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# Temporal indication of cannabis use by means of THC glucuronide determination

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**According to the regulations of the World Anti-Doping Agency (WADA), the use of cannabinoids is forbidden in competition. In doping controls, the detection of cannabinoid misuse is based on the analysis of the non-psychoactive metabolite 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (carboxy-THC). The determination of values greater than 15 ng/mL in urine represents an adverse analytical finding; however, no accurate prediction of the time of application is possible as the half-life of carboxy-THC ranges between three and four days. Consequently the detection of carboxy-THC in doping control urine samples collected in competition might also result from cannabis use in out-of-competition periods. The analysis of the glucuronide of the pharmacologically active delta 9-tetrahydrocannabinol (THC-gluc) may represent a complementary indicator for the detection of cannabis misuse in competition.**

**An assay for the determination of THC-gluc in human urine was established. The sample preparation consisted of liquid-liquid extraction of urine specimens, and extracts were analysed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Authentic doping-control urine samples as well as specimens obtained from a controlled smoking study were analysed and assay characteristics such as specificity, detection limit (0.1 ng/mL), precision (>90%), recovery (~80%), and extraction efficiency (90%) were determined.** 

**Keywords:** cannabis; doping; urine; liquid chromatography mass spectrometry; sport

## Introduction

Estimating the time of cannabis (marijuana, hashish) or tetrahydrocannabinol (THC) use is important in assessing accident investigations, in verifying accuracy of court testimony and for clinical evaluations. Using Huestis's[1] concept of the normalization of drug excretion to urine creatinine concentration, Fraser and Worth monitored the ratio of the non-psychoactive metabolite 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (carboxy-THC, Figure 1(4)) to creatinine in several studies in order to determine whether consecutive urine specimens (collected at least 24 h apart), which tested positive for cannabinoids, would allow differentiation between new marijuana use and the excretion of residual carboxy-THC.[2–4] Further improvement of the method was attempted by including the active cannabinoid metabolite 11-hydroxy-THC (Figure 1(3)) in the evaluation.[5] Manno and co-workers estimated the temporal indication of marijuana use in plasma and urine from concentrations of THC, 11-hydroxy-THC and carboxy-THC. A urinary concentration of THC greater than 1.5 ng/mL was suggested as an indicator for marijuana use during the previous 8 h time period.[6] Skopp *et al*. investigated free and glucuronidated cannabinoids in 135 urine specimens collected from 49 volunteers up to 10 days after abstaining from cannabis use to assess the extent of cannabis use.[7] For frequent users, THC-glucuronide (THC-gluc, Figure 1(2)) and 11-hydroxy-THC glucuronide were found up to one and three days after abstinence, respectively.[7] Recently, Lowe and co-workers showed the presence of THC for up to 24 days in urine obtained from chronic cannabis users; 11-hydroxy-THC and carboxy-THC were detectable for more than 28 days.[8] With occasional users, neither THC-gluc nor 11-hydroxy-THC glucuronide were detectable.[7] On the one hand, the determination of conjugated THC and 11-hydroxy-THC in addition to free and bound carboxy-THC was suggested as an aid in assessing the frequency of cannabis use but this is limited to samples obtained within a few days after the last consumption.[7] On the other hand, Skopp's investigations concerning the stability of carboxy-THC glucuronide in spiked and authentic urine and plasma samples[9,10] showed a high lability of the glucuronide at a storage temperature of 4 °C and above. Consequently interpretations based on the presence of carboxy-THC and carboxy-THC glucuronide in stored urine samples appears difficult.[10]

Matrices, including saliva,[11] sweat[12] and hair are being utilized for monitoring cannabis use in treatment, employment, and criminal justice settings.

