Determination of the Cross-Reactivity of the Biological Metabolite (–)-trans- Δ^9 -Tetrahydrocannabinol-Carboxylic Acid-Glucuronide (THC-COOH-Gluc) for Cannabinoid Immunoassays

Katharina Elisabeth Grafinger^{1,2} and Wolfgang Weinmann^{1,*}

¹Institute of Forensic Medicine, Forensic Toxicology and Chemistry, University of Bern, Bülhlstrasse 20, 3012 Bern, Switzerland and ²Institute of Forensic Medicine, Forensic Toxicology, Medical Center, University of Freiburg, Faculty of Medicine, University of Freiburg, Alberstr. 9, 79104 Freiburg im Breisgau, Germany

*corresponding author: Wolfgang Weinmann, Wolfgang.weinmann@irm.unibe.ch Institute of Forensic Medicine, Bülhlstrasse 20, 3012 Bern, Switzerland

Orcid:

Katharina Elisabeth Grafinger <https://orcid.org/0000-0002-3647-7455>

Wolfgang Weinmann <https://orcid.org/0000-0001-8659-1304>

Keywords: 11-nor-9-carboxy-(–)-trans- Δ^9 -tetrahydrocannabinol- β -D-glucuronide, enzyme immunoassay EIA, cross-reactivity, cannabinoids in urine

Abstract

The highest concentrated metabolite of (–)-trans- Δ^9 -tetrahydrocannabinol (THC) in urine, the main psychoactive constituent of *Cannabis sativa*, is 11-nor-9-carboxy-(–)-trans- Δ^9 -tetrahydrocannabinol- β -D-glucuronide [(–)-trans-THC-COOH-Gluc]. Even though reference standards for THC, 11-hydroxy-THC (11-OH-THC) and THC-COOH are commercially available as the biological (–)-trans-stereoisomers, the reference standard of THC-COOH-Gluc is only available as the racemic 11-nor-9-carboxy-($\pm$)-cis- Δ^9 -tetrahydrocannabinol- β -D-glucuronide. This poses the problem for immunoassays, because different stereoisomers may have different cross-reactivity (CR).

The aim of the current study was to extract the biological stereoisomer (–)-trans-THC-COOH-Gluc from a urine sample of two marijuana consumers by solid-phase extraction with a Chromabond® C18 cartridge. The cannabinoids in the obtained extract were quantified by LC–MS–MS and used after dilution for further testing of the CR of (–)-trans-THC-COOH-Gluc with a homogenous enzyme immunoassay assay (hEIA) (Urine HEIA® Cannabinoids (THC), Immunalysis™, Pomona, CA, USA). The CR was determined as the measured HEIA® signal (ng/mL) per THC-COOH-Gluc concentration (ng/mL) in percentage.

Results showed that the CR (determined in concentration ratios) is concentration dependent and is 72 to 87% in the calibration range (20–50 ng/mL). At the cut-off of the hEIA (40 ng/mL) the CR was determined to be 75%. With a molecular weight quotient of 1.51 ($MW_{\text{THC-COOH-Gluc}}/MW_{\text{THC-COOH}} = 520.568 \text{ g/mol} / 344.451 \text{ g/mol}$), this means that cross-reactivity (in molar ratios) is 106–131%. This finding is important, since the major metabolite of THC in urine is (–)-trans-THC-COOH-Gluc and not (–)-trans-THC-COOH, which is used for calibration and no hydrolysis is performed during the determination by hEIA.

Introduction

Cannabis is the most widely abused drug world-wide (1), with nearly 15% of young adults (15–34 years) and 7% of adults (15–64 years) having reported to use cannabis in 2018 in Europe (2). The main psychoactive compound of *Cannabis sativa* is (–)-trans- Δ^9 -tetrahydrocannabinol (THC) (3), which is hydroxylated during phase I metabolism in the human liver to the psychoactive metabolite 11-hydroxy-(–)-trans- Δ^9 -tetrahydrocannabinol (11-OH-THC) (4). 11-OH-THC is then further oxidised to the inactive 11-nor-9-carboxy-(–)-trans- Δ^9 -tetrahydrocannabinol (THC-COOH) (5, 6). During phase II metabolism THC-COOH is glucuronidated to 11-nor-9-carboxy-(–)-trans- Δ^9 -tetrahydrocannabinol- β -D-glucuronide [(–)-trans-THC-COOH-Gluc] (7), which is the main metabolite of THC excreted in urine (8, 9).

