandem mass spectrometry (LC-MS-MS) [16–21], but immunoassays are also available [22].

All the methods incorporate a hydrolysis step utilizing beta-glucuronidase prior to analysis because of the low concentrations of unconjugated metabolites involved, together with the lack of reference materials for conjugates. On the other hand, the extraction or preparation of the urine differs among the methods. More traditional extraction methods using solid-phase extraction [16] or liquid/liquid extraction [17, 18] have been used, as well as simpler dilution/precipitation methods that employ methanol [19, 20]. Yanes and Lovett [21] also present a salting-out extraction with acetonitrile that can be considered a mixture of regular solvent extraction and dilution.

Most of the methods published so far are quantitative, but a recent methodological paper by Wohlfarth et al. [20] suggests that a qualitative approach is a good compromise between information and flexibility. They suggest that a qualitative method needs less validation when adding a new analyte, and that this is a favorable aspect considering that new compounds are quickly emerging.

Owing to the high potency of the synthetic cannabinoids, the expected concentrations in urine are low and thresholds must therefore be adjusted appropriately. Typically, thresholds have been below 1 ng/ml when an extraction has been performed [16, 17], and significantly higher when sample preparation has been minimized [19–21]. Based on the few studies that have been performed where subjects have smoked "Spice" products [10, 17], it is clear that only a few ng/ml will be present 1 or 2 days after administration.

Arntson et al. [22] described two immunoassays that were developed for the analysis of metabolites from JWH-018 and JWH-250. Compared to a confirmation method using LC-MS-MS, they performed very well, with sensitivities and specificities of >95 %. Cross-reactivity with other synthetic cannabinoids was limited.

Due to rapid changes in the compounds used, methods must be updated and new analytes added, or they will soon become less useful. An interesting analytical technique that has excellent screening capabilities is high-resolution mass spectrometry using the time-of-flight methodology (TOF) [23–31]. This has been used for metabolite profiling as well as for identifying new synthetic cannabinoids in botanical materials and powders [32–34]. Recently, Sundström et al. [35] published a TOF screening method that included metabolites from synthetic cannabinoids, and they concluded that the method could easily be updated with new substances to keep up with the current ever-changing drug situation.

However, when a high number of samples—especially negative samples—are expected, the use of an immunoassay is much preferred. The aim of the study reported in the present paper was to compare a commercial immunoassay with a newly developed liquid chromatography quadrupole time-of-flight mass spectrometric (LC-QTOF-MS) method used for confirmation.

Experimental

The screening method was the Immunalysis (Pomona, CA, USA) Spice K2 homogeneous enzyme immunoassay (HEIA) for synthetic cannabinoids, which was set up on an ADVIA 1850 system with parameters provided by the manufacturer of the HEIA. Accordingly to the manufacturer, the HEIA was developed to detect metabolites from JWH-018, JWH-073, and AM-2201. The proposed cutoff was 20 ng/ml using JWH-018 pentanoic acid as calibrator.

The confirmation method was based on a salting-out liquid-liquid extraction (SALLE) of the analytes from urine using ammonium acetate and acetonitrile [21]. An aliquot of the acetonitrile supernatant was analyzed using ultra high-performance LC-QTOF-MS to qualitatively identify analytes.

Chemicals, reagents, and reference compounds

Optima®-grade acetonitrile, formic acid, methanol, water, and 2-propanol for the LC-QTOF-MS mobile phases and wash solutions were purchased from Fisher Scientific (Gothenburg, Sweden). Acetonitrile gradient grade and ammonium acetate were purchased from Merck (VWR, Stockholm, Sweden), ammonium formate was purchased from Fluka (Sigma–Aldrich, Stockholm, Sweden), and β-glucuronidase (*E. coli*) was purchased from Roche (Mannheim, Germany). High-purity water was produced in-house in a MilliQ Gradient 10 production unit from Millipore (Billerica, MA, USA). The ESI-L low-concentration tuning mix (acetonitrile solution) and the API-TOF reference mass solution kit (consisting of 5 mM purine and 2.5 mM HP-921 solution in acetonitrile) were all from Agilent Technologies (Kista, Sweden).

All metabolite reference substances were purchased as 1.0 mg of powder or in solution from Cayman Chemicals (LGC Standards, Boras, Sweden) with the exception of the JWH-019 5-hydroxyhexyl metabolite, which was from Cerilliant (LGC Standards, Boras, Sweden), and JWH-081 pentanoic acid, which was from Chiron (LGC Standards, Boras, Sweden). See Table 1 for all analyte standards.