Carboxy-THC is the non-psychoactive major urinary metabolite of cannabis.[13] Its terminal urinary excretion half-life time was estimated as 1.3–2.5 and 3–4 days in heavy marijuana users[14,15] and substantial intersubject variability between subjects and drug doses was noted.[16]

The use of cannabinoids is prohibited in sports competition. Carboxy-THC is presently the target analyte for the detection of cannabis misuse in doping control-urine samples. The

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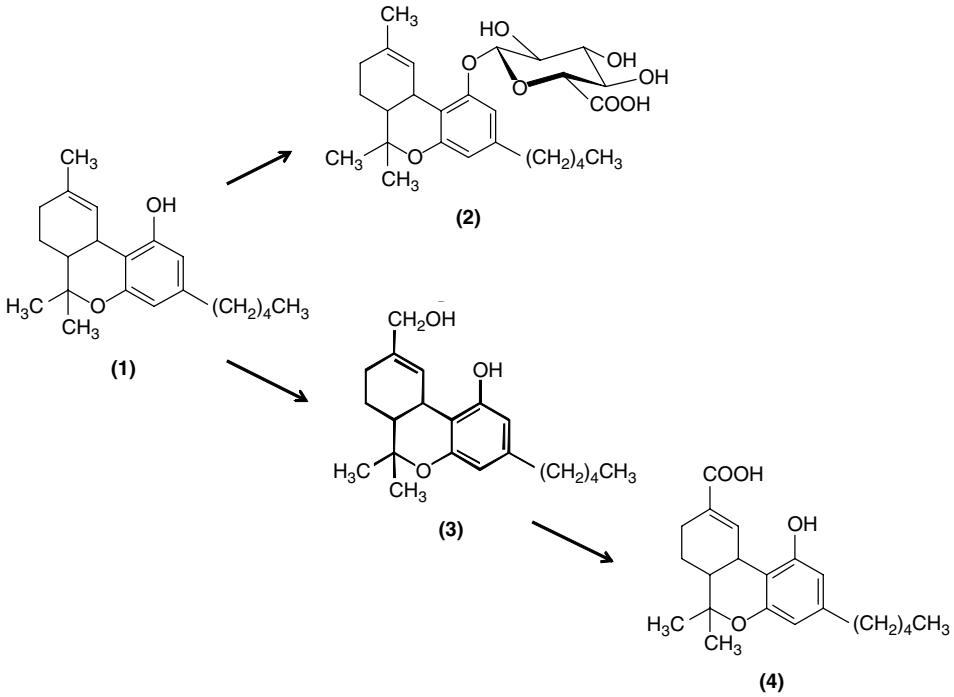


**Figure 1.** Major metabolic route for delta-9-tetrahydrocannabinol (THC) **(1)** including THC-glucuronide **(2)**, the active metabolite 11-hydroxy-THC **(3)** and the inactive metabolite carboxy-THC **(4)**.

reporting threshold is fixed at 15 ng/ml.[17] It is well known that concentrations higher than 15 ng/ml for urinary carboxy-THC are possible for a long time period after the use of cannabis. Occasional users of marijuana have had positive urine specimens for 3–4 days after receiving a standard dose of marijuana.[6,16,18] Urine specimens of heavy marijuana users have remained positive for cannabinoids for 7–10 days after the last drug use.[6,16,18] Accordingly, in frequent users[7] or following controlled multiple drug administration studies,[6,19] the detection of carboxy-THC was possible over a much longer time period. Consequently, predicting time of last cannabis use based on the carboxy-THC analysis by gas chromatography-mass spectrometry (GC-MS) in single urine specimens is complicated. This also means that an adverse finding of an in-competition doping control urine sample may derive from cannabis use out of competition. During the period 2003–2008[20–25] cannabis was reported as one of the most frequently detected substances by the WADA-accredited anti-doping laboratories worldwide with a percentage of adverse analytical findings ranging between 9% and 15.7%.

For a temporal indication of marijuana use, the pharmacologically active THC metabolite 11-hydroxy-THC or THC seem to be the more suitable target analytes, both excreted in urine mainly as glucuronic acid conjugates. Methods for the determination of THC and 11-hydroxy-THC using liquid chromatography/tandem mass spectrometry[26] or gas chromatography/mass spectrometry[6,7,27–32] in urine[6,7,27,28] and plasma[26,29–32] were established but the completeness of the hydrolysis was not proved due to the lack of the reference standards THC-gluc and 11-hydroxy-THC glucuronide.[7]

As the reference material delta-9-THC glucuronide is now commercially available, an assay for the temporal indication of cannabis was attempted by determining THC-gluc as target analyte in urine specimens using liquid chromatography/tandem mass spectrometry (LC-MS/MS). Various aspects such as detection limit, precision, recovery, extraction efficiency and specificity were validated.