While the reference standards for THC, 11-OH-THC and THC-COOH are commercially available as the biological (–)-trans-stereoisomers, the reference standard of THC-COOH-Gluc is only available as the racemic 11-nor-9-carboxy-(+/-)-cis- Δ^9 -tetrahydrocannabinol- β -D-glucuronide or as a cis/trans mixture of stereoisomers (10, 11), because their production is more cost-efficient (12). This does not pose a visible problem when used as reference standard for quantification by (rapid) liquid chromatography–mass spectrometry (LC–MS), even though the cis- and trans- isomers may be separated and may also influence the ion ratios of product ions. In contrast, for immunoassays, the cross-reactivity (CR) of a substrate can differ significantly between stereoisomers. Therefore, suppliers such as Cerilliant® label the racemic mixture of ($\pm$)-11-nor-9-carboxy- Δ^9 -THC not to be suitable as reference material for calibrators for immunoassays (13) and should only to be used as reference material for LC–MS–MS and GC–MS. Immunoassays are biochemical tests using selective antigen-antibody binding to qualitatively or semi-quantitatively determine the presence of an analyte (14) and can be differentiated into non-competitive and competitive assays (15). In an competitive assay a known amount of labelled antigen is competing for a limited number of binding sites, hence, the amount of bound labelled antigen is inversely proportional to the amount of antigen present in the sample (16).

Most utilized immunoassays for cannabinoids in urine are calibrated with (–)-trans-THC-COOH (17). In general, different stereoisomers may have different cross-reactivity. Cross-reactivity is the ability of a chemically different compound—most often with structural similarities to the target substrate—to cross-react with the antibody of the assay, which results in a "false" positive (or "false" negative) result of the immunoassay (18).

In order to determine the CR of the urine cannabis immunoassay currently used in our laboratory, we isolated the biological metabolite (–)-trans-THC-COOH-Gluc from a urine sample of marijuana consumers by fractionated solid-phase extraction (SPE), which had been optimised for separation from non-glucuronidated THC-COOH and other major cannabinoids. The THC-COOH-Gluc concentration was determined by LC–MS–MS after enzymatic hydrolysis. Then this extract was used as a stock solution for cross-reactivity testing after severe dilution (1:500 v/v) using an AU 480 analyser (Beckman Coulter, Nyon, Switzerland) with a competitive immunoassay Cannabinoids Urine Enzyme Assay (HEIA[®], Immulysis[™], Pomona, California, United States). The principle of the assay is the use of monoclonal and polyclonal cannabinoid antibodies and is based on the competition of cannabinoids labelled enzyme glucose-6-phosphate dehydrogenase (G6PDH) and the free drug in the urine sample for the fixed amount of antibody binding sites. The enzyme G6PDH activity is determined at 340 nm spectrophotometrically by the conversion of NAD to NADH. The assay is calibrated with THC-COOH (17).

Materials and Methods

Materials

Acetic acid (LiChrosolv purity), β -glucuronidase/aryl sulfatase (*Helix pomatia*), di-sodiumhydrogenphosphate-dihydrate and potassium-dihydrogen-phosphate were purchased from Merk AG (Zug, Switzerland). Acetonitrile (HPLC gradient grade, 99.9%) was obtained from Acros Organics (Chemie Brunschwig, Basel, Switzerland), methanol (absolute, HPLC grade) from Bisolve (Chemie Brunschwig, Basel, Switzerland), butylacetate (HPLC grade, 99.7%) from Chromasolv Plus

