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Short communication

On-line solid-phase extraction combined with liquid chromatography–tandem mass spectrometry for high throughput analysis of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in urine

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ABSTRACT

A simple, rapid and highly sensitive method for the analysis of THC-COOH in urine, using automated on-line solid-phase extraction (SPE) combined with liquid chromatography (LC)–mass spectrometry (MS/MS), is developed and fully validated according to international guidelines. Chromatographic separation was achieved on an Atlantis dC_{18} column with an isocratical gradient, ensuring the elution of THC-COOH within 4.1 min. The total process time was 6 min and 500 μL of sample was required. SPE using C_8 cartridges was highly effective, reproducible and led to significant decreases in the interferences present in the matrix. The method showed an excellent intra- and inter-assay precision (relative standard deviation (RSD) <7% and bias <13%) for four external quality control (QC) samples and three 'in house' QCs. Responses were linear over the investigated range ($r^2 > 0.99$, 5–200 μg/L). Limits of quantification (LOQ) and detection (LOD) were determined to be 5 μg/L and 0.25 μg/L, respectively. Furthermore, the analyte and the processed samples were demonstrated to be stable. Moreover, no carryover was observed after the analysis of high concentrated urine samples (5000 μg/L THC-COOH)). The method was subsequently applied to authentic samples previously screened by a routine immunoassay method.

1. Introduction

High-throughput analysis is becoming increasingly important in all areas of science; the forensic sciences being no exception. Moreover, due to the development of more potent drugs, drug concentrations in biological samples are often present at much lower levels than before. Therefore, fast analytical techniques with higher sensitivity and selectivity are needed. One of the bottlenecks in bio analysis is sample preparation, especially if the method requires manual extraction techniques. An elegant system for the rapid analysis of complex samples can be obtained by on-line coupling of SPE to LC–MS detection. With this procedure, the sample is injected directly into the SPE-MS system and the rate-limiting off-line extraction step is eliminated. As a result, automation leads to higher sample throughput and increased sensitivity as the whole sample extract is analyzed and not a fraction. Other advantages are safer sample handling and improved precision as operator intervention is minimized [1].

Cannabis is one of the most widely used illicit drug in the world, being the most frequently detected drugs in cases of driving under the influence of drugs (DUID) in several countries [2]. Cannabis use is detected by identifying the presence of the major psychoactive constituent of marijuana, Δ^9-tetrahydrocannabinol (THC) or its metabolites in biological fluids. The major metabolite found in urine is 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), which exists in both the free and glucuronide form [3–5]. Analytical procedures are well-documented for determining the presence of THC-COOH in urine using gas chromatography–mass spectrometry (GC–MS(/MS)) [6–10], liquid chromatography–mass spectrometry (LC–MS(/MS)) [1,6,11–13] and immunoassays [14–17]. However, to date, no report has been published dealing with the analysis of THC-COOH in urine by any type of on-line SPE-LC–MS/MS.

Therefore, the aim of this study was to develop and validate a simple, rugged and high-throughput on-line SPE-LC–MS/MS method for quantification of THC-COOH in urine.

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2. Materials and methods

2.1. Reagents

Individual stock solutions of THC-COOH (1 mg/mL in methanol), and the internal standard (IS) [$^9H_3$] THC-COOH (THC-COOH-d_9) (0.1 mg/mL in methanol) were from LGC Promochem (Molsheim, France).

Water (HPLC grade), methanol (LC–MS grade), 0.1% formic acid in water (UPLC/MS grade) and acetonitrile (LC–MS grade) were purchased from Biosolve (Valskenswaard, The Netherlands). Potassium hydroxide (powder), triethylamine, (puriss.p.a.) and trifluoroacetic acid (puriss.p.a.) were purchased from Sigma–Aldrich (Steinheim, Germany). HySphere C_8 cartridges were from Cofraz (Elsene, Belgium). Glacial acetic acid was from VWR (Leuven, Belgium).

External quality control (QC) urine samples (Medidrug U-screen cut-off −25% and +25%) were obtained from Medichem World (Steinenbronn, Germany). Liquicheck external quality controls C1 and C3 for urine were purchased from Bio-Rad Laboratories (Irvine, CA).

2.2. Specimens

Blank urine samples were obtained from drug-free volunteers. Authentic urine samples were obtained from forensic toxicology cases.

2.3. Preparation of standard solutions for calibrators and QC samples

Two different working solutions of the non-deuterated compound at 10 mg/L in methanol were prepared. The first was used for preparation of the calibrators and the second for the 'in house' QC samples. The internal standard (IS) working solution of 1 mg/L was prepared in methanol. Working solutions were prepared monthly and stored at 4 °C. The 'in house' QCs were stored at −20 °C until use.

