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Accurate identification and quantification of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in urine drug testing: Evaluation of a direct high efficiency liquid chromatographic–mass spectrometric method

Nikolai Stephanson[a], Martin Josefsson[b], Robert Kronstrand[b], Olof Beck[a,*]

[a] Department of Medicine, Division of Clinical Pharmacology, Karolinska Institute and University Hospital, SE-171 76 Stockholm, Sweden
[b] Department of Forensic Genetics and Forensic Toxicology, The National Board of Forensic Medicine, Linköping, Sweden

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ABSTRACT

A direct liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method for measurement of urinary Δ^9-tetrahydrocannabinol carboxylic acid (THCA) was developed. The method involved dilution of the urine sample with water containing $^2H_9$-deuterated analogue as internal standard, hydrolysis with ammonia, reversed phase chromatography using a Waters ultra-performance liquid chromatography (UPLC™) equipment with gradient elution, negative electrospray ionization, and monitoring of two product ions in selected reaction monitoring mode. The measuring range was 2–1000 ng/mL for THCA, and the intra- and inter-assay imprecision, expressed as the coefficient of variation, was below 5%. Influence from urine matrix on ionization efficiency was noted in infusion experiments, but was compensated for by the internal standard. Comparison with established gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry methods in authentic patient samples demonstrated accuracy in both qualitative and quantitative results. A small difference in mean ratios (~15%) may be explained by the use of different hydrolysis procedures between methods. In conclusion, the high efficiency LC–MS/MS method was capable of accurately identify and quantify THCA in urine with a capacity of 14 samples per hour.

1. Introduction

Cannabinoids belong to the primarily detected class of illegal drugs in clinical and forensic settings, including workplace drug testing. It is well known that Δ^9-tetrahydrocannabinol (THC) is the main psychoactive component present in *Cannabis sativa* [1]. After cannabis intake, THC is metabolized mainly by cytochrome P450 enzymes in the liver and other tissues to numerous metabolites [2], and its most relevant metabolite for drug testing is 11-hydroxy-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCA) [3,4]. In phase II metabolism several glucuronides of cannabinoid metabolites are formed [5-7], including the ester-linked β-glucuronide of THCA [6], which is being eliminated in urine as a major metabolic end product of THC [8].

The first step in urine drug testing is immunochemical screening, which detects samples containing free and conjugated THCA. The screening positive samples are usually confirmed by a second, more specific technique, such as gas chromatography–mass spectrometry (GC–MS) [9–12]. The GC–MS methods require sample preparation by liquid–liquid (LLE) or solid-phase extraction (SPE) and derivatization following chemical hydrolysis of conjugates.

The possibility of using the alternative liquid chromatography–mass spectrometry (LC–MS) technology for analysis of illicit drugs (e.g., methamphetamine, amphetamine, ephedrine, methylephedrine, morphine, morphine-3-glucuronide, morphine-6-glucuronide, 6-acetylmorphine, cocaine, and benzoylecgonine) was first demonstrated using a solid-phase extraction together with thermospray ionization [13]. With the advent of increased selectivity and sensitivity when using LC–tandem MS (LC–MS/MS), the possibility of omitting derivatization for THCA analysis has been demonstrated [3,14,15]. Nevertheless, these procedures still included sample preparation with SPE or LLE.

The possibility of using direct injection of urine with LC–MS/MS was shown for cocaine and benzoylecgonine analysis [16]. However, this procedure was not validated using authentic urine samples and

* Corresponding author at: Karolinska University Hospital Solna, L7:05 Clinical Pharmacology, SE-171 76 Stockholm, Sweden. Tel.: +46 8 51773026; fax: +46 8 333819.
E-mail address: olof.beck@karolinska.se (O. Beck).

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in the later published method the same authors included SPE to minimize matrix effect [17]. The usefulness of direct injection of urine in combination with LC–MS/MS in performing confirmation analysis in urine drug testing has since been shown in several publications for the opiate and amphetamine class of drugs [18–20]. These studies have shown that direct injection of urine is viable in combination with LC–MS/MS. The application of this analytical strategy for THCA was even more challenging because of the more than 10-fold lower concentrations that must be measured.

Recent technological development of liquid chromatography equipment, which is operated at higher pressures has made reversed phase chromatography material with <2 μm particle size available. This technology offers highly efficient chromatography with significant advantages in resolution, speed and sensitivity for bioanalytical applications, particularly when coupled with high speed acquisition mass spectrometers [21]. This has already been used in pharmaceutical development [22,23], and for direct measurement of two serotonin metabolites in urine [24], and should also enable direct measurement of THCA in urine.

The aim of the present study was to develop a sensitive and specific direct LC–MS/MS method and validate it for identification and quantification of total urinary THCA for use in urine drug testing.

2. Materials and methods

2.1. Urine samples

Randomly selected and anonymous urine specimens were obtained from patient samples sent to the laboratory for routine drug testing. Blank urine was collected from healthy volunteers. The urine specimens were stored at +4 °C until analysis (maximum storage time, 7 days).

2.2. Chemicals

(±)-11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCA; molecular weight, 344.45 g/mol) and (±)-11-nor-9-carboxy-Δ^9-tetrahydrocannabinol-$^2H_9$ (THCA-$^2H_9$, internal standard; molecular weight, 353,38 g/mol) were obtained as methanolic stock solutions from Cerilliant Co. (Round Rock, TX, USA). All other chemicals were of analytical grade and ultra-pure water (>18 MΩ/cm) was used.