(Sigma-Aldrich, Buchs, Switzerland) and formic acid (analytical grade, 08%) from Fluka (Sigma-Aldrich, Buchs, Switzerland). Ultrapure water was produced in-house using the direct-Q purification system from Millipore (Zug, Switzerland). Ethyl acetate was obtained from Grogg Chemie AG (Stettlen, Switzerland). The reference standards THC (1 mg/mL), 11-OH-THC (100 µg/mL), THC-COOH (100 µg/mL) and internal reference standards, THC-d₃ (100 µg/mL), 11-OH-THC-d₃ (100 µg/mL) and THC-COOH-d₃ (100 µg/mL) were obtained from Cerilliant (Fluka, Sigma-Aldrich, Buchs, Switzerland). Two urine samples positive for THC (routine in-house screening; no other drugs were present) were taken from routine forensic case work and drug-free blank urine, from a volunteer.

Methods

Solid-Phase-Extraction (SPE)

The extraction of THC-COOH-Gluc was performed with solid-phase-extraction on non-encapped Chromabond® C18 cartridges 3 mL/500 mg (Macherey-Nagel™, Oensingen, Switzerland) columns. The cartridges were conditioned with 2 mL methanol and subsequently with 2 mL 0.1 M acetic acid, followed by loading of 3 mL of urine sample positive for cannabinoids and washing with 2 mL 0.1 M acetic acid. For extraction, 2 mL each of four different eluents were used, with 40% (E1), 60% (E2), 70% (E3) and 100% (E4) acetonitrile (v/v), respectively. Extracts were stored at -20°C until further analysis. Each urine was extracted at least twice.

Quantitative Analysis

Quantification of THC, 11-OH-THC, THC-COOH and THC-COOH-Gluc was performed using an previously published fully-validated online-SPE method (19) by an LC-MS-MS system consisting of an Dionex Ultimate 3000 HPLC (Thermo Scientific, Basel, Switzerland) and a Qtrap 4500 (AB Sciex, Baden, Switzerland). In short: Separation was performed on a Kinetex C8 2.6 µm column (50 x 2.10 mm, Phenomenex, Basel, Switzerland) with a MercuryMS Synergi Polar RP trapping column (20 x 2.0 mm, Phenomenex, Basel, Switzerland). Mass spectrometric detection was performed using

selective reaction monitoring (SRM) scan mode. Each analyte was measured with two transitions (quantifier and qualifier). Compare Table I for SRM parameters.

Calibrators and Sample preparation

A seven-point calibration curve was prepared in blank urine, containing THC (0.5, 1, 1.5, 2, 5, 10 and 20 ng/mL), 11-OH-THC (0.5, 1, 1.5, 2, 5, 10 and 20 ng/mL) and THC-COOH (2.5, 5, 7.5, 10, 25, 50 and 100 ng/mL). For the quantification of residual free cannabinoids the SPE fractions (E1-E4) were diluted 1:5 (v/v) (without previous hydrolysis) with deionized water. Calibration and SPE fractions were extracted simultaneously as follows: 20 μ L ISTD-mix [consisting of THC- d_3 (40 ng/mL), 11-OH-THC- d_3 (10 ng/mL) and THC-COOH- d_3 (50 ng/mL)] was added to 200 μ L sample followed by dilution with acetonitrile (1:3, v/v). Samples were mixed for 10 minutes followed by centrifugation at 17,000 g at 8°C for 10 min. The liquid phase was transferred to a new vial and evaporated at 50°C under nitrogen until dryness. All samples were reconstituted in acetonitrile/water/formic acid (60:40:0.1; v/v/v) (200 μ L) for analysis.