The external QCs were prepared following the indications of the manufacturer. Each vial of the Medidrug U-screen controls was reconstituted exactly with 5.0 mL of bidistilled water, swirled gently and allowed to equilibrate for 20 min at room temperature (RT). Before sampling, the vial was gently homogenized for 5 min using a rotation mixer. After reconstitution, the QCs were stable for 7 days (at 2–8 °C in the dark). The Liquicheck controls were equilibrated to RT and swirled gently to ensure homogeneity before sampling. Once the control was opened, it was stable for 30 days when stored tightly capped at 2–8 °C.

2.4. SPE-LC–MS/MS

2.4.1. Sample preparation: hydrolysis

Fifty microliters of potassium hydroxide 10 M and 50 μL of the IS working solution (0.25 mg/L) were added to 500 μL of urine and the samples were incubated at 60 °C for 15 min. Similar hydrolysis procedures have been described by other authors [1,18]. Samples were cooled to RT before the addition of 300 μL distilled water. Before injection onto the on-line SPE system, the samples were acidified by adding 600 μL of glacial acetic acid.

2.4.2. XLC (on-line SPE)

Sample extraction was performed using the on-line SPE Symbiosis™ Pharma System (Spark Holland™, Emmen, The Netherlands). The entire system was operated by SparkLink for Masslynx™ software (version 4.1, Waters).

The following XLC program was used: after conditioning with 2 mL of methanol and 1 mL of water, 200 μL of the diluted and acidified urine sample was applied to the SPE cartridge using 2 mL of water as transport solvent. Clean-up was accomplished through successive 2 mL washes of 0.1% formic acid, and methanol:0.1% formic acid (50:50, v/v) in order to remove salts and endogenous interferences present in the biological samples. Elution of the analytes from the cartridge was achieved by application of the LC mobile phases (0.1% formic acid (A) and acetonitrile (B)) (standard (gradient pump) elution mode) during the chromatographic run. Whilst the elution step was being performed, a new cartridge was conditioned, loaded and washed in the left clamp. Following the elution step, several automated clamp and valve washes with water, 0.2% triethylamine and 0.1% trifluoroacetic acid were performed to avoid carryover between samples.

2.4.3. Chromatographic conditions

Analytes were separated using an Atlantis dC_{18} column, 3 μm, 3 mm × 100 mm (Waters). Separation was carried out in isocratic mode (0.1% formic acid:acetonitrile, 20:80, v/v). The complete run time was 6 min.

2.4.4. Tandem mass spectrometry

A Quattro Premier tandem mass spectrometer (Waters) was used. Ionization was achieved using electrospray in positive ionization mode (ESI+). Nitrogen was applied as nebulisation and desolvation gas at a flow rate of 600 L/h and heated to 350 °C. Capillary voltage and source block temperature were 3 kV and 120 °C, respectively.

In order to establish the appropriate multiple reaction monitoring (MRM) conditions for the individual compounds, solutions of standards (200 μg/L, in 0.1% formic acid–acetonitrile (20:80, v/v)) were infused into the mass spectrometer and the cone voltage (CV) was optimised to maximise the intensity of the protonated molecular species $[M+H]^+$. Collision-induced dissociation (CID) of each protonated molecule was performed. The collision gas (argon) pressure was maintained at 0.35 Pa (3.5×10^{-3} mbar) and the collision energy (eV) adjusted to optimise the signal for the most abundant product ions, which were subsequently used for MRM analysis (Table 1).

2.5. On-line SPE-LC–MS/MS assay validation

Validation was performed based on the FDA guidelines and recent publications concerning validation of bio analytical methods [19,20].

Table 1
MRM transitions and conditions for THC-COOH and its deuterated analogue. Underlined transition was used for quantification.

Compound	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
THC-COOH	345.2	<u>299.3</u> 193.1	30	20 30
THC-COOH-d_9	354.2	308.4	30	20

2.5.1. Linearity, limit of quantification (LOQ), limit of detection (LOD), precision and bias

It is known that the concentration of drugs in urine can vary considerably (e.g. from some μg/L to few thousands of μg/L of THC-COOH) depending on the individual and the time of collection, In routine urine toxicological analysis, it is necessary to fix the quantification range depending on the cut-off established in the laboratory. The aim in our toxicological laboratory is to determine the presence of THC-COOH in urine with respect to the Belgian legal cut-off of 15 μg/L. Thus, the established quantification range has been determined to guarantee a good quantification around this concentration.