2.3. Instrumentation

The LC–MS/MS system consisted of a Waters Acquity UPLC (ultra-performance liquid chromatograph) with a vacuum degasser, binary pump, and sample manager at ambient temperature connected to a Quattro Premier XE tandem mass spectrometer with MassLynx™/Target Lynx™ Software version 4.1 (Waters Co., Milford, MA, USA). The electrospray interface was used with the instrument operating in the negative ion mode. Nitrogen was used as nebulizer, desolvation and cone gas, and argon as collision gas.

The liquid chromatography system was operated in a gradient mode with a flow rate of 200 μL/min (Table 1), giving a typical back pressure of 10,000–12,000 psi. Chromatography was performed using a 1.7-μm 100 mm × 1.0 mm (inner diameter) ethylene bridged hybrid (BEH) C_{18} column (Waters Co.), preceded by a 0.2 μm column filter (Waters Co.). Solvent A consisted of 0.1% (26.5 mmol/L) formic acid (pH 2.85) and Solvent B was 100% acetonitrile. The injection volume was 2 μL and the column oven temperature 60 °C. The strong injector wash solvent wash was 1200 μL of acetonitrile/water/formic acid (90/9.5/0.5, v/v/v) and the weak needle wash solvent was 700 μL of mobile phase A (0.1% formic acid). The total run time of the method was 4.2 min. The following conditions were used in the mass spectrometer: source temperature, 125 °C; desolvation gas temperature, 350 °C; capillary voltage, −3.7 kV; multiplier voltage, 650 V; extractor voltage, −3 V; RF lens voltage, −0.5 V; cone gas flow, 50 L/h; desolvation gas flow, 800 L/h; ion energy – 1, 0.5 V; ion energy – 2, 1.2 V; entrance and exit potential, −1 V and 1 V, respectively; collision gas flow, 0.20 mL/min. The selected ions, cone voltage, collision energy and dwell time used for each compound are presented in Table 2. The monitoring time was 1.5–3.0 min. Cone voltage and collision energy were optimized separately for THCA and the internal standard, using the autotune feature of the MassLynx software during direct infusion into the ion source. In order to obtain maximal sensitivity for THCA collision gas flow, ES source- and analyzer parameters were optimizes manually by injection of urine samples, containing low level of THCA.

Table 1
Gradient profile used for the separation of urinary THCA with the LC–MS/MS method

Time (min)	Mobile phase	
	Solvent A[a] (%)	Solvent B[a] (%)
0	80	20
2.0	10	90
3.0	10	90
3.1	80	20
4.2	80	20

[a] Solvent A consisted of 0.1% formic acid (pH 2.85) and solvent B of acetonitrile.

2.4. Analytical procedure for THCA

A 120-μL aliquot of each urine specimen was added to autosampler vials together with 60 μL of a water solution containing 200 ng/mL of the internal standard THCA-$^2H_9$. Thereafter 30 μL of 10 mol/L ammonia was added. The vials were capped, vortexed for ~30 s, and placed in thermo blocks to carry out hydrolysis at 100 °C for 15 min. After cooling to room temperature the vials were loaded on the sample manager. Mass spectrometric detection was performed using selected reaction monitoring (SRM) of two product ions for THCA and one product ion for the internal standard from the respective deprotonated molecules (Table 2).

Calibrators and controls of THCA were prepared by dilution of stock solutions with blank urine (pH 8.4 to secure maximal stability) [25]. Calibration samples and controls were stored at +4 °C until analysis (maximum storage time was 2 months). Three calibration levels in duplicate (0, 6 and 100 ng/mL THCA) and 3 control samples of low, medium and high levels (10, 150 and 800 ng/mL) to secure the linearity range were routinely used. Working solution of THCA-$^2H_9$ was prepared in water and stored at +4 °C until use (maximum storage time, 2 months). The THCA concentrations of unknown samples were determined from the peak area ratio by reference to the calibration curve between THCA and THCA-$^2H_9$. The criteria for identification was a relative ion intensity between qualifier and quantifier ions within ±20% of the target value and a relative retention time between analyte (both ions) and deuterated internal standard within ±1% of the target value. Target values for

Table 2
Mass spectrometric parameters for THCA in the UPLC–MS/MS method

Compound	Parent ion (m/z)	Product ion (m/z)	Cone (V)	Collision energy (eV)	Dwell time (s)
THCA	343.3	299.1	−40	27	0.07
	343.3	245.1	−40	20	0.07
THCA-$^2H_9$	352.3	308.2	−43	21	0.07

ion ratios and relative retention times were taken from the calibration standards and updated for each batch. The quantifier and qualifier detected transitions had a signal-to-noise ratio of >10 and >3, respectively.

2.5. Immunochemical assay

Urine samples were analyzed for THCA using CEDIA reagents (Microgenics, Passau, Germany). Assays were performed on a Hitachi 917 (Roche, Mannheim, Germany) according to the manufacturers instructions with 25 ng/mL as cut-off limit. Quality control was conducted using CEDIA Specialty Control Set (Microgenics, Passau, Germany). The inter-assay CV at the 13, 37.7, 30 and 61.4 ng/mL THCA control levels was <7.15% ($n > 20$).