Enzymatic hydrolysis with β -glucuronidase/aryl sulfatase

In order to quantify THC-COOH-Gluc, the extract was analyzed after deglucuronidation with β -glucuronidase/aryl sulfatase. The extract was diluted 1:500 (v/v) with deionized water before hydrolysis, in order to get results in the calibration range. For hydrolysis, phosphate buffer pH 6 was mixed with 200 μ L diluted extract in a ratio of 1:1 (v/v), 20 μ L ISTD-mix [consisting of THC- d_3 (10 ng/mL), 11-OH-THC- d_3 (10 ng/mL) and THC-COOH- d_3 (50 ng/mL)] and 10 μ L β -glucuronidase/aryl sulfatase, followed by incubation for 2 hours at 47°C. Extraction was performed by addition of butylacetate (1 mL), followed by 10 min of shaking and 10 min of centrifugation at 17,000 g at 8°C. The supernatant was transferred to an autosampler vial and evaporated to dryness under nitrogen at 50°C. The samples were reconstituted (200 μ L) in acetonitrile/water/formic acid (60:40:0.1; v/v/v).

Qualitative Analysis

Qualitative analysis was performed using a previously published gas chromatography coupled to mass spectrometry (GC–MS) method with electron impact ionisation (EI) (20). In short: instrumentation-6890 N GC with a 7683B autosampler coupled to a 5973 inert MS (Agilent, Basel, Switzerland); chromatographic separation: on a 5% phenyl methyl polysiloxane capillary column (30 m, 0.25-mm i.d., film thickness 0.25 µm; Perkin Elmer, Schwerzenbach, Switzerland); total run time: 30.5 min; temperature gradient: 0–3 min: 80°C, 3–7 min: 20°C/min to 150°C, 7–22 min: 10°C/min to 300°C, held at 300°C for 8.5 min; carrier gas: helium; flow rate: 1 mL/min; injection volume: 1 µL (splitless); solvent delay: 3 min; ionisation energy: 70 eV; MS scan range: m/z 25 to 700; scan time: 1 sec/scan.

For sample preparation 200 µL of E2 fraction were evaporated to dryness under a stream of nitrogen at 50 °C, followed by reconstitution in 50 µL ethyl acetate.

Compound identification was performed using the mass spectral libraries “Pfleger, Maurer, Weber”, “Wiley Registry of Mass Spectral Data with NIST” and “SWGDRUG MS Library” Version 3.5.L (September 23, 2019).

Homogenous enzyme immunoassay HEIA

AU 480 analyser

Immunological screening was performed on an AU 480 analyser (Beckman Coulter, Nyon, Switzerland) using the ImmualysisTM Cannabinoids Urine Enzyme Assay (Pomona, California, United States), measuring the enzyme activity spectrophotometrically at 340 nm. The manufacturer provided negative control and two calibrators (20 and 50 ng/mL). The cut-off for THC-COOH in urine is 40 ng/mL for urine samples.

In order to determine the cross-reactivity different concentrations of THC-COOH-Gluc in the calibration range from 20 to 50 ng/mL were tested. Extracts were stored in acetonitrile and water (60/40, v/v). Dilutions were prepared using blank urine by diluting first the extracts E2 working

solution to a concentration of 1000 ng/mL and preparing further dilutions from it with concentrations of 20, 25, 30, 35, 40, 45 and 50 ng/mL. The two urine samples were extracted two times and their cannabinoid concentration quantified before each immunoassay testing. The CR was determined as the measured HEIA[®] signal (ng/mL) per THC-COOH-Gluc concentration (ng/mL) in percentage.