Quantification was performed by integration of the area under the specific MRM chromatograms in reference to the integrated area of the deuterated analogue. Freshly prepared working solutions of 1000 μg/L and 100 μg/L in water were used to prepare urine calibrators at a concentration of 200, 100, 50, 20, 10 and 5 μg/L using HPLC-grade water. Standard curves, freshly prepared with each batch of QC samples and authentic samples, were generated using a least-squares linear regression, with a $1/x$-weighting factor.

The limit of quantification (LOQ) was estimated by replicate analysis ($n = 2$) over 8 different days and was defined as the concentration of the lowest calibrator that was calculated within ±20% of the nominal value and with a relative standard deviation (RSD) less than 20%.

The limit of detection (LOD) was estimated from blank urine samples, spiked with decreasing concentrations of the analyte. It was defined as the concentration for which the response of the qualitative ion could reliably be differentiated from background noise, i.e. signal-to-noise ratio (S/N) equal to or greater than 3:1. The acceptance criteria for ion ratios equal to or lower than 20% and retention time deviations lower than 3.5% relative to that of the corresponding control or calibrator.

Seven QCs were analyzed, four external QCs (two each from Medichem and Bio-Rad Laboratories) and three 'in house' QCs.

Intra- and inter-assay imprecision was evaluated by replicate ($n = 2$) analysis of the QC samples performed over eight different days. Imprecision (expressed as $\%RSD_r$ for intra-assay imprecision and $\%RSD_t$ for inter-assay imprecision) was determined by performing the analysis of variance: a 'single factor' ANOVA test (significance level (α) of 0.05). Bias of the method was determined by comparison of the mean of calculated concentrations of QC samples to their respective nominal values.

2.5.2. Selectivity and specificity

The selectivity and specificity of the method against endogenous interferences was verified by examination of the chromatograms obtained after the extraction of eight different blank urine samples from healthy volunteers, and after the analysis of authentic urine samples from cocaine and amphetamine users. Moreover, blank urine samples ($n = 3$) spiked with amphetamine, methamphetamine, MDA, MDMA, ephedrine, PMA, mCPP, morphine, codeine, benzoylecgonine, codeine, 6-MAM, fentanyl, pholcodine, hydromorphone, hydrocodone, norcodeine, dihydrocodeine, oxycodone, oxymorphone, cocaine, methadone, EDDP, 27 benzodiazepines, zolpidem, zopiclone, zaleplon, THC and 11-OH-THC were also analyzed to check for interferences.

2.5.3. Stability

The autosampler stability of processed samples at concentrations of 160 μg/L and 15 μg/L ($n = 6$ at each concentration) was monitored as follows; one pool of samples were determined immediately, while another pool of samples was analyzed after remaining in the autosampler at 6 ± 2 °C for 24 h and at RT for 72 h (a weekend). All samples were spiked with the IS just before analysis.

Stability of THC-COOH in the matrix was determined through spiked blank urine samples with concentrations of 160 μg/L and 15 μg/L ($n = 6$ at each concentration). Stability was checked after storage at 2–6 °C for 72 h (weekend) and after three freeze/thaw cycles.

All the stability experiments were tested against a lower percentage limit corresponding to 90–110% of the ratio (mean value of stability samples/mean value control samples) with a 90% of the confidence interval of the stability samples between 80 and 120% of the mean of the control samples.

2.5.4. Assessment of matrix effects

To assess any potential suppression or enhancement of ionization due to the sample matrix, two different experiments were carried out.

The first one involved a post-column infusion experiment [21]. This experiment was based on a continuous post-column infusion of THC-COOH and its internal standard (10 μg/L at a flow rate of 10 μL/min) to produce a constant response in the MRM channels. This constant response was monitored throughout the whole run following the injection of urine samples from different origin ($n = 6$) and compared to the response following the injection of mobile phase only.

The second experiment consisted of a comparison between the peak responses of THC-COOH spiked to a blank urine sample at concentrations of 160 μg/L and 15 μg/L ($n = 6$, for each concentration) with those obtained after being spiked in the mobile phase at the same concentration levels [22].

2.5.5. Recovery

Extraction recoveries were estimated by performing the following experiments: blank urine samples spiked at 160 μg/L and 15 μg/L ($n = 6$, for each concentration) were loaded and washed in a first SPE cartridge while a second cartridge was placed in series to determine the breakthrough of the first one. Both cartridges were subsequently eluted independently. Recovery was considered to be the ratio between the response obtained after elution of the first cartridge and the total response (sum of both, the first and the second SPE cartridge).