The internal standards d_5-JWH-018 N-pentanoic acid, d_9-JWH-018 6-hydroxyindole, d_9-JWH-018 7-hydroxyindole, d_5-(±)-JWH-073 N-(3-hydroxybutyl), d_7-JWH-073 6-hydroxyindole, and d_5-JWH-250 N-(4-hydroxypentyl) were all from Cayman Chemicals (LGC Standards, Boras, Sweden).

Solutions

Ammonium acetate solution (10 M) for SALLE was prepared by dissolving ammonium acetate salt in MilliQ® water. In order to minimize background signal and interferences, Optima® ultrapure solvents were used to prepare mobile phases and instrument wash solutions. Ammonium formate stock solution (1 M) was prepared by dissolving ammonium formate salt in Optima® water. Ammonium formate mobile phase buffer A (10 mM, 0.05 % formic acid) was prepared by dilution of ammonium formate stock solution and formic acid with Optima® water. Mobile phase solution B (0.05 % formic acid in methanol) was prepared by dilution of formic acid in Optima® methanol. A needle wash solution was prepared by adding 0.2 % formic acid to a mixture consisting of Optima® acetonitrile, isopropanol, methanol, and water (25:25:25:25 v/v/v/v).

Stock solutions of the reference compounds were prepared at 1 mg/ml and were further diluted to 10 μg/ml working solutions in Optima® acetonitrile or methanol. Mixed working solutions (n=4) of a maximum of 10 test compounds were prepared for validation. Compounds were combined in order to avoid coeluting critical pairs during validation and determination of the threshold level.

Calibration solutions (A–D, 10 ml) were prepared in Optima® acetonitrile to a final concentration of 0.08 μg/ml of each compound, and were stored in 2.5-ml aliquots at −20 °C.

A solution of the six internal standards was prepared in Optima® acetonitrile to a final concentration of 0.025 μg/ml of each compound, and stored in 100-ml aliquots at −20 °C for long-term storage and at +4 °C for daily use.

The TOF tuning mixture used for instrument tuning and mass calibration was prepared by the dilution of 10 mL ESI-L low-concentration tuning mix and 1.5 mL Optima® water to a final volume of 100 mL with Optima® acetonitrile. For internal mass calibration, a solution was prepared by diluting one ampoule of 5 mM purine, one ampoule of 2.5 mM HP-921, and 5 mL Optima® water to a final volume of 500 mL with Optima® acetonitrile.

Sample preparation

Daily urine calibrators (n=4) were freshly prepared by adding 25 μL calibration solution (A–D) to 1 ml blank urine. Aliquots of 600-μL urine samples, urine calibrators, and a negative control were transferred to secondary tubing by a Tecan pipetting robot (Männerdorf, Switzerland). Samples were hydrolyzed with 30 μL β-glucuronidase for 20 min at ambient temperature before the extractions. Metabolites were extracted from urine by SALLE. A volume of 400 μL of cold acetonitrile solution of the internal standards (+4 °C) was added and the samples were vortex mixed for 5 s. A volume of 200 μL of 10 M ammonium acetate was added, the samples were mixed, and they were then centrifuged at 5000 rpm. Aliquots of 100 μL of the acetonitrile were transferred to vials with inserts for further analysis by LC-QTOF-MS.

Instrumentation

LC-QTOF-MS analysis was performed on an Agilent 6540 quadrupole time-of-flight mass spectrometer equipped with a Jet Stream interface for electrospray ionization (ESI) in combination with an Agilent 1290 Infinity LC instrument. Mobile phase A consisted of 0.05 % formic acid in 10 mM ammonium formate and mobile phase B of 0.05 % formic acid in methanol. A high-resolution separation was achieved within 15 min by stepwise linear gradient chromatography [2 % mobile phase (B) for 0.5 min, 50-70 % (B) for 11.5 min, 100 % (B) for 1.5 min, 2 % (B) for 1.5 min] at a flow rate of 0.6 ml/min on an ACE Excel 2 C18-AR (100×2.0 mm, 2 μm) maintained at 60 °C.