To test if THC-COOH-Gluc is stable during the Immunoanalysis[™] Cannabinoids Urine Enzyme Assay, and hence no hydrolysis of THC-COOH-Gluc to THC-COOH takes place, the extract E2 working solutions were incubated with the assay solutions in a similar way as in the AU 480 analyzer, extracted and THC-COOH was quantified using the described LC–MS–MS method (experiment A). As a control, aliquots of E3 working solutions were diluted and extracted without incubation (experiment B). For experiment A 125 µL assay reagent 1 (antibody solution, TRIS buffer pH 5.5–6.5, 0.5% sodium azide) and 6 µL extract E2 working solution were mixed, followed by incubation at 37°C for 3.5 min. Then 50 µL reagent 2 (TRIS buffer pH 7.7–8.7, 0.5% sodium azide) were added, the solution was incubated at 37°C for another 2 min and 19 µL of water added to have a final volume of 200 µL. For experiment B (without incubation) 194 µL phosphate buffer pH 6 were added to 6 µL extract E2 working solution. Further sample preparation for solutions from experiments A and B was as follows: 20 µL ISTD-mix [consisting of THC-d₃ (10 ng/mL), 11-OH-THC-d₃ (10 ng/mL) and THC-COOH-d₃ (50 ng/mL)] and 200 µL phosphate buffer pH 6 were added to either solution and shortly mixed. For extraction 1 mL n-butylacetate was added, followed by 10 min of shaking and 10 min of centrifugation at 17,000 g at 8°C. The supernatant was transferred to an autosampler vial and evaporated to dryness under nitrogen at 50°C. The samples were reconstituted in 200 µL acetonitrile/water/ formic acid (60:40:0.1; v/v/v) and ready for analysis using the described LC–MS–MS method.

Results

In order to obtain the biological stereoisomer (–)-*trans*-THC-COOH-Gluc, urine samples from two marijuana consumers were extracted using SPE with different composition of eluents. THC-COOH was quantified in the four fractions (E1–E4) using LC–MS–MS, in order to identify the fraction

containing exclusively THC-COOH-Gluc and further to obtain a stock solution of THC-COOH-Gluc, which was used for further immunological analysis by HEIA[®] testing. Qualitative analysis of this fraction was performed to exclude the presence of other cannabinoids. Results of the HEIA[®] testing, which were used to determine the CR of THC-COOH and THC-COOH-Gluc are presented in Table II and Figure 1.

Quantitative analysis of extracts

For the determination of the THC-COOH-Gluc concentration, THC-COOH was quantified before and after enzymatic hydrolysis, and the molecular weight difference between THC-COOH (344.451 g/mol) and THC-COOH-Gluc (520.568 g/mol) was taken into consideration (factor 1.511: $MW_{\text{THC-COOH-Gluc}} / MW_{\text{THC-COOH}} = 520.568 \text{ g/mol} / 344.451 \text{ g/mol}$). Completeness of hydrolysis was proved by absence of mass spectrometric transitions for THC-COOH-Gluc after the enzymatic hydrolysis.

Quantitative analysis of the four extracts E1 to E4 showed that the highest concentration of THC-COOH-Gluc was present in the second extract E2 (acetonitrile/water 60:40; v/v). The THC-COOH concentration was in the range from 6.1 to 152.5 ng/mL and the THC-COOH-Gluc concentration was from 13,000 to 22,600 ng/mL. THC and 11-OH-THC could not be found. However, THC-Gluc (35.4–43.5 ng/mL) and 11-OH-THC-Gluc (311.6–317.0 ng/mL) could be quantified, via hydrolysis with β -glucuronidase/aryl sulfatase. These are 0.16–0.33% (THC-Gluc) and 1.38–2.44% (11-OH-THC-Gluc) in comparison to THC-COOH-Gluc and was therefore neglect able. Extract E3 (acetonitrile/water 70:30; v/v) contained more THC-COOH than THC-COOH-Gluc. Hence, extract E2 was used for all further analyses.

The THC-COOH concentration did not change by the incubation of extracts E2 with the reagents of the ImmualysisTM Cannabinoids Urine Enzyme Assay under simulated conditions as used by the AU 480 analyzer. This showed that no THC-COOH was formed from the THC-COOH-Gluc and its stability during the assay conditions could be demonstrated.

Qualitative analysis of extracts

Qualitative analysis of the E2 extracts using GC–MS (20) showed that both fractions were free of any cannabinoids besides THC-COOH-Gluc.