2.6. GC–MS reference method

The THCA results were compared with an established GC–MS method, which involved sodium hydroxide hydrolysis, solid-phase extraction (SPE) and formation of silyl derivative prior to GC–MS analysis. A 2 mL aliquot of urine was mixed with an internal standard THCA-$^2H_9$ solution (0.15 μg) and hydrolyzed by addition of 100 μL 10 mol/L sodium hydroxide at 22 °C for 20 min. Subsequently, 1 mL of concentrated acetic acid was added and the pH was adjusted to between 3.0 and 4.0. The SPE cartridges (Bond Elut-Certify 3CC, C_8-strong cathion exchanger, 130 mg/3 mL) were used on an automated SPE Gilson Aspec XL4 (Gilson Inc., Middleton, WI, USA) and conditioned with 3 mL methanol followed by 3 mL deionized water and 1 mL 0.1 mol/L hydrochloric acid. The specimens were applied followed by washing with 2 mL deionized water and 2 mL 0.1 mol/L hydrochloric acid/acetonitrile (70:30, v/v). The cartridges were eluted with 3 mL of freshly prepared hexane/ethylacetate (85:15, v/v). After evaporation the dried eluates were treated with 60 μL of MSTFA at 60 °C for 30 min.

The GC–MS system was an Agilent Technologies series 6890 GC consisting of an autosampler, connected to a quadrupole 5973MS with chemstation Software version D.01.00 (Agilent Technologies Inc., GmbH, Germany). Split injection (1:15) was performed using 1 μL injection volume. Chromatographic separation of THCA was achieved on a 0.25 μm 30 m × 0.25 mm (i.d.) J&W DB-1701 column (Agilent Technologies Inc., St. Clara, CA, USA) with initial oven temperature at 260 °C for 1 min followed by temperature increase at a rate of 10 °C up to 300 °C. The total run time was 5.5 min. Electron ionization (70 eV) was used and ions monitored in the selected ion monitoring mode were (m/z) 488.2, 473.3, 371.2 for THCA and 497.2, 380.2 for THCA-$^2H_9$. The measuring range of the GC–MS method was 2–3000 ng/mL (by dilution) THCA and the intra- and inter-assay coefficients of variation (CV) were <9% and <13%, respectively at levels of 8.8 ng/mL ($n = 10$) and of 87 ng/mL ($n = 17$), respectively. The limit of detection (LOD) of the method was 0.6 ng/mL (signal-to-noise ratio of 3). This method was in routine use and approved by SWEDAC (www.swedac.se) for accreditation according to ISO 17025 and by CAP (College of American Pathologists, www.cap.org) for FUDT. The applied reporting limit for the present study was 6 ng/mL for THCA. The THCA calibration standards from the GC–MS method were analyzed with the UPLC–MS/MS method, to ensure comparable calibration of both methods.

2.7. LC–MS reference method

Comparison was also made with an LC–MS method established at the Forensic Toxicology laboratory. The method involved sodium hydroxide hydrolysis and solid-phase extraction (SPE) prior to quantification. Using a TECAN Genesis 150 sample robot (Mannedorf/Zurich, Switzerland) a 0.95 mL aliquot of urine was mixed with 0.05 mL of the internal standard THCA-$^2H_9$ solution (0.1 μg) and 0.5 mL of 2 mol/L sodium hydroxide. Samples were then hydrolyzed in oven at a temperature of 60 °C for 20 min. Subsequently, 0.5 mL of concentrated acetic acid in water (50:50 v/v) was added (pH 5). The SPE cartridges (Isolute C_8, 100 mg/3 mL) were used on an automated SPE Gilson ASPEC XL4 robot (Gilson Inc., Middleton, WI, USA) and conditioned with 2 mL acetonitrile followed by 2 mL 1% formic acid in water. The specimens were applied as two aliquots of 0.5 mL followed by washing with 2 mL 1% formic acid in water and 2 mL acetonitrile/acetone/1% formic acid in water (15:15:70, v/v/v). The cartridges were eluted with 0.6 mL of acetonitrile/diethyleter (50:50, v/v) directly into 1.5 mL Micro-V vials (National Sci., Duluth, GA, USA) prepared with 100 μL 1% formic acid in water. The excess of solvents (i.e., diethylether) was evaporated with a gentle stream of nitrogen to a final volume of about 150 μL in each vial.

The injection volume was 10 μL. The chromatographic separation was performed using a Zorbax SB-Phenyl column 50 mm × 2.1 mm I.D. with 3.5 μm particles size (Rockland Technologies, USA). The LC–MS system consisted of a Perkin Elmer 200 HPLC system (Norwalk, CT, USA) connected to a Sciex API 150 EX single quadrupole instrument equipped with a Turbo Ion-Spray interface (Toronto, Canada). The liquid chromatography system was operated in an isocratic mode with a flow rate of 0.25 mL/min and the column oven temperature at 50 °C. The mobile phase consisted of an acetonitrile and water mixture in a ratio of 50:50 (v/v) containing 0.2% formic acid. Mass spectrometric detection was performed using electrospray ionization in negative mode at an ion-spray voltage of −3500 V and nebulizer gas temperature of 400 °C. Ions, formed by in-source collision induced dissociation (CID), monitored in the selected ion monitoring mode, were (m/z) 343.2, 299.2 for THCA and 352.3, 308.2 for THCA-$^2H_9$. The measuring range of the LC–MS method was 5–1000 ng/mL THCA. The intra- and inter-assay coefficients of variation (CV) for THCA at levels; 5, 20 and 100 ng/mL were <11%. The limit of detection (LOD) of the method was 0.9 ng/mL (signal-to-noise ratio of 3). This method was in routine use and approved by SWEDAC (www.swedac.se) for accreditation according to ISO 17025. The applied reporting limit for the present study was 6 ng/mL for THCA.