The Jet Stream conditions applied for positive ionization were: capillary voltage 3,500 V, fragmentor voltage 150 V, gas temperature 300 °C, drying gas flow 6 L/min, nebulizer pressure 35 psi, sheath gas temperature 375 °C. Samples were detected in the low mass range (m/z 1700) and at 2 GHz (extended dynamic range) in auto MS/MS mode with an MS scan rate of 2 spectra/s (m/z range 50–1,000) and with an MS/MS scan rate of 3 spectra/s (m/z range 50–500) with a fixed collision energy of 20 V. For internal mass calibration, two reference masses were constantly infused into the ion source, and mass correction was performed in every single scan (121.050873 and 922.009798 in positive ionization mode). Data acquisition and evaluation was performed using MassHunter software: acquisition module (Acq) B.05.01, qualitative analysis module (Qual) B.06.00, and quantitative analysis module (Quant) B.05.01.

Spectral library

The in-house personal compound database and library (PCDL) comprising 38 metabolites was generated by flow injection analysis in mobile phases A and B of each substance

Table 1 Method analytes divided into calibration groups A–D, including performance characteristics for the library search score and threshold calibrator imprecision

Compound	Peak	I.S.	Auto MS/MS			Quantitation			
			Score (Lib)			R_t (min)	Transition [M+1]	Area ratio (N=10)	
			Mean	CV	N			A/I.S.	CV
Standard A									
RCS-4 5-OH-pentyl	1	I	94	1.5 %	6	5.01	338.2	0.039	19 %
JWH-250 5-OH-pentyl	2	I	83	9.1 %	8	5.48	352.2	0.075	11 %
JWH-073 3-OH-butyl	3	II	93	4.0 %	10	6.66	344.2	0.114	7 %
JWH-018 5-OH-pentyl	4	III	94	3.8 %	10	7.50	358.3	0.053	10 %
UR-144 4-OH-pentyl	5	III	82	4.7 %	10	7.70	328.2	0.110	12 %
MAM-2201 4-OH-pentyl	6	IV	91	3.6 %	10	8.31	390.2	0.096	6 %
JWH-019 5-OH-hexyl	7	IV	92	2.7 %	9	8.68	372.2	0.092	10 %
JWH-122 5-OH-pentyl	8	IV	91	4.3 %	8	9.04	372.2	0.045	9 %
JWH-210 4-OH-pentyl	9	V	90	2.8 %	10	10.46	386.2	0.098	3 %
JWH-398 4-OH-pentyl	10	V	89	2.9 %	10	10.55	392.1	0.050	6 %
Standard B									
RCS-4 4-OH-pentyl	11	I	94	1.6 %	10	4.92	338.2	0.109	5 %
JWH-250 4-OH-pentyl	12	I	89	2.7 %	9	5.42	352.2	0.075	9 %
JWH-073 butanoic acid	13	II	88	8.7 %	10	6.37	358.3	0.057	9 %
AM-2201 4-OH-pentyl	14	II	92	4.6 %	9	6.83	376.2	0.112	6 %
JWH-018 4-OH-pentyl	15	III	93	1.7 %	9	7.39	358.3	0.119	6 %
UR-144 5-OH-pentyl	16	III	88	3.4 %	10	7.84	328.2	0.122	8 %
JWH-081 5-OH-pentyl	17	IV	89	5.5 %	6	8.63	388.2	0.111	12 %
JWH-122 4-OH-pentyl	18	IV	91	5.8 %	10	8.90	372.2	0.071	9 %
JWH-210 pentanoic acid	19	V	91	2.3 %	10	10.47	400.2	0.103	11 %
JWH-398 5-OH-pentyl	20	V	91	1.3 %	10	10.73	392.1	0.031	6 %
Standard C									
RCS-4 pentanoic acid	21	I	93	2.5 %	9	4.86	352.2	0.094	17 %
JWH-250 pentanoic acid	22	I	87	5.6 %	10	5.33	366.2	0.048	8 %
JWH-073 4-OH-butyl	23	II	93	3.7 %	8	6.36	344.1	0.099	20 %
JWH-018 pentanoic acid	24	III	90	4.1 %	10	7.39	372.2	0.049	5 %
UR-144 pentanoic acid	25	III	93	3.9 %	10	7.60	342.3	0.065	5 %
JWH-081 pentanoic acid	26	IV	87	8.2 %	10	8.49	402.2	0.101	30 %
JWH-019 6-OH-hexyl	27	IV	91	4.5 %	7	8.90	372.2	0.051	7 %
MAM-2201 pentanoic acid	28	IV	91	2.9 %	10	8.92	386.2	0.127	3 %
JWH-398 pentanoic acid	29	V	93	1.5 %	10	10.51	406.3	0.085	20 %
JWH-210 5-OH-pentyl	30	V	90	3.9 %	10	10.66	386.2	0.141	19 %
Standard D									
AM-2201 6-OH-indole	31	III	93	4.2 %	10	7.77	376.2	0.122	3 %
JWH-073 6-OH-indole	32	IV	95	3.4 %	10	8.55	344.2	0.122	12 %
JWH-073 5-OH-indole	33	IV	94	1.4 %	8	8.94	344.2	0.110	6 %
JWH-073 7-OH-indole	34	V	93	1.7 %	10	9.89	344.2	0.089	5 %
JWH-018 6-OH-indole	35	V	92	6.0 %	10	10.33	358.3	0.121	4 %
JWH-018 5-OH-indole	36	V	93	2.6 %	10	10.82	358.3	0.116	6 %
JWH-018 7-OH-indole	37	VI	92	2.9 %	10	11.66	358.3	0.112	5 %
AM-2201 7-OH-indole	38	VI	90	3.4 %	10	12.00	376.2	0.123	3 %
Internal standards (I.S.)									
d5-JWH-250 4-OH-pentyl	I	–				5.33	357.2		
d5-JWH-073 3-OH-butyl	II	–				6.59	349.2		