Authentic urine samples and specimens obtained from a controlled smoking study were analysed and the suitability of THC-gluc as target analyte for the temporal indication of cannabis use with a suggested threshold value of 2.3 ng/mL (corresponding to 1.5 ng/mL for THC – based on Manno[6]) was discussed. The metabolism following cannabis ingestion shows in general strong variation between individuals, which is also connected with consumption frequency. The threshold value should be a useful tool to avoid 'false positive' findings for cannabis in in-competition doping control urine samples (derived from cannabis use out-of-competition), whereas 'false negative' findings are also possible and cannot be excluded.

No specific gravity correction of the threshold for THC-gluc was taken into consideration. This was in accordance with the currently effective WADA Technical Document TD2009MRPL, where no adjustment of the threshold for carboxy-THC is recommended.

Resulting from the fact that heavy cannabis use is probably not compliant with high performance sport, this item is explicitly excluded.

## Experimental

### Delta-9-THC glucuronide

Delta-9-THC glucuronide (a controlled substance) was obtained from ElSohly Laboratories, Inc. (Oxford MS, USA).

### Internal standard

Deuterated THC-gluc is not currently commercially available. **$D_9$**-carboxy-THC was chosen as internal standard because of similar physiochemical properties to THC-gluc. The diagnostic specific ion

transition *m/z* 352/308 was used for the detection of the internal standard.

### Chemicals and reagents

All solvents and reagents were of analytical grade purity. The internal standard **$d_9$**-carboxy-THC was purchased from LGC Promochem (Wesel, Germany). All solutions and buffers were prepared using deionized water (Water Lab System, Millipore, Eschborn, Germany).

### Sample preparation

Urine samples (2 mL) were fortified with 2 ng of the internal standard **$d_9$**-carboxy-THC and 1 mL of 5 mM ammonium acetate containing 0.1% glacial acetic acid (pH 3.5). Five millilitres of *tert*-butyl methyl ether (TBME) were added, the mixture shaken for 5 min and subsequently centrifuged at 600 *g* for 5 min. The organic layer was transferred to a fresh glass tube, evaporated to dryness under reduced pressure at 50 °C using a rotary evaporator and the dry residue was reconstituted in 100 µL of a mixture of acetonitrile/ammonium acetate buffer (1 : 4) and transferred into HPLC vials. A volume of 10 µL was injected into the LC-MS/MS.

### Liquid chromatography/tandem mass spectrometry

All samples were analysed by LC-MS/MS employing an Agilent 1200 series liquid chromatograph coupled to an Applied Biosystems API 4000 Qtrap mass spectrometer. The LC was equipped with an Agilent Zorbax Eclipse XDB-C8 column (4.6 × 150 mm, 5 µm particle size), and the eluents used were (1) 5 mM ammonium acetate buffer containing 0.1% of glacial acetic acid (pH = 3.5), and (2) acetonitrile. A gradient was employed from 10% B to 100% B within 8 min, and the column was re-equilibrated at 10% B for 2.5 min. The flow rate was set to 800 µL/min.

The ion source was operated in the negative mode (5500 V) at an interface temperature of 450 °C and THC-gluc was detected by means of characteristic product ions at *m/z* 113, *m/z* 175 and *m/z* 313, formed from the deprotonated molecule by electrospray ionization (ESI) utilizing the multiple reaction monitoring mode (MRM). The ion transition *m/z* 352/308 was used for the detection of the internal standard **$d_9$**-carboxy-THC.

The collision gas was nitrogen at a nominal pressure of $3.5 \times 10^{-3}$ Pa (obtained from a K75-72 Whatman nitrogen generator) and collision offset voltages were optimized for each product ion.