Determination of cross-reactivity (CR)

Different concentrations of THC-COOH-Gluc (range 20–50 ng/mL) were tested using the Immunalysis Cannabinoids Urine Enzyme Assay HEIA[®]. Due to validation in our laboratory, the cut-off for THC-COOH in urine was set to 40 ng/mL and the calibration range is from 0 to 50 ng/mL. Dilutions of the E2 with a THC-COOH-Gluc concentration higher than 50 ng/mL gave a positive result. Table II and Figure 1 depict the CR in the calibration range. The CR was determined as the measured HEIA[®] signal (ng/mL) per THC-COOH-Gluc concentration (ng/mL) in percentage. Each of the two urine samples was extracted at least two times, from which duplicates were prepared for the immunoassay testing, resulting in 8 measurements per concentration (calibrator). Table II depicts the arithmetic mean of measured HEIA[®] signal (ng/mL). The relative standard deviation for the HEIA signal of eight measurements ranged from 14.25 to 20.02%.

The CR is concentration dependent and increases at higher concentrations from 72% at 20 ng/mL THC-COOH-Gluc to 86% at 50 ng/mL THC-COOH-Gluc.

Discussion

The Immunalysis™ THC immunoassay test is calibrated for the isomer (–)-trans-THC-COOH (17) (personal communication, Stephan Dormeier). The data sheet of Immunalysis™ states the CR of THC-COOH (100%), 11-OH-THC (50%), 11-nor-carboxy- Δ^8 -THC (125%), cannabinol (66.7%), cannabidiol (< 0.005%) and THC (100%) (see Table III) (17). However, no CR is given for the major THC metabolite THC-COOH-Gluc. Other manufacturers such as Thermo Scientific (Cedia®) or Alere™ (Alere Triage®) give the CR (%) for THC-COOH-Gluc to be 78% and 100%, respectively, but do not specify which isomer was tested (Table III). Immunoassays are performed as preliminary tests, in order to determine which confirmatory tests and quantification methods are necessary (14). These confirmatory tests are required to confirm legally a substance consumption. Nevertheless, immunoassays are a crucial tool as it is neither feasible nor economically to test a sample for every potential medical and recreational drug (14, 21). Any analyte having a CR > 0% can facilitate a higher additive hEIA signal than the calibrated analyte. This effect is beneficial for detecting a potential cannabis consumption, because due to our enzymatic diversity each individual produces a different ratio of metabolites.

THC-COOH-Gluc is the major metabolite of THC, therefore it is expected to be found in high concentrations in urine after marijuana consumption. A high CR of THC-COOH and THC-COOH-Gluc for immunoassays is therefore desirable, as it facilitates the identification of potential marijuana consumption, which needs to be confirmed using methods such as GC–MS or LC–MS–MS.

Conclusion

The fractionated extraction of THC-COOH-Gluc by solid-phase extraction is a simple and fast procedure. The extraction solvent consisting of acetonitrile/water 60:40 (v/v) contained the highest THC-COOH-Gluc concentration. Other cannabinoids or metabolites were not present or only in minor concentrations (max. 2.44% of THC-COOH-Gluc concentration). Results showed that the CR (determined in concentration ratios) is concentration dependent and is 70 to 87% in the calibration range (20–50 ng/mL). The CR at the cut-off of the hEIA (40 ng/mL) was determined to be 87%. Considering the molecular weight quotient of 1.51, the cross-reactivity (in molar ratios) is 106–131%.

This finding is important, since the major metabolite of THC in urine is (–)-trans-THC-COOH-Gluc and not (–)-trans-THC-COOH, which is used for calibration.

UNCORRECTED MANUSCRIPT

Acknowledgments

Katharina Elisabeth Grafinger would like to acknowledge a Postdoctoral fellowship from the Swiss National Science Foundation (Grant SNSF_P2BEP3_191780). The authors would like to thank Nadja Utiger, Sidonia Guggisberger and Thomas Wüthrich for their support in the laboratory and Dr. Stephan Dormeier for personal communications about the Immunalysis™ Cannabinoids Urine Enzyme Assay.

Funding

None

Conflict of Interest

The authors declare no conflict of interest.