Table 1 (continued)

Compound	Peak	I.S.	Auto MS/MS			Quantitation			
			Score (Lib)			R_t (min)	Transition [M+1]	Area ratio (N=10)	
			Mean	CV	N			A/I.S.	CV
d5-JWH-018 pentanoic acid	III	–				7.28	377.2		
d7-JWH-073 6-OH-indole	IV	–				8.47	351.2		
d9-JWH-018 6-OH-indole	V	–				10.24	367.2		
d9-JWH-018 7-OH-indole	VI	–				11.55	367.2		

CV coefficient of variation, *A* analyte, *I.S.* internal standard, R_t retention time

individually at 1 μg/ml in methanol or acetonitrile, according to the procedure previously described by Broecker et al. [36, 37]. The protonated molecules $[M+H]^+$ were selected by the quadrupole, and three MS-MS spectra were generated in product-ion scan mode at collision-induced dissociation (CID) energies of 10, 20, and 40 eV.

Data analysis for targeted screening by Auto MS/MS

Data was acquired in auto MS/MS mode. MS data were extracted by the "Find By Formula" (FBF) algorithm, and only those compounds with retention time differences and mass errors that were within the criteria set in the data analysis method were reported. A presumptive positive result included a match tolerance for mass error of ±10 ppm, a retention time deviation of ±0.15 min, and an area of ≥10,000 counts. The absolute peak areas were measured and analyses were considered acceptable when the area was ≥2,000,000 counts for the internal standards.

Primary MS identification was based on retention time scoring, accurate mass measurement, and the isotopic pattern (mass, abundance, and spacing). This was followed by a secondary MS/MS identification in which the obtained spectrum was matched with accurate mass CID spectra from the PCDL generated at 20 eV. Hits with an accurate mass deviation of within ±5 ppm, a retention time deviation of within ±0.1 min, and a confirmed isotopic pattern were considered positive and an overall MS score was calculated. A weighted average was obtained of the individual scores calculated by individual algorithms in the data analysis software. An overall score of ≥80 out of 100 was considered a positive result in MS, and a score (Lib) of ≥80 out of 100 was considered a positive result in MS/MS.

A peak that was found to be positive in the MassHunter Qual software was confirmed to be a true positive finding above the defined threshold level of 2 ng/ml in the MassHunter Quant software by comparison with the results obtained by analyzing the daily calibrators (A–D).

Validation of the LC-QTOF-MS method

Selectivity

Urine specimens with creatinine concentrations of between 0.28 and 2.25 mg/ml from ten donors were used for selectivity testing. Drug-free urine samples were spiked with relevant substances; these substances were selected from among the amphetamines, the benzodiazepines, cocaine, the opiates, cannabis, and from among close to a hundred analogs of designer drugs from the phenylethylamine, cathinone, and piperazine groups. Drug-free urine was also spiked with the internal standards to investigate potential contaminants.

Matrix effects

Initial qualitative studies of matrix effects were performed with post-column infusion of the first eluting analyte, RCS-4 pentanoic acid. Both urine samples as well as water samples with different volumes of 10 M ammonium acetate (used for the salting out) were tested.