### Excretion study urine samples

In a one-session clinical trial, 11 healthy, male volunteers (age 26 ± 3 years, BMI 24 ± 2 kg/m$^2$) with cannabis experience (≤ once/month), who had given written consent, smoked a cannabis cigarette standardized to 70 mg THC/cigarette (Bedrobinol® 7%, Dutch Office for Medical Cannabis) following a paced-puffed procedure. Plasma and urine were collected up to 8 h and 11 days, respectively.

The volunteers left the closed clinical research unit 8 h after the study was started. The issue of self-administration was considered and taken care of by selecting the subjects very carefully regarding reliability, thus minimizing the potential risk of smoking cannabis when not under observation. In addition, uncontrolled cannabis consumption would have been detected by altered urine excretion profiles, such as concentration spikes.

The urine specimens were stored frozen until analysis. The excretion study was performed in agreement with the ethical commission of the University of Bern (Switzerland).

### Authentic doping control urine samples

A total of 83 doping-control urine samples were analysed, received between 2007 and 2009 from national and international federations, taken in- and out-of-competition, indicating suspicious screening results for carboxy-THC. The specimens were stored frozen until analysis.

### Determination of carboxy-THC in human urine[33]

Carboxy-THC was analysed by means of GC-MS according to established and accredited procedures, following enzymatic hydrolysis (*E.coli*), extraction with *tert*-butyl methyl ether and derivatization with MSTFA/NH4I/ethanethiol; **$d_9$**-carboxy-THC was used as the internal standard. The estimation of the concentration was performed using one-point calibration utilizing the peak area ratios of analyte (*m/z* 371.20) and internal standard (*m/z* 374.20). The LLOQ for carboxy-THC was estimated at 4.4 ng/mL.

### Assay validation

*Specificity*

Ten different blank urine specimens obtained from five female and male healthy volunteers each were prepared and analysed as described above in order to probe for interfering peaks in the selected ion chromatograms at the expected retention time of THC-gluc.

*Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)*

The LLOD was defined as the 'lowest content that can be measured with reasonable statistical certainty' at a signal-to-noise ratio ≥ 3, while the LLOQ represents the lowest quantifiable amount of an analyte, which is characterized by a signal-to-noise ratio ≥ 9.[34] Ten blank urine samples spiked with the internal standard (ISTD) only, and ten blank urine specimens fortified with 0.5 ng of THC-gluc per mL were prepared and analysed according to the established protocol providing the data necessary to estimate the LLOD and LLOQ.

*Linearity*

Two calibration curves for THC-gluc were generated using six calibration points at 0.3, 1, 2, 3, 4 and 5 ng/mL, respectively and ten calibration points at 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 ng/mL. The peak area ratios of analyte and ISTD were used to calculate the correlation coefficient, intercept and slope. The source of the spiked urine specimens was a blank urine obtained from a healthy female volunteer.

*Intraday precision*

On one day, six urine samples of LLOQ (0.3 ng/mL), low (1 ng/mL), medium (5 ng/mL) and high (10 ng/mL) concentrations of THC-gluc were prepared and analysed and the intraday precision was calculated for each concentration level.

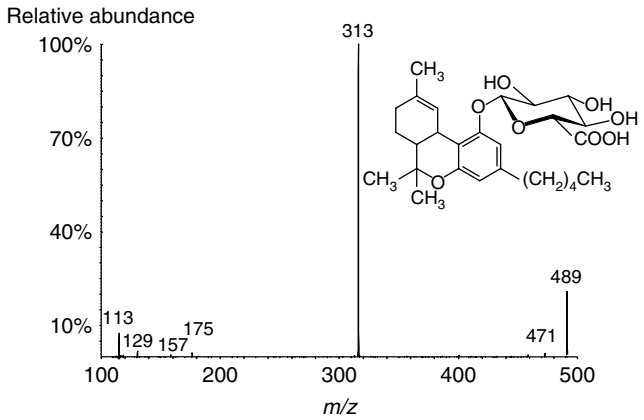


**Figure 2.** Product ion mass spectrum of THC-glucuronide $[M-H]^- = m/z$ 489 recorded on an Applied Biosystems API 4000 triple quadrupole mass spectrometer using a CE of −35 eV.