2.8. Method validation

Calibration curves covering 0–1000 ng/mL for THCA at concentration levels of 0, 10, 50, 100, 300, 500, 700, 1000 ng/mL in two replicates were studied in the validation study.

The influence of matrix was evaluated using post column continuous infusion of a water solution of THCA (10 μg/mL) at a constant flow rate of 10 μL/min. Urine samples were injected with either gradient (Table 1) or isocratic (20% solvent B) elution at a flow rate of 200 μL/min. A second experiment involved comparison of the response for internal standard (THCA-$^2H_9$) in 4 calibrators (water matrix) with the response for 26 randomly selected urine samples containing the same amount of internal standard.

The following substances were spiked into blank urine (5 mg/mL) to study possible in reference: morphine, codeine, ethylmorphine, amphetamine, methamphetamine, MDMA, MDA, MDEA, MBDB, benzylpiperazine, phenylethylamine, ephedrine, methcathinone, cathinone, phenmetrazine, phentermine, ritalinic acid, methylphenidate, fenfluramine, methadone, benzoylecgonine, propoxyphene, phenylpropanolamine, desmethyldiazepam, oxazepam, temazepam, alprazolam, 7-amino-flunitrazepam and 7-amino-nitrazepam.

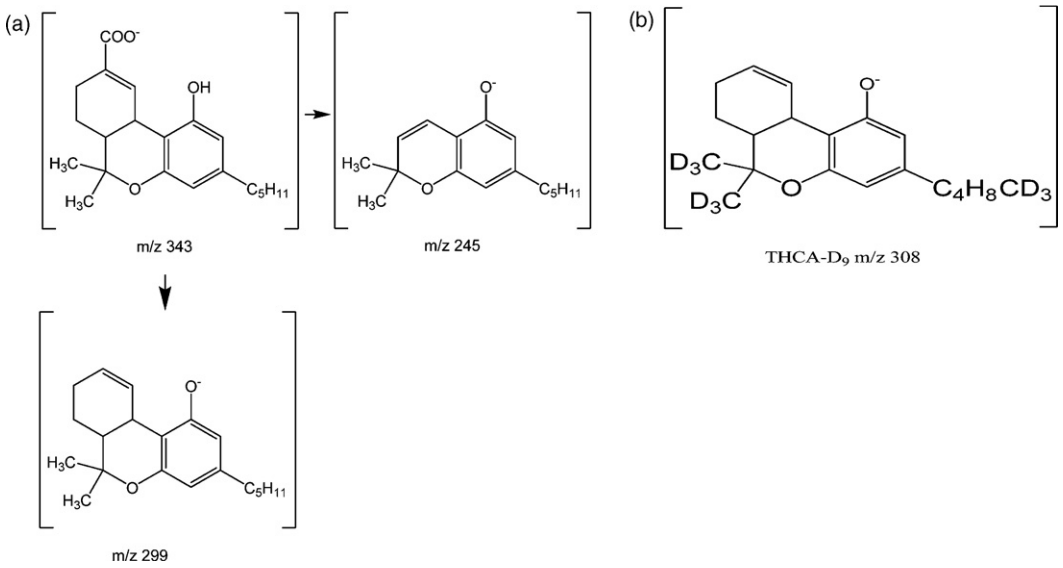

Fig. 1. Proposed chemical structures of the product ions for THCA (a) and internal standard (b) monitored in the method.

Three blank urine samples collected from the healthy subjects were used to study the analytical recovery of THCA. The samples were spiked with known amounts of THCA and the concentrations were measured.

The limit of detection (LOD; signal-to-noise ratio of 3) and the limit of quantification (LOQ; signal-to-noise ratio of 10) was determined by analyzing a series of prepared urine samples containing THCA between 0.5, 1.0, 2.0, 5.0 and 10 ng/mL.

Urine extracts were prepared in duplicate from three authentic patient urine samples (THCA levels, 14.2, 72.2 and 297.1 ng/mL) and were used to study the stability of THCA during storage at 22 °C. After storage for 3 days, the extracts were reanalyzed.

3. Results

3.1. Mass spectrometric conditions

Initial experiments indicated that electrospray ionization in the negative mode, detecting the deprotonated molecule $[M-H]^-$ for THCA, gave a stronger response than positive electrospray ionization. The two most intense product ions for THCA and the most intense product ion for the internal standard were used for identification and quantification. Other product ions were observed at *m/z* 258, 203, 169, 163 and 147 (Fig. 1). Furthermore, the presence of ammonia in the injected urine extract reduced the background signal by about 30%. Thereby, an increased signal-to-noise ratio was obtained.