Quantitative matrix effects were studied by pre- and post-extraction spiking. Ten urine samples from different donors were used in the experiment, and the results were compared to those obtained from a directly injected standard.

Determination of thresholds

Based on initial experiments, a general threshold of 2 ng/ml seemed appropriate from an analytical perspective. This was verified by analyzing ten urine samples from different donors spiked with standards at 2 ng/ml.

Stability

The stabilities of the analytes in urine were investigated at room temperature over 2 days, at +4 °C over 2 weeks, and at −20 °C over 15 weeks. Processed sample stability was investigated over 96 h. The experiments at room temperature and +4 °C were carried out as 6 replicates that were compared to

Table 2 Imprecision and accuracy of the Spice K2 HEIA at three proposed cutoffs

Control	5 ng/ml		10 ng/ml		20 ng/ml	
	Imprecision (%)	Accuracy (%)	Imprecision (%)	Accuracy (%)	Imprecision (%)	Accuracy (%)
−50 %	19.7	108	9.0	97	5.8	129
+50 %	6.4	103	5.0	95	2.9	83

freshly prepared control samples. The experiments at −20 °C were performed as triplicates and compared to freshly prepared controls.

Hydrolysis

Enzymatic hydrolysis was evaluated using JWH-018 5-hydroxy pentyl glucuronide. Samples (N=5) were spiked with the glucuronide to give a concentration of free analyte of 100 ng/ml. These underwent the procedure and were then analyzed using a calibration curve for 2–150 ng/ml. The between-day precision of the hydrolysis was estimated by analyzing a spiked sample on ten different days.

Validation of the HEIA method

Response curves

Response curves for JWH-018 pentanoic acid were obtained in triplicate on three different days (N=9). Levels were 0, 2.5, 5, 7.5, 10, 15, 20, and 30 ng/ml.

Precision and accuracy

Controls of ±50 % around three proposed cutoff concentrations (5, 10, and 20 ng/ml) were run, with 10 replicates of each. Controls around the final 5 ng/ml cutoff were also run for 1 month (N=14) to estimate the between-day precision.

Cross-reactivity

Cross-reactivity of the Spice K2 HEIA has already been described for several metabolites, but not for some of the newer synthetic cannabinoids. Therefore, we performed a limited cross-reactivity test with the following metabolites: MAM-2201 4-hydroxy pentyl, MAM-2201 pentanoic acid, UR-144 5-hydroxy pentyl, UR-144 pentanoic acid, JWH-122 4-hydroxy pentyl, and JWH-122 5-hydroxy pentyl. The metabolites were evaluated at 2–4 concentrations (7.5, 20, 50, and 100 ng/ml; N=3), depending on their cross-reactivities.

Table 3 Method comparison between the Spice K2 HEIA and the LC-QTOF-MS method (N=87)

		LC-QTOF-MS	
		NEG	POS
HEIA	NEG	40	5
	POS	9	33

Method comparison

Eighty-seven authentic urine samples were analyzed with both the HEIA and the LC-QTOF-MS methods and the results were compared. The method comparison was performed with a screening cutoff of 5 ng/ml and a confirmation threshold of 2 ng/ml.

Routine samples

All requests for synthetic cannabinoids during 1 month (April 2013, N=204) were analyzed using both methods to evaluate routine performance. Approximately 90 % of the samples were from cases involving suspected petty drug offences or from drugs and driving cases, while 10 % were from criminal justice cases.

Results and discussion

The response curve experiments showed that the assay was linear between 2.5 and 15 ng/ml, with measured concentrations of within 10 % of the expected value. The precision and accuracy of controls around different cutoffs are presented in Table 2. The accuracy was not acceptable at the proposed 20 ng/ml cutoff, probably because of the curvature of the response curve. The imprecision increased with decreased concentration but was acceptable at the lowest cutoff. A recent publication presenting an immunoassay using ELISA proposed a cutoff of 5 ng/ml to be able to readily detect the use of synthetic cannabinoids [22]. The few controlled studies that have measured metabolites after the administration of synthetic cannabinoids have shown that individual metabolites are present at low nanogram concentrations within a day after administration [10, 17]. Therefore, we chose the 5 ng/ml cutoff for subsequent analysis. The between-day imprecisions at 2.5 ng/ml and 7.5 ng/ml were 23.7 % and 9.5 %, while the accuracies were 108 % and 90 %, respectively. The HEIA method showed no cross-reactivity with the UR-144