*Interday precision*

On three consecutive days, six urine samples of low (1 ng/mL), medium (5 ng/mL) and high (10 ng/mL) concentrations of THC-gluc were prepared and analysed randomly. The assay precision was calculated for each concentration level.

*Recovery*

The recovery of the method was determined from urine aliquots, which were prepared and analysed for the interday precision. The results obtained were correlated with the calibration curve.

*Extraction efficiency*

The extraction efficiency of the method was calculated by comparison of the peak area ratios from six commonly fortified urine samples (at 5 ng/mL) and six urine samples that were fortified with the same amount after sample preparation prior to evaporation of the solvent.

*Ion suppression*

Ion-suppression studies were performed using post-column continuous infusion of the analyte THC-gluc and the internal standard **$d_9$**-carboxy-THC at a concentration of 1 µg/mL via a syringe pump and observation of the ESI response during injection of six different urine specimens into the HPLC-MS system.[35]

## Results and Discussion

### Mass spectrometry

The product ion mass spectrum of THC-gluc was recorded from reference material at a collision energy of −35 eV (Figure 2). Diagnostic and abundant fragment ions were generated from the deprotonated molecule of THC-gluc $[M-H]^- = m/z$ 489 and found at *m/z* 471, 313, 175, 157, 129 and 113. The elimination of water (−18 Da) led to the formation of *m/z* 471, and it is suggested that the base peak observed at *m/z* 313 may have resulted from the loss of the glucuronic acid moiety (−176 Da). It is suggested that the product ions at *m/z* 175, 157, 129 and 113 may have resulted from the decomposition of the glucuronic acid residue as reported e.g., for steroid glucuronides.[36]

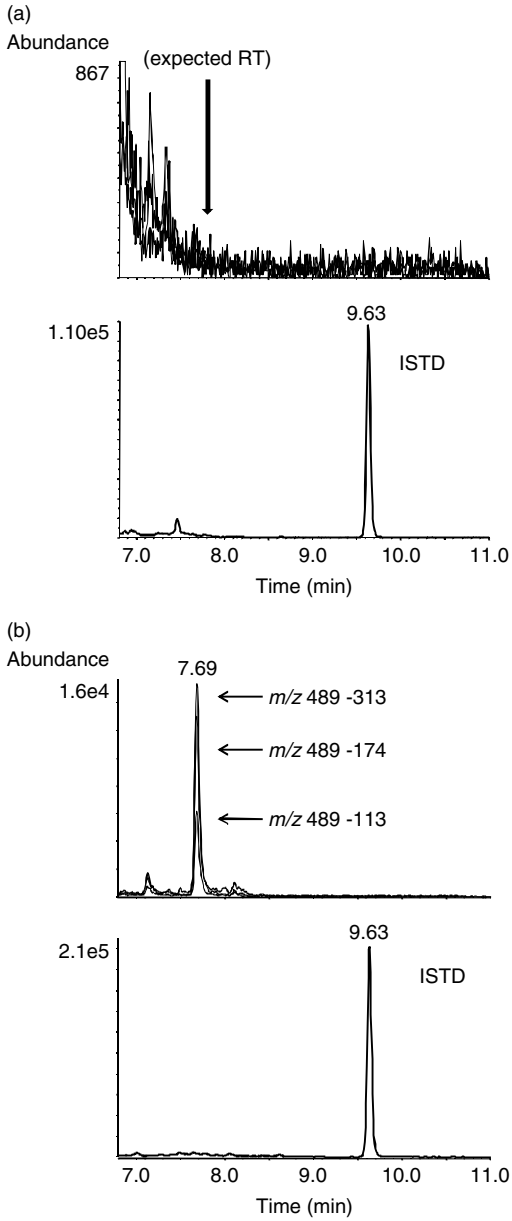


**Figure 3.** Extracted ion chromatograms of a) blank urine containing the internal standard (**$d_9$**-carboxy-THC) only, and b) urine specimen (authentic doping control urine sample) yielding approx. 0.54 ng/mL THC-gluc measured on an Applied Biosystems API 4000 triple quadrupole mass spectrometer using a CE of −35 eV. The analyte THC-gluc was determined using the diagnostic ion transitions *m/z* 489-313, 489-175 and 489-113 employing multiple reaction monitoring (detection at retention time 7.69 min).