3.2. Chromatographic conditions

A suitable chromatography was achieved on a C_{18} analytical column with a capacity factor of about 5.6 for THCA (Fig. 2). THCA eluted at a retention time of about 2.25 min with a peak width of 0.06 min (4 s). Gradient elution was chosen over isocratic elution, because of shorter retention times and sharper peaks, which resulted in higher sensitivity and less interference from neighboring peaks (Fig. 2). In routine use, a total analysis time of 4.2 min was used to achieve column equilibration between

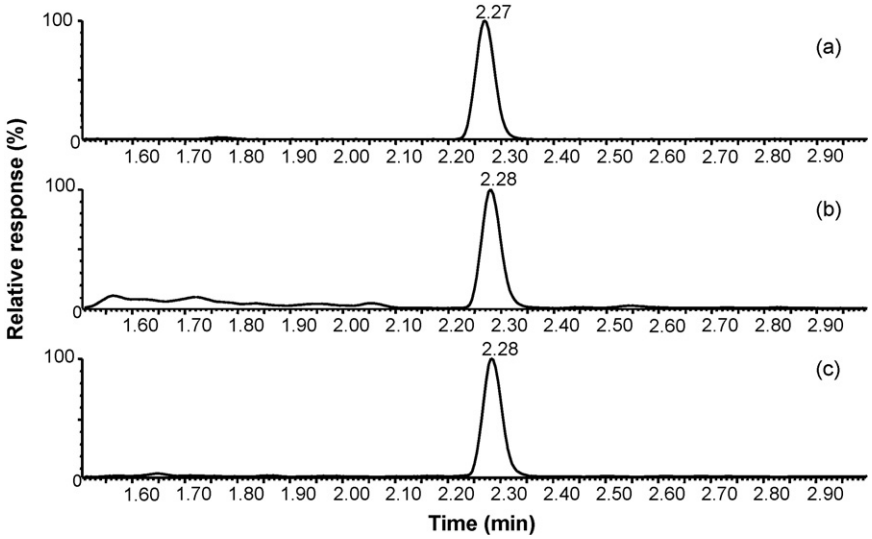

Fig. 2. UPLC–MS/MS chromatograms showing the peaks for THCA-$^2H_9$ ((a) *m/z* 352.25–308.2) and THCA ((b) *m/z* 343.3–299.1; (c) *m/z* 343.3–245.1) on the UPLC acquity C_{18} analytical column for a human urine sample found to contain 10 ng/mL THCA.

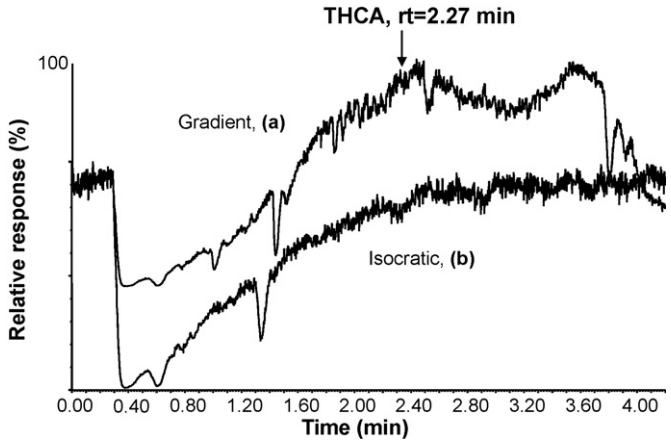

Fig. 3. Effect of injecting a prepared blank urine sample with gradient (a) and isocratic (b) elution on the MS response of an infused THCA solution.

injections. The capacity of the method was thereby 14 injections per hour. The column could be used for between 3000–5000 injections without any loss in performance.

3.3. Evaluation of matrix effects

The maximum loss in response (~50% for gradient elution and ~99% for isocratic elution) occurred at the time corresponding to the column void volume (Fig. 3). The time required for the ion intensity of THCA to return to its pre-sample injection level was about 1.5 min in gradient elution and further ion enhancement up to 40% occurred as the acetonitrile amount was increased up to 90% at 2 min. In contrast, for isocratic elution, the time required for the ion intensity to return to its pre-sample injection level was about 2.4 min.

In the addition experiment the peak area response for the 26 authentic urine samples were on average 36% lower for THCA-$^2H_9$ than in the water matrix calibrators, demonstrating an ion suppression effect (Table 3).

3.4. Method validation

A linear correlation between the concentration and the area ratio to the internal standard was obtained for THCA up to 1000 ng/mL ($r^2 = 0.999$; equation: $y = 0.00645x - 0.034$, where y is the area ratio between THCA and internal standard, x is the THCA concentration). The LOD and LOQ were estimated to be 0.2 and 0.7 ng/mL, respectively and the same results were obtained at all concentrations from 0.5 to 10 ng/mL. The values obtained for intra- and inter-assay CV for THCA were <5% (Table 4), and were documented according to CLSI guidelines [26]. The values of quality control (QC) samples observed over 4 weeks ($N = 30$ for each level) for the method in routine use for THCA were 10.0% (mean, 8.12 ng/mL); 11.2% (mean, 107.6 ng L/mL) and 6.7% (mean, 614.7 ng/mL). The carry-over in the LC–MS/MS system, as determined after injection of a urine sample spiked with a high concentration of THCA (1000 ng/mL), was <0.03%.

No interference was observed from other possibly occurring drugs of abuse (see list above).

The analytical recoveries calculated from spiking blank urine with THCA at four different levels were 99.4% at 10 ng/mL, 100.9% at 150 ng/mL, 98.2 at 800 ng/mL and 105% at 1000 ng/mL ($N = 3$–5).