Table 4 Metabolite findings in the 22 positive samples from consecutive cases during April 2013

Case	JWH-018				JWH-073	JWH-122			MAM2201	UR-144			JWH-210
	4-OH-pentyl	5-OH-pentyl	6-OH-indole	Pentanoic acid	Pentanoic acid	4-OH-pentyl	5-OH-pentyl	Pentanoic acid	4-OH-pentyl	4-OH-pentyl	5-OH-pentyl	Pentanoic acid	5-OH-pentyl
1	X											X	
2							X						
3							X						
4							X						
5												X	
6												X	
7												X	
8						X	X	X					
9							X						
10										X	X		
11	X												
12						X							
13							X	X	X	X			
14												X	
15											X	X	
16													X
17													X
18				X									
19							X	X	X				
20	X	X	X	X	X	X							
21							X	X				X	
22							X						

metabolites. For MAM-2201 pentanoic acid, the mean cross-reactivity was 52 %, for MAM-2201 4-hydroxy-pentyl it was 36 %, for JWH-122 4-hydroxy-pentyl it was 40 %, and for JWH-122 5-hydroxy-pentyl it was 39 %. The results from the method comparison during validation are summarized in Table 3. The sensitivity of the HEIA method was 87 % and its specificity was 82 %. The five false-negative samples contained UR-144 metabolites only or in combination with JWH-122 metabolites. When using the LC-TOF-method, the 87 authentic cases yielded 80 positive findings in 38 cases. The most common finding was JWH-122 5-hydroxy-pentyl, with 21 findings, followed by JWH-018 5-hydroxy-pentyl, with 15 findings. There were 9 findings of UR-144 metabolites and 3 of JWH-073 metabolites. These samples were retrospectively selected and analyzed based on requests from the police as well as positive findings of synthetic cannabinoids in blood.

To better reflect the current situation, we also prospectively analyzed 204 consecutive samples for which synthetic cannabinoids had been requested. Among those samples, 170 came out negative with both methods while 19 were positive with both methods. There were 15 discrepancies, 12 false positives, and 3 false negatives. Again, this showed a good agreement between the methods. The 22 samples that were confirmed positive are summarized in Table 4.

Figure 1 shows a chromatogram for each calibrator as well as a positive sample. Chromatographic selectivity for isobaric positional isomers was achieved by employing a bifunctional (C18 and phenyl) stationary phase and a slowly increasing gradient over 11 min. Complete resolution could not be achieved for all positional isomers. The method characteristics are shown in Table 1. There were no positive findings in any of the selectivity experiments. The qualitative matrix effect studies showed that there were matrix effects at the beginning of the chromatogram but that the signal had returned to baseline by approximately 4 min. The first-eluting compound eluted at 4.9 min. There was no visual difference in matrix effect depending on the volume of 10 M ammonium acetate added, but there were two unknown peaks that eluted at 8.8 and 9.4 min in all samples. These probably originated from the salt, since they were present in both urine samples as well as water with salt added but not in the blank sample.

The quantitative matrix effect studies showed that matrix effects were <15 % for all analytes, except for JWH-250 pentanoic acid (22 % ion suppression), JWH-081 pentanoic acid (21 % ion suppression), and JWH-073 6-OH-indole