References

1. United Nations Office on Drugs and Crime .(2019) World Drug Report 2019- Global Overview of Drug Demand and Supply
2. European Monitoring Centre for Drugs and Drug Addiction (2019) European Drug Report 2019: Trends and Developments.
3. Costa, B. (2007) On the pharmacological properties of Delta9-tetrahydrocannabinol (THC). *Chemistry & biodiversity*, 4, 1664-1677.
4. Lemberger, L., Crabtree, R.E. and Rowe, H.M. (1972) 11-hydroxy- 9 -tetrahydrocannabinol: pharmacology, disposition, and metabolism of a major metabolite of marihuana in man. *Science*, 177, 62-64.
5. Levine, B. (2013) Principles of Forensic Toxicology, 4th edition. AACC Press, Washington DC.
6. Saenz, S.R., Lewis, R.J., Angier, M.K. and Wagner, J.R. (2017) Postmortem Fluid and Tissue Concentrations of THC, 11-OH-THC and THC-COOH. *Journal of analytical toxicology*, 41, 508-516.
7. Lucas, C.J., Galettis, P. and Schneider, J. (2018) The pharmacokinetics and the pharmacodynamics of cannabinoids. *British journal of clinical pharmacology*, 84, 2477-2482.
8. Desrosiers, N.A., Himes, S.K., Scheidweiler, K.B., Concheiro-Guisan, M., Gorelick, D.A. and Huestis, M.A. (2014) Phase I and II cannabinoid disposition in blood and plasma of occasional and frequent smokers following controlled smoked cannabis. *Clin Chem*, 60, 631-643.
9. Skopp, G.P., Pötsch, L., Ganßmann, B., Mauden, M., Richter, B., Aderjan, R., and Mattern, R. (1999) Free and glucuronidated cannabinoids in urine – investigations to assess the extent of cannabis use. *Rechtsmedizin*, 10, 21-29.
10. Kailua, M.A.T. and Hagadone, M.R. (1994) Synthesis of 11-nor-delta-9-Tetrahydrocannabinol-9carboxylic acid glucuronide. *International Patent Number: C07D 311/80: C07D 311/00*.
11. Hanisch, S., Paulke, A. and Toennes, S.W. (2017) 11-nor-9-carboxy-Delta(9)-tetrahydrocannabinol glucuronide exhibits acyl-migration isomers. *Journal of pharmaceutical and biomedical analysis*, 146, 261-265.
12. Aijaz, S.W., A. Dilek, and S. Sreenivasan U. Impact of isomeric differences between internal standard and analyte for the LC-MS/MS quantification of 11-Nor-delta9-THC-9-carboxylic acid glucuronide. <https://www.cerilliant.com/Shoponline/OpenDocument.aspx?DocumentId=427>, (access date: 23.01.2020)
13. Cerrilant® (2020) (±)-11-nor-9-Carboxy-Δ9-THC (Racemic mixture - not to be used for immunoassay) https://www.cerilliant.com/shoponline/Item_Details.aspx?itemno=b8af16dc-51e7-4be6-8d0c-85636e3024fe&item=T-006 (14.04), (access date: 14.04.2020)
14. Graziano, S., Anzillotti, L., Mannocchi, G., Pichini, S. and Busardo, F.P. (2019) Screening methods for rapid determination of new psychoactive substances (NPS) in conventional and non-conventional biological matrices. *Journal of pharmaceutical and biomedical analysis*, 163, 170-179.
15. Coulter, C., Tuyay, J., Taruc, M. and Moore, C. (2010) Semi-quantitative analysis of drugs of abuse, including tetrahydrocannabinol in hair using aqueous extraction and immunoassay. *Forensic science international*, 196, 70-73.
16. Moser, A.C. and Carlson, T. L. (2014) General principles of immunoassays. *Novel Approaches in Immunoassays*, 13,6-19. doi.org/10.2217/ebo.13.617
17. Immunalysis™ (2017) Product insert: Cannabinoids Urine Enzyme Immunoassay.
18. Tate, J. and Ward, G. (2004) Interferences in immunoassay. *Clin Biochem Rev*, 25, 105-120.
19. König, S., Aebi, B., Lanz, S., Gasser, M. and Weinmann, W. (2011) On-line SPE LC-MS/MS for the quantification of Delta9-tetrahydrocannabinol (THC) and its two major metabolites in human peripheral blood by liquid chromatography tandem mass spectrometry. *Anal Bioanal Chem*, 400, 9-16.