No change (<6.5%) in the THCA concentration (THCA levels, 15.1, 74.1 and 305.1 ng/mL) could be observed after storage of extracts for 3 days in room temperature. However, the mean THCA peak area decreased by 7.8%. Stability of THCA during storage at 4 °C was documented for the calibrators and control samples for at least 2 months. The concentration decrease observed was within the method imprecision (<7%).

Table 3
The MS relative response in 26 urine samples for the internal standard THCA-$^2H_9$

	THCA-$^2H_9$ urine	THCA-$^2H_9$ water
N	26	4
Range (%)	49–87	94–103
Mean (%)	64	100
Median (%)	60	102
S.D.	11.7	4.1
CV (%)	18.1	4.1

Table 4
Quantification imprecision for urinary THCA

Mean THCA concentration (ng/mL)	Within-run CV[a] (%)	Between-run CV[a] (%)	Total CV[a] (%)
14.7	4.2	2.1	4.7
153.6	4.4	0	4.4
975.8	2.4	0	2.4

[a] Analysis was performed with three replicates per run over 5 days.

3.5. Method comparison

A comparison with the reference GC–MS method was done using 116 authentic patient urine samples, which were positive in the immunochemical screening assay for THCA. The qualitative results, using reporting limit of 6 ng/mL, had an overall 98.3% agreement (Table 5). The reporting limit of 6 ng/mL was applied to fit the screening cutoff at 25 ng/mL and not due to method limitation. Two deviating samples were over this reporting limit (6.1 and 6.0 ng/mL) with the LC–MS/MS method and the same samples were classified negative (5.7 and 5.0 ng/mL) with the GC–MS method. The results for 107 urine samples, covering the concentration range of 2–2624 ng/mL of THCA showed a high correlation ($r^2 = 0.962$; $y = 1.005x - 7.907$, where y = LC–MS/MS and x = GC–MS; Fig. 4a) between LC–MS/MS and GC–MS methods. The mean ratio for THCA between LC–MS/MS and GC–MS results was 0.93 (95% CI, 0.89–0.98). The mean ratio between product ions m/z 245/299 was 0.28 ± 6.1% (CV), $N = 107$.

A comparison with the reference LC–MS method was performed using 70 forensic urine samples. The qualitative results for all 70 samples using a reporting limit of 6 ng/mL were in total agreement (Table 6). For 57 samples covering the concentration range between 2 and 1000 ng/mL of THCA a high correlation ($r^2 = 0.991$; $y = 0.812x + 2.514$, where y = LC–MS/MS and x = LC–MS; Fig. 4b) between the LC–MS/MS and the LC–MS methods was obtained. The mean ratio for LC–MS/MS over LC–MS was 0.86 (95% CI, 0.82–0.89).

A number of 17 patient samples were selected by being negative in the immunochemical screening assay and confirmed not to

Table 5
The qualitative results between UPLC–MS/MS and GC–MS using 116 authentic patient urine samples, positive in the immunochemical assay for THCA

GC–MS	UPLC–MS/MS	
	Negative	Positive
Negative	11	2[a]
Positive	0	103

[a] Positive with the UPLC–MS/MS method (over reporting limit) and negative with the GC–MS method (under reporting limit).