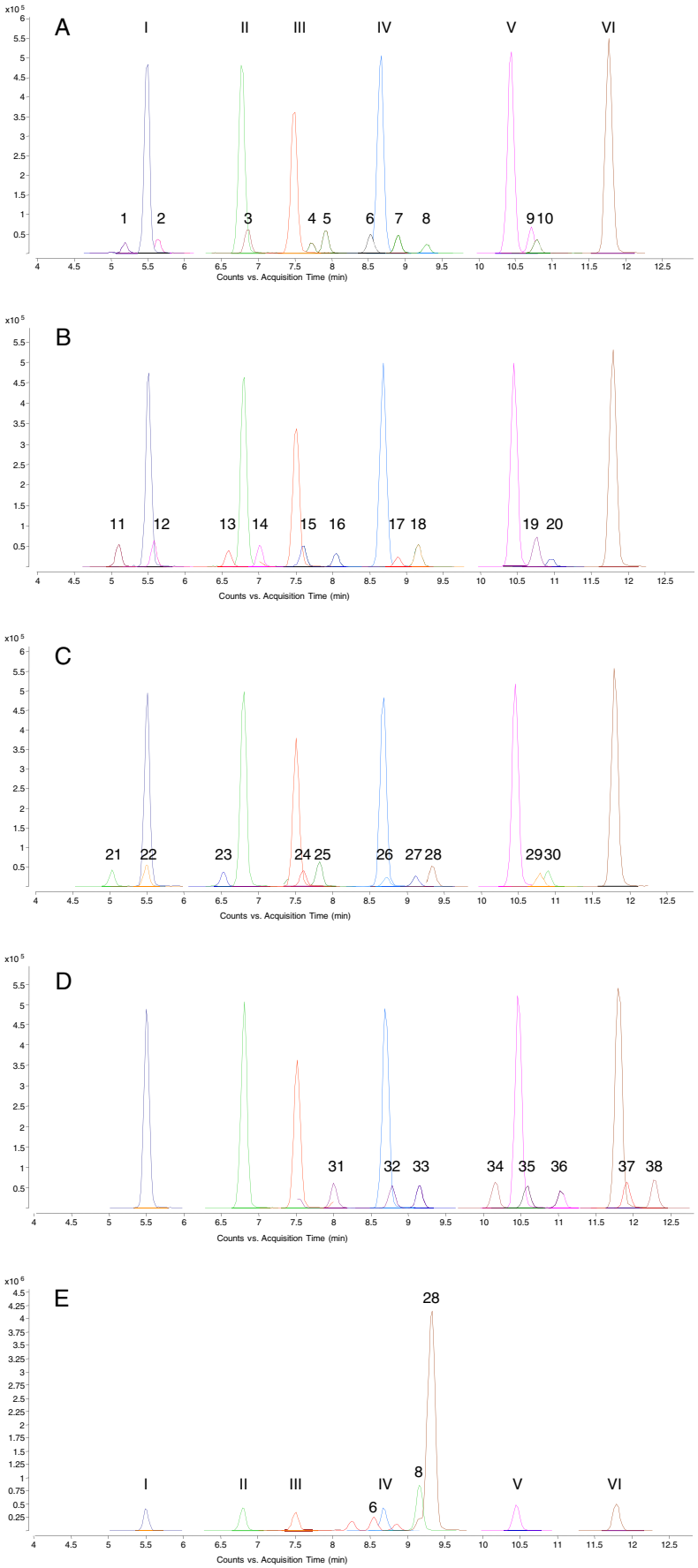

Fig. 1A–E Chromatograms from the four different urine calibrators at 2 ng/ml and a sample. **A** RCS-4 5-OH-pentyl (*1*), JWH-250 5-OH-pentyl (*2*), JWH-073 3-OH-butyl (*3*), JWH-018 5-OH-pentyl (*4*), UR-144 4-OH-pentyl (*5*), MAM-2201 4-OH-pentyl (*6*), JWH-019 5-OH-hexyl (*7*), JWH-122 5-OH-pentyl (*8*), JWH-210 4-OH-pentyl (*9*), JWH-398 4-OH-pentyl (*10*), and the internal standards: d5-JWH-250 4-OH-pentyl (*I*),d5-JWH-073 3-OH-butyl (*II*), d5-JWH-018 pentanoic acid (*III*), d7-JWH-073 6-OH-indole (*IV*), d9-JWH-018 6-OH-indole (*V*), d9-JWH-018 7-OH-indole (*VI*). **B** RCS-4 4-OH-pentyl (*11*), JWH-250 4-OH-pentyl (*12*), JWH-073 butanoic acid (*13*), AM-2201 4-OH-pentyl (*14*), JWH-018 4-OH-pentyl (*15*), UR-144 5-OH-pentyl (*16*), JWH-081 5-OH-pentyl (*17*), JWH-122 4-OH-pentyl (*18*), JWH-210 pentanoic acid (*19*), JWH-398 5-OH-pentyl (*20*). **C** RCS-4 pentanoic acid (*21*), JWH-250 pentanoic acid (*22*), JWH-073 4-OH-butyl (*23*), JWH-018 pentanoic acid (*24*), UR-144 pentanoic acid (*25*), JWH-081 pentanoic acid (*26*), JWH-019 6-OH-hexyl (*27*), MAM-2201 pentanoic acid (*28*), JWH-398 pentanoic acid (*29*), JWH-210 5-OH-pentyl (*30*). **D** AM-2201 6-OH-indole (*31*), JWH-073 6-OH-indole (*32*), JWH-073 5-OH-indole (*33*), JWH-073 7-OH-indole (*34*), JWH-018 6-OH-indole (*35*), JWH-018 5-OH-indole (*36*), JWH-018 7-OH-indole (*37*), AM-2201 7-OH-indole (*38*). **E** Authentic urine sample that was positive for MAM-2201 4-OH-pentyl (*6*), JWH-122 5-OH-pentyl (*8*), and MAM-2201 pentanoic acid (*28*)

(17 % ion suppression). Salting out resulted in 1.5–2-fold increase in sample concentration, depending on the analyte.