2.5.6. Carryover

Carryover was evaluated by the analysis of blank urine samples spiked with the IS after the analysis of the upper calibrator (200 μg/L, $n = 8$), after the analysis of authentic urine samples from cannabis users ($n = 8$) and after the analysis of a highly concentrated sample (5000 μg/L).

2.5.7. Dilution integrity

Spiked blank urine samples at 3200 μg/L and 600 μg/L were rediluted 1:20 (v/v) with blank urine ($n = 6$) and analyzed to evaluate the dilution integrity.

3. Results and discussion

The method was validated for selectivity, linearity, LOQ, LOD, imprecision, bias, analyte and processed sample stability, matrix effect, recovery, carryover and dilution integrity.

The applied chromatographic method ensured the elution of THC-COOH within 4.1 min and produced peaks of acceptable symmetry. Selectivity of the method was achieved by a combination of retention time, precursor and two product ions. The most prominent precursor-product transition was used for quantification and the next most abundant as qualifier (Table 1). For the corresponding deuterated analogue, only one transition was monitored. Fig. 1 shows the MRM chromatograms obtained following the analysis of the urine

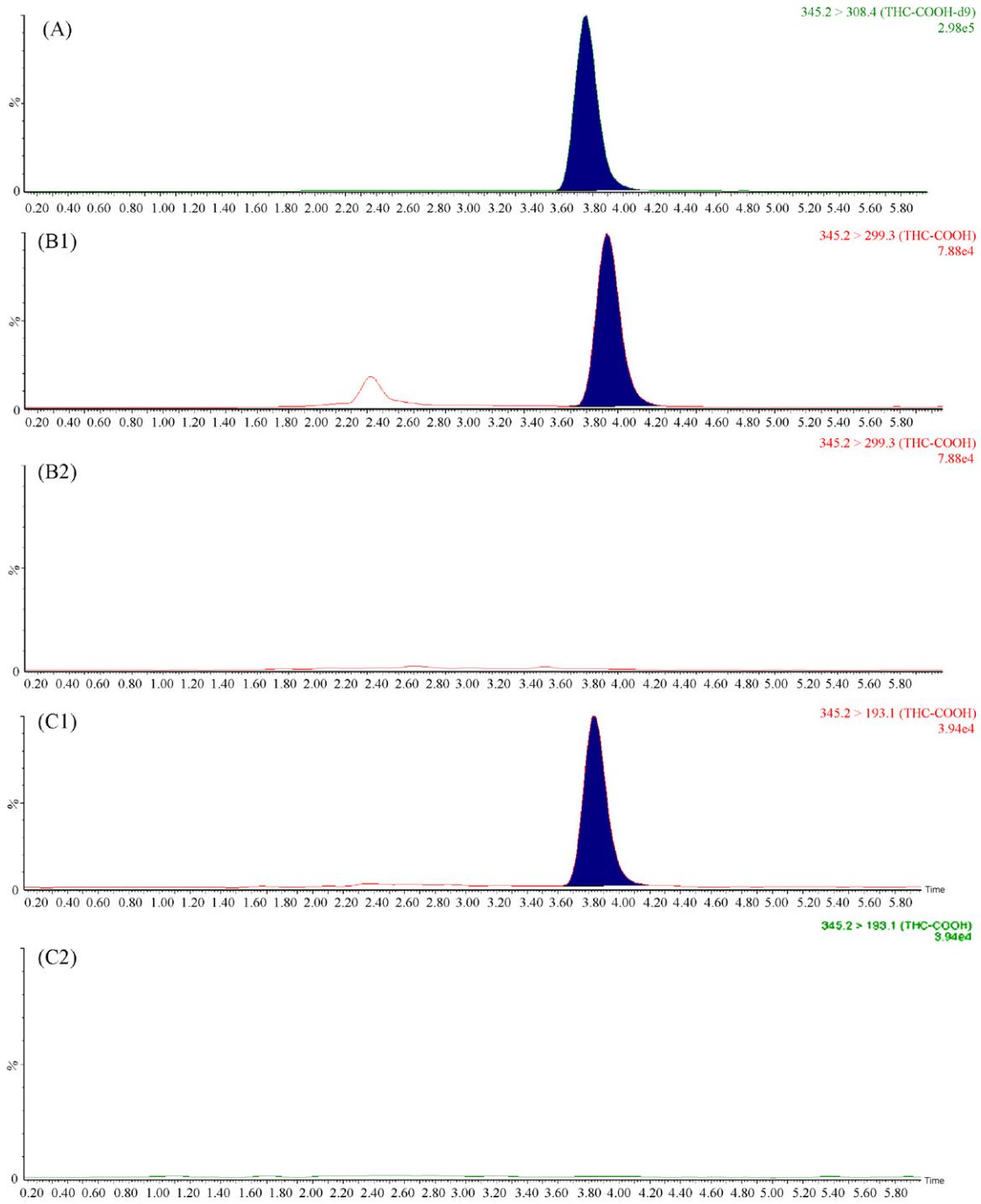

Fig. 1. MRM chromatograms obtained following the analysis of a spiked urine sample with 5 μg/L (LOQ) and a blank urine injected after the highest calibrator: (A) IS, (B1) quantifier of THC at LOQ, (B2) quantifier of THC in the blank urine, (C1) qualifier of the LOQ and (C2) qualifier of the blank urine. Peak intensity is shown in the top right-hand corner of each trace. Blank urine samples are displayed at the same peak intensity of the LOQ.

lowest calibrator (5 μg/L) and a blank injected after a high concentrated sample. No interferences were observed after the analysis of blank urine samples spiked with over—the counter-drugs and cannabinoids, ensuring the selectivity of the method.

During pre-validation experiments, the linearity was tested up to 5000 μg/L ($r > 0.99$). However, due to the legal cut-off in Belgium for THC-COOH, which is 15 μg/L, the quantification range applied in the laboratory was 5–200 μg/L. Correlation coefficients of the weighed ($1/x$) linear regressions for this range were higher than $r^2 = 0.99$.

The LOQ was determined at 5 μg/L as a S/N > 10:1 was observed for the qualifier and the criteria for LOQ were satisfied. LOD was 0.25 μg/L.

The intra- and inter-assay imprecision were satisfactory, with all RSDs lower than 7% (Table 2). The results indicated that the bias of the assay was <13%.