20. Grafinger, K.E., Kronert, S., Broillet, A. and Weinmann, W. (2020) Cannabidiol and tetrahydrocannabinol concentrations in commercially available CBD E-liquids in Switzerland. *Forensic science international*, doi: 10.1016/j.forsciint.2020.11026
21. Grafinger, K.E., Liechti, M.E. and Liakoni, E. (2020) Clinical value of analytical testing in patients presenting with new psychoactive substances intoxication. *British journal of clinical pharmacology*, 86, 429-436.
22. Thermo Scientific® (2016) CEDIA® Multi-Level THC Assay, <http://tools.thermofisher.com/content/sfs/manuals/10006559-CEDIA-Multi-Level-THC-Assay-EN.pdf> (access date: 14.04.2020)
23. Alere Triage® (2016) TOX Drug Screen Product Insert , <https://www.quidel.com/sites/default/files/612022C30F7F904D79.pdf> (access date: 14.04.2020)

Figure caption

Figure 1. Cross-reactivity of THC-COOH and THC-COOH-Gluc (middle line) for HEIA[®] Immunalysis[™] Cannabinoids Urine Enzyme Assay determined using an AU 480 Beckman Coulter. For comparison the theoretical CR of 50% (upper dotted line) and 100% (lower dashed line) are indicated.

Table I. Parameters of the SRM Experiments for Each Analyte, Including the Quantifier and Qualifier Transition, Retention Times, and the Corresponding Internal Standard with the Quantifier Transition

Substance	Quantifier	Qualifier	Retention time [min]	ISTD	Quantifier
THC	315.2/193.2	315.2/123.2	5.81	THC-d ₃	318.3/196.3
11-OH-THC	331.2/313.3	331.2/193.2	4.57	11-OH-THC-d ₃	334.3/316.3
THC-COOH	345.2/327.3	345.2/299.2	4.63	THC-COOH-d ₃	348.2/330.3
THC-COOH-Gluc	521.2/345.3	521.2/327.3	3.75	THC-COOH-d ₃	348.2/330.3

Table II. Cross-Reactivity of THC-COOH with THC-COOH-Gluc Determined by Correlating a Defined THC-COOH-Gluc Concentration with the HEIA[®] Signal of Two Extracted Urine Samples*

THC-COOH-Gluc [ng/mL]	HEIA [®] signal [ng/mL]	Relative Standard Deviation HEIA [®] signal [%]	CR [%] neu
20.0	14.3	14	72
25.0	17.5	13	70
30.0	21.5	19	72
35.0	28.6	22	82
40.0	34.8	21	87
45.0	38.8	19	86
50.0	43.2	20	86

* Samples were extracted twice and all HEIA[®] testing was performed in duplicates.

Table III: Cross Reactivity of Different Cannabinoids for the Calibration Compound of Three Different Commercially Available Immunoassays*

Analyte	Test		
	Immunalysis TM CR [%] (17)	Cedia [®] CR [%] (22)	Alere Triage [®] CR [%] (23)
11-nor-carboxy- Δ^9 -THC	100	100	-
11-hydroxy- Δ^9 -THC	50 (+/-)	43	-
11-nor-carboxy- Δ^8 -THC	125	125	-
Cannabinol	66.7	2.9	<0.025
Cannabidiol	<0.005	<0.1	-
Δ^9 -THC	100	10.4	-
8 β -hydroxy- Δ^9 -THC	-	2.8	-
8 β -11-di-hydroxy- Δ^9 -THC	-	8.4	-
11-nor- Δ^9 -THC-COOH-Gluc	-	78	100

* All tests were calibrated for THC-COOH at 50 ng/mL.