Because of the extensive conjugation of metabolites of synthetic cannabinoids in human urine, a hydrolysis step is necessary prior to analysis [14, 19, 38]. Unfortunately, at the time of the study, only one glucuronidated metabolite was available for testing. The recovery of JWH-018 5-hydroxy pentyl glucuronide following hydrolysis was estimated to be higher than 80 % based on a quantitative analysis against a prepared calibration curve. The imprecision of the hydrolysis over 10 days was 9.5 % based on the ratio of the analyte area to the internal standard area.

The stability of metabolites of synthetic cannabinoids has not been extensively studied. Among the methodological papers published so far, some have performed processed sample stability experiments [20, 35]. Stability is usually evaluated in qualitative methods using area ratios. In our study, we compared the analyte area and the internal standard area obtained from stored samples to those obtained from freshly prepared samples. All but one of the analytes showed variations of <15 % over 2 days at room temperature. The metabolite JWH-073 7-OH-indole showed a decrease of 25 % after 2 days. At +4 °C, all but two of the analytes showed good stability, with changes of <20 %. JWH-019 6-OH-indole increased by 46 % and RCS-4 5-OH-pentyl increased by 21 %. At −20 °C, all of the analytes were stable for up to 15 weeks (i.e., changes were <20 %), and all of the analytes were stable in the processed samples for up to 96 h when stored in the autosampler. Sundström et al. [35] reported instability in another hydroxylated JWH-073 indole in their processed sample experiments, whereas Wohlfarth et al. [20] did not report degradation for any of their analytes. To ensure method performance, samples should be analyzed within 2 weeks after arrival at the laboratory.

Our strategy utilizing a qualitative LC-QTOF-MS method has several advantages. Firstly, it will be less labor intensive to include new analytes as the drug panorama changes. The number of validation experiments required is reduced, and the method is more flexible [20]. Secondly, employing only a threshold calibrator allows daily calibration to be performed even though the chromatography takes a long time, so variations in instrument performance will be corrected for. The use of a daily calibrator to confirm a positive result has been suggested before for scenarios involving low concentrations and where small changes in instrument performance may affect results [26]. Thirdly, the TOF technique combines the capacity to perform untargeted data acquisition with excellent selectivity, which is important when many analogs and isobars are available as designer drugs and the potential for isomeric metabolites increases.

The 204 consecutive samples from April 2013 showed a positivity rate of 11 %, with a predominance of JWH-122 metabolites. This reflects the use of JWH-122 as well as the use of MAM-2201, since they may have metabolites in common (analogously to JWH-018 and AM-2201) [10]. In two cases, the unique MAM-2201 metabolite MAM-2201 4-hydroxy pentyl was found, confirming the use of MAM-2201. Similar reasoning can be applied to the 9 positive cases for UR-144 metabolites, since it has been shown that XLR-11 metabolizes to the UR-144 pentanoic acid as well as the 5-hydroxy metabolite [13]. In these samples, it is also clear that the use of JWH-018 or AM-2201 has decreased.

Conclusions

We conclude that there was fairly high cross-reactivity with MAM-2201 and JWH-122 metabolites for the immunoassay, but there was no cross-reactivity with the UR-144 metabolites at all. The rapid exchange of one cannabinoid for another may pose problems for any method, but immunoassays are generally becoming less useful for the screening of newly emerging drugs. We believe that the strategy of using a qualitative method and a sensitive and selective technique such as quadrupole time of flight mass spectrometry yields a flexible method that can be adjusted to the analytes that are currently used and available on the market. A concern is that good forensic practice is to have positive results from two different methods before reporting a positive sample. When moving straight to the confirmation method, that important step is omitted. Therefore, one aim is to develop a screening method using only accurate mass acquisition with an orthogonal, shorter chromatographic method.

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