Stability of the processed samples in the autosampler was monitored after 24 (at 2–6 °C) and 72 h (RT). No instability was observed during this period of time. Moreover, THC-COOH spiked to blank urine samples was also stable after the three freeze/thaw cycles and after 72 h in the fridge.

Table 2
Intra-assay (expressed as RSD_r (%)) and inter-assay precision (expressed as RSD_t (%)) and bias of the LOQ and QC urine samples. Intra-assay, inter-assay precision and bias were evaluated by replicate ($n = 2$) analysis of the QC samples performed over eight different days.

	Nominal value (μg/L)	Average (μg/L) ($n = 16$)	RSD_r (%) ($n = 16$)	RSD_t (%) ($n = 16$)	Bias (%) ($n = 16$)
C1	6.0	6.4	2.9	4.1	7.2
C3	18.5	20.1	1.9	2.8	8.8
Medichem cutoff −25%	37.5	34.3	5.2	5.8	−8.5
Medichem cutoff +25%	62.5	55.1	1.9	5.1	−11.8
'In house' QC low	15.0	13.1	6.2	6.8	−12.3
'In house' QC medium	80.0	79.8	2.0	4.4	−0.3
'In house' QC high	160.0	156.8	2.9	5.4	−2.0

Post-column infusion experiments were performed to provide information of the matrix effect throughout the course of the elution time for the analyte and its IS. No significant changes in response were observed. The second experiment performed to assess matrix effects compared the peak area responses, obtained when the compound was spiked into blank urine samples, with the responses obtained when the compound was added to mobile phase at the same concentration. No significant matrix effect (mean 3.6%) was observed with this on-line SPE procedure.

Moreover, for the recovery studies, no breakthrough was observed in the second cartridges placed in series, so recovery was 100%.

No carryover was observed in the analysis of a blank urine sample injected after the analysis of the upper calibrator (200 μg/L), neither after the analysis of authentic urine samples or after a highly concentrated sample (5000 μg/L).

The dilution integrity test demonstrated a bias <2% and an RSD (%) of 13.4% and 11.6% for the diluted blank urine samples at 3200 μg/L and 600 μg/L, respectively.

4. Samples

Thirty-four urine samples from cannabis users were analyzed in one run with the present method. Concentrations varied considerably. Those samples with concentrations above the upper calibrator were diluted 1/20 with blank urine and reanalyzed. The median was 652 μg/L with minimum–maximum range of 12.1–3681.

5. Conclusions

In this report a fully validated and highly sensitive automated LC–MS/MS method is described for the analysis of THC-COOH in urine. The method combined on-line SPE with LC–MS/MS and provided a thorough clean-up of the matrix in combination with high recovery, excellent precision and bias within the investigated linear range. The method was successfully applied to authentic samples from cannabis users.

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