For screening purposes the most suitable ion transitions were *m/z* 489/313, followed by *m/z* 489/175 and *m/z* 489/113. The retention time of the target compound under the chosen conditions was 7.7 min. (Figure 3a and b).

### Assay validation

In order to test for assay suitability, the parameters specificity, LLOD, LLOQ, linearity, intraday and interday precision, recovery and extraction efficiency were determined according to International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)

**Table 1.** Summary of assay validation for the THC glucuronide determination by means of LC-MS/MS

| | | Intraday precision (n = 6 + 6 + 6 + 6) | | Interday precision (n = 18 + 18 + 18) | | Recovery (n = 18 + 18 + 18) | |
|---|---|---|---|---|---|---|---|
| **LLOD (ng/mL)** | **LLOQ (ng/mL)** | **Concentration (ng/mL)** | **CV [%]** | **Concentration (ng/mL)** | **CV [%]** | **Concentration (ng/mL)** | **%** |
| 0.1 | 0.3 | 0.3 | 11.3 | | | | |
| | | 1 | 5.4 | 1 | 4.0 | 1 | 78 |
| | | 5 | 2.3 | 5 | 4.2 | 5 | 82 |
| | | 10 | 6.0 | 10 | 4.6 | 10 | 77 |

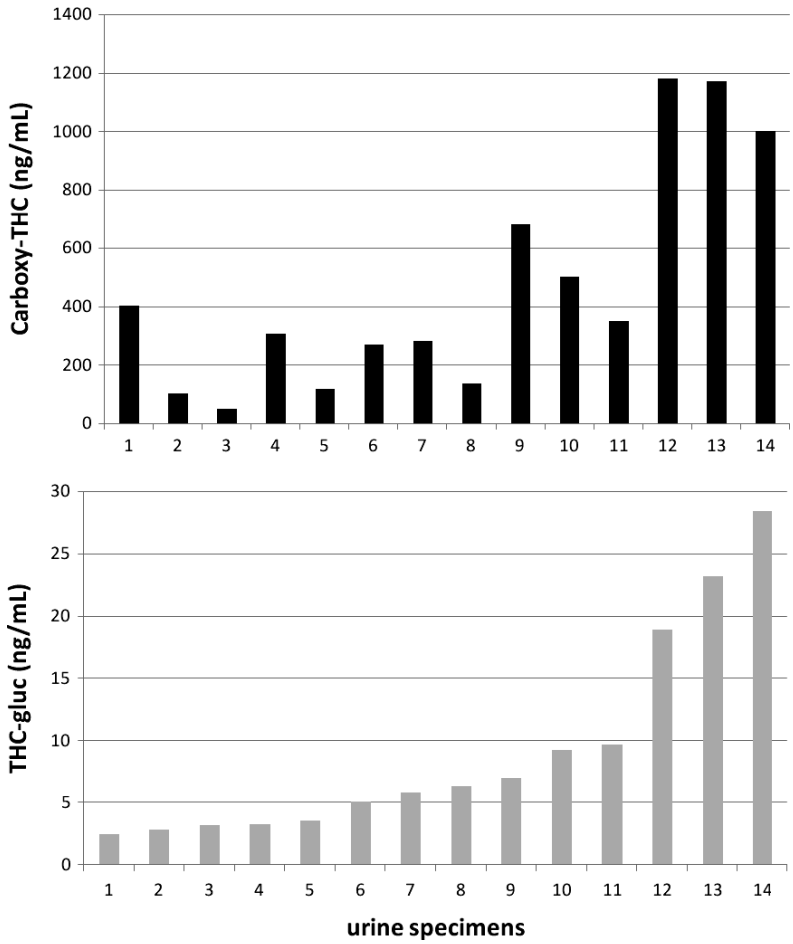


**Figure 4.** Authentic doping control urine samples containing carboxy-THC > 15 ng/mL and corresponding THC-gluc > 2.3 ng/mL. Top: carboxy-THC > 15 ng/mL Bottom: THC-gluc > 2.3 ng/mL.

guidelines[37] (Table 1). Regarding specificity, the investigation of 10 different blank urine samples generated no interfering signals at the expected retention time for THC-gluc. At the required signal-to-noise ratio of 3, the LLOD for THC-gluc was estimated at 0.1 ng/mL and the corresponding LLOQ at 0.3 ng/mL.