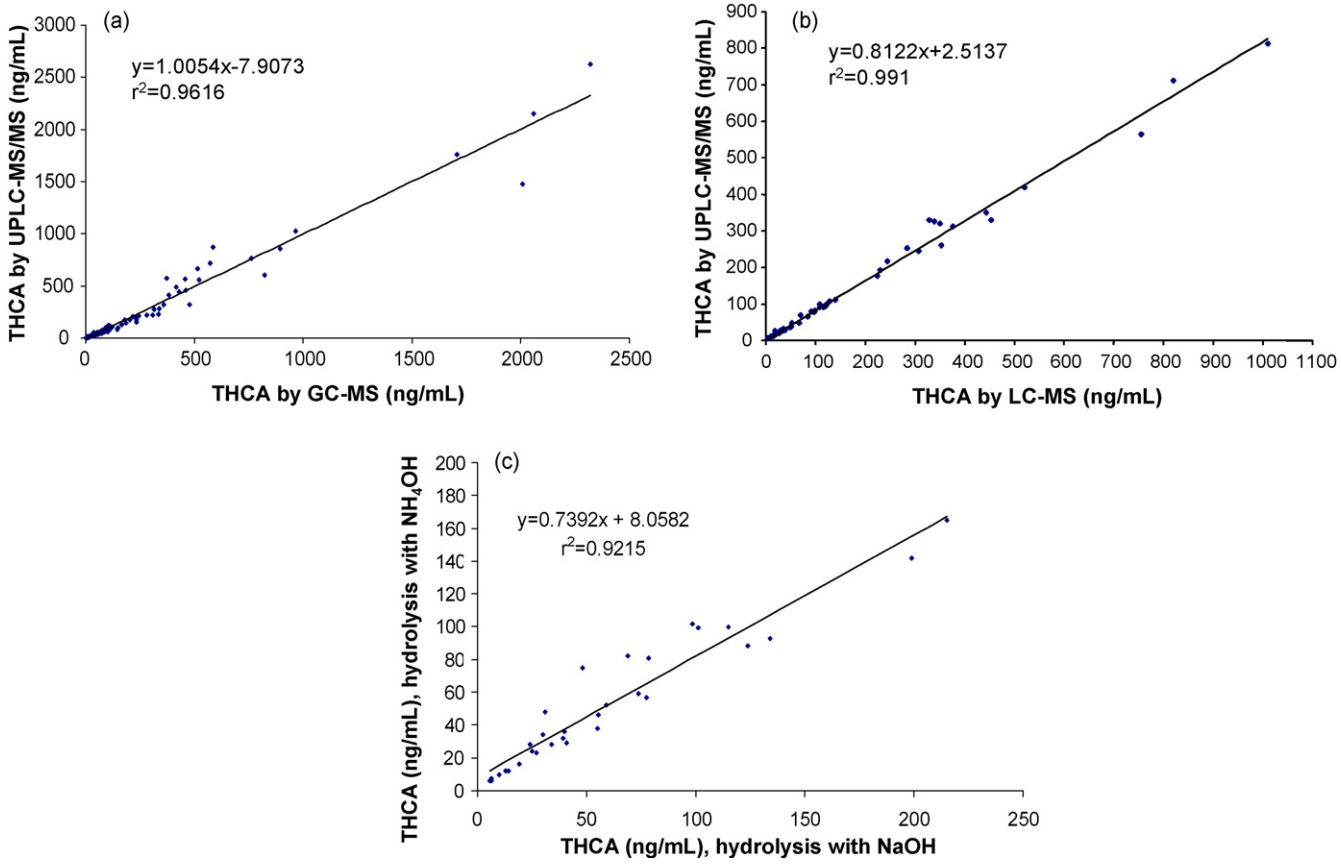

Fig. 4. (a) Correlation between the THCA values obtained with the GC–MS method and the UPLC–MS/MS method for 107 human urine samples. (b) Correlation between the THCA values obtained with the LC–MS method and the UPLC–MS/MS method for 57 human urine samples. (c) Correlation between the THCA values obtained after hydrolysis with sodium hydroxide and ammonia in 31 human urine samples using the GC–MS method.

contain any detectable THCA levels by GC–MS. No detected THCA or chromatographic interferences were observed in the LC–MS/MS analysis of these samples.

3.6. Hydrolysis efficiency

Thirty-one authentic urine samples were analyzed by GC–MS with either ammonia or sodium hydroxide hydrolysis. The qualitative results for all 31 samples covering the concentration range 13–215 ng/mL were similar with both hydrolysis methods. The results obtained after each of the hydrolysis methods showed a high correlation ($r^2 = 0.922$; $y = 0.739x + 8.058$, where y is the hydrolysis with ammonia and x is the hydrolysis with sodium hydroxide; Fig. 4c). The mean ratio between ammonia and sodium hydroxide hydrolysis procedures was 0.94 (95% CI: 0.86–1.02).

THCA was analyzed in triplicate in an authentic patient urine sample either without hydrolysis or with hydrolysis after different time intervals at 100 °C. Maximum hydrolysis efficiency was obtained after 15 min (Fig. 5).

Table 6
The qualitative results between UPLC–MS/MS and LC–MS using 70 authentic patient urine samples, positive in the immunochemical assay for THCA

LC–MS	UPLC–MS/MS	
	Negative	Positive
Negative	2	0
Positive	0	68

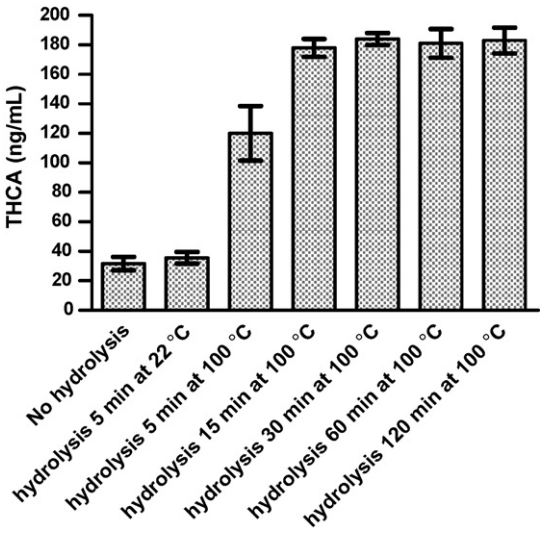

Fig. 5. The THCA values in triplicate of a human urine, obtained either without hydrolysis or with hydrolysis after different time intervals using ammonia and the UPLC–MS/MS method.

4. Discussion

This study demonstrates that direct injection of urine in combination with electrospray ionization LC–MS/MS is a possible analytical approach for confirmatory purposes of preliminary pos-

itive results from the immunochemical screening of THCA in urine drug testing.

The availability of atmospheric ionization techniques, such as electrospray and chemical ionization, and the direct coupling of liquid chromatography to mass spectrometry, has resulted in increased analytical sensitivity and selectivity with great potential for clinical laboratories [27]. LC–MS/MS techniques have already been applied for a wide array of substances, including illicit and therapeutic drugs [18–20,28,29]. Furthermore, the strategy for direct injection of diluted urine into LC–MS/MS offers great potential for routine drug testing laboratories as the turn-around time can be shortened by the elimination of extraction and derivatization procedures [19,20,24,30]. The benefit of the UPLC technology was helpful in this study, particularly for enabling determination of THCA in the lower concentration levels. In comparison, the previous LC–MS/MS methods [3,14,15] for measurement of urinary THCA required an initial sample preparation using LLE or SPE. However, the procedure still included chemical hydrolysis but this could be smoothly incorporated into procedure as it was performed directly in autosampler vials.

