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DETERMINATION OF TETRAHYDRO-
CANNABINOL-CARBOXYLIC ACID IN
URINE BY LIQUID CHROMATOGRAPHY
WITH MASS SPECTROMETRY

ODREĐIVANJE TETRAHIDROKANABINOL-
KARBOKSILNE KISELINE U URINU
PRIMENOM TEČNE HROMATOGRAFIJE SA
MASENOM-SPEKTROMETRIJOM

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Ključne reči

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Abstract

Detection of most abusing drug - marijuana in urine specimens is performed by immuno-chromatographic tests. However, each positive test result has to be confirmed by mass spectrometric detection after analyzing samples with some of chromatographic techniques. We developed liquid chromatography-mass spectrometric (LC-MS) method for determination of 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THCA) as a major THC metabolite in urine. Because of low concentration of free THCA in urine, it has to perform hydrolysis of its glucuronide. Hydrolysis of glucuronide from urine samples was performed with KOH on 60°C. THCA and internal standard (THCA-D3) were extracted from urine after adjusting of pH on 3 with mixture n-hexan-ethylacetate (7:1).

THCA was determined by LC-MS method in single ion monitoring mode (SIM) at m/z 345, 327 and 299 for THCA, and 348, 330 and 302 for IS. The chromatographic separation was performed on XTerra®RP18 column, using a gradient of acetonitrile/acetic acid 1% and acetate buffer pH 3.5 as the mobile phase.

Linearity was achieved in the range from 10-100 ng/mL. Retention times of THCA and IS were 21.54 and 21.52 minutes respectively. Limit of detection and limit of quantitation for THCA were 2.65 and 8.82 ng/mL respectively. Coefficient of variation was 7.62%.

Described LC-MS method of analysis THCA in urine is precise, accurate, reproducible and reliable method for confirmation of cannabis abuse.

INTRODUCTION

Cannabis is the most abusing psychoactive substance in recent years. The most used methods for identification of cannabinoids in urine are immuno-tests⁽¹⁾ which major advantage is quick performance of 5 minutes. However, as potentially forensic evidence, each of the positive result has to be confirmed by mass-spectrometry. The major metabolite of main cannabis ingredient, tetrahydrocannabinol, in urine is 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THCA) and in urine is present in a form of glucuronide.

Confirmation of THCA in urine commonly implies gas-chromatography with mass spectrometry after extraction from the sample and derivatization⁽²⁻⁵⁾. Recently, liquid chromatographic method with mass spectrometric detection is in use for determination of THCA from urine samples⁽⁶⁻¹⁶⁾.

The aim of our study was to develop precise, accurate, reproducible and reliable LC-MS method for determination of THCA in urine as an extra proof of cannabis abuse, after first confirmatin with immuno-tests.

MATERIALS AND METHODS

Material

Analytical standards of THCA and THCA-D3 obtained by Cerilliant, Round Rock, Texas, USA. Methanol, acetic acid, n-hexane, ethyl acetate, potassium hydroxide and acetonitrile, were of HPLC purity, obtained from Merck, Darmstadt, Germany. Ammonium acetate and water (HPLC grade), were obtained from Fluka, Buchs, Switzerland and JT Baker, Phillipsburg NJ, USA.

Method

A Micromass ZQ2000 ESI-LC/MS System, consisted of Waters Alliance 2695 separations module and Waters micromass ZQ mass selective detector equipped with an electrospray ion source, was used for quantitative determination of THCA in urine sample. Chromatographic separation was performed on XTerra® MS C18, 3,5 μ m, 2,1x150 mm. The column temperature was maintained at 30°C. The injection volume was 50 μ L. The mobile phase consisted of 5 mmol/L ammonium acetate in water (A) and 0.1% of acetic acid in acetonitrile (B) in ratios given in Table 1.

Table 1. Content of mobile phase for determination of THCA and THCA-D3 in urine

| t (min) | %A | %B |
|---------|------|------|
| 0,00 | 95,0 | 5,0 |
| 2,00 | 95,0 | 5,0 |
| 16,00 | 10,0 | 90,0 |
| 20,00 | 95,0 | 5,0 |
| 26,00 | 95,0 | 5,0 |

The flow rate of mobile phase was 0.2 mL/min.

An ESI source in positive ion mode was used for determination. The optimized ionization conditions were: capillary voltage 3.8 kV; cone voltage 30 V; extractor 3 V. The source and desolvation temperatures were 125 and 430°C, respectively. Nitrogen was used as a cone and desolvation gas at flow rates of 50 and 400 L/Hr, respectively. For detection of THCA and THCA-D3 were used ions: m/z 345, 327 i 299, and m/z 348, 330 i 302 respectively. The LC/MS system was controlled using Masslynx 4.0 software system.

Sample preparation