Linear calibration curves (linearity was proven by Mandel test[38]) for THC-gluc were obtained with calibration equations of $y = 0.1543\,x + 0.0076$ with $r^2 = 0.9947$ (range 0.3–5 ng/mL) and $y = 0.2535\,x + 0.0073$ with $r^2 = 0.9705$ (range 5–50 ng/mL). The intraday and interday precisions were determined at three concentration levels and show variations of less than 10% for low (1 ng/mL), medium (5 ng/mL), and high (10 ng/mL) concentration. At three concentration levels (1, 5, 10 ng/mL) the recovery was determined by means of measured calibration curves and was found to be between 77% and 82%. The extraction efficiency was specified with 90%.

For THC-gluc no suppression of the ESI response was observed while the urinary matrix was injected. The preparation and inclusion of quality-control samples yielding 2.3 ng/mL THC-gluc is recommended for the confirmatory analysis of single authentic doping-control urine samples.

### Stability of THC-gluc

Data from Skopp *et al*. indicate an instability of carboxy-THC-gluc.[7] Hence, the stability of THC-gluc must be tested to ensure accurate analytical results. At present, no stability data are available in the literature. The specimens of the controlled drug administration study and authentic doping-control urine samples containing

more than 15 ng/mL carboxy-THC were analysed for presence of free THC. No unconjugated THC was detected indicating no deconjugation as a degradation process. Nevertheless, further investigations have to be performed concerning the stability of THC-gluc.

### Controlled clinical smoking study

A controlled clinical smoking study was performed with 11 occasional cannabis users and the collected urine specimens were analysed for THC-gluc. No THC-gluc was detected in any of the samples, which is in accordance with earlier reports, e.g. by Skopp *et al*.[7] who stated that neither THC-gluc nor 11-OH-THC is commonly found in urine of light or occasional cannabis users. However, all volunteers delivered at least one urine sample containing detectable amounts of carboxy-THC, with four volunteers reaching urinary concentrations between 15 and 40 ng/mL within the first 8 h post-administration.

### Authentic doping control urine samples

Forty-six urine samples containing more than 15 ng/mL of carboxy-THC were analysed for THC-gluc. In 32 specimens, the concentration of THC-gluc was less than 2.3 ng/mL with corresponding carboxy-THC values between 15 and 100 ng/mL. In 14 specimens, more than 2.3 ng/mL of THC-gluc (max. 29 ng/mL) was found and great variations for the corresponding carboxy-THC values (Figure 4) were observed.

The relevant literature deals mainly with inhalation application routes, but ingestion of cannabis has also been reported as a common method.[39] Following slow absorption and reduced bioavailability (4–20%, primarily due to the degradation of the drug in the stomach), a significant first-pass metabolism to active and inactive metabolites was specified. Consequently, an oral administration study with cannabis is required to address this issue further.

## Conclusion

The assay presented allows for the quantitative determination of THC-gluc in human urine. The implementation of THC-gluc (with a reporting threshold of 2.3 ng/mL) as additional parameter for the interpretation of urine specimens containing carboxy-THC is suggested based on the data presented from authentic doping-control urine samples.

Analysis of THC-gluc is more suitable for interpreting whether cannabis was used in or out of competition than the analysis of carboxy-THC alone. This suggestion is, however, valid only for the current situation, where the use of cannabinoids is forbidden in competition. If cannabis is prohibited at all times (in and out of competition) the long-term metabolite carboxy-THC would be the more adequate analyte.

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