A major disadvantage of direct injection of urine samples into LC–MS is that analyte signal can be affected by the sample matrix components [31]. Earlier, it was generally assumed that the highly selective LC–MS/MS technique permits the use of short chromatographic retention times and minimal or eliminated sample clean-up procedures [32]. Contrary to this common belief, those conditions can easily cause serious matrix effect problems affecting analyte response and sensitivity [33]. Suppression of electrospray ionization response caused by matrix components often limits the level of lower quantification, precision and accuracy [33,34]. As a consequence, assessment and elimination of matrix effects for a bioanalytical method is recommended [33]. Several experiments were performed to assess the matrix effect in this study. The post column infusion of THCA into the MS detector with a simultaneous injection of a urine matrix showed that the most intense response loss (50% in gradient elution and 99% in isocratic elution) occurred immediately after the column void had eluted. This experiment allowed determining the extent of the effect of endogenous components present in the matrix on the analyte response as a function of chromatographic retention time [34]. The recovery time was, however, much shorter in gradient elution than in isocratic. Furthermore, gradient gave ion enhancement of THCA compared to isocratic elution. That may be caused by increased ion transfer efficiency from the liquid to gas phase in the ES ion source at 90% acetonitrile concentration with gradient elution [35]. The results from the second experiment in this study, where the response of internal standards in 26 patients urine samples were compared to the area of internal standards in water, demonstrated the variable influence of different matrix components on the analyte signal. To correct for variations in ion response and to minimize influence from matrix effects a stable isotope labeled analogue was used as internal standard [36]. A potential disadvantage by this approach is that the internal standard may contain traces of the non-labeled compound as an impurity [36] and therefore the purity of the internal standard was determined by analyzing blank samples. Due to the presence of THCA as an impurity (0.2%) in the internal standard, the calibration level of blank urine sample was used to correlate the calibration curve for analyte presence in the internal standard.

As LC–MS methods become more frequent for quantitative confirmatory analysis of drugs of abuse, guidelines for criteria for identification of compounds have been published by several organizations [37]. Several different rules for the identification of analytes based on identification points (IP) from the diagnostic ions and requirement for correct intensities between these have been suggested [38]. In previous LC–MS methods describing THCA analysis in urine [39,40] electrospray ionization, solid-phase extraction with C_{18} cartridges and selected ion monitoring mode was used. In these methods monitoring of three ions per analyte with the suggested criteria for ion intensity ratio [38] were used allowing for confidence in identification, as a minimum of three IP is required [38]. Subsequently, with more frequent use of LC–MS/MS, a number of other issues, e.g. requirement of sample preparation, chromatographic resolution and detection of multiple analytes (metabolites) is very important to support identification [37,38]. It has been observed that monitoring of two product ions in LC–MS/MS may lead to false identification indicating that sample preparation, chromatographic resolution and criteria for ion intensity ratios are critical elements [41]. The identification of THCA in the present study fulfilled the recommendation for monitoring of at least two transitions for substance detection (the second as a qualifier) in SRM which is required by several guidelines [38]. The chromatogram from blank urine, spiked with substances, which are likely to be present in patient samples, did not show any interfering peaks, neither was the chromatographic resolution affected. Furthermore, patient samples with negative results from immunochemical screening assay were in agreement with both GC–MS and LC–MS/MS results.

The high correlation obtained between LC–MS/MS and GC–MS and between LC–MS/MS and LC–MS, respectively, and an overall 98.3% agreement in qualitative results between LC–MS/MS and GC-/MS and a 100% agreement between LC–MS/MS and LC–MS, respectively, indicates that the LC–MS/MS method is equivalent to the both reference methods. The two deviating samples, classified as negative with the GC–MS method (5.0 and 5.7 ng/mL) and positive with the LC–MS/MS method ($\geq$6 ng/mL) were related to the imprecision of the two methods. This study also confirms the usefulness of LC–MS when combined with sample preparation for enabling accurate identification in urine drug testing.

Investigations of THCA excretion in humans [42] have shown that only a small amount of THCA (about 1%) is excreted as unconjugated. However, there may be inter-individual variations in the excretion profiles between conjugated and unconjugated THCA. Since glucuronide conjugated THCA is not available as reference material it cannot be used as analyte for routine measurement at present. In addition, instability of the conjugate form might also refrain its use as analyte. For GC–MS and LC–MS analysis of the unconjugated THCA in human urine, it is necessary to include a procedure for cleaving the conjugated (glucuronide) moiety. In both reference methods alkaline hydrolysis, followed by SPE was used. Since sodium hydroxide is less suitable for direct injection to LC–MS, ammonia was chosen for LC–MS/MS. THCA concentrations obtained with both GC–MS and LC–MS were repeatedly found to be slightly higher than the THCA concentrations by LC–MS/MS. This difference in the measurements must be due to a difference in hydrolysis efficiency between sodium hydroxide and ammonia. The difference in result between procedures may be related to the fact that two different glucuronide conjugates of THCA occur [43] and that they differ in resistance to hydrolysis.

5. Conclusion

The present study demonstrated for the first time that direct analysis of THCA in urine, being used as a marker for cannabis intake, can be performed by the validated LC–MS/MS method. The simplicity of this method, achieved by direct injection of urine, should have advantages over earlier GC–MS and LC–MS methods using SPE procedures [3,14,37,39,40] or LLE procedures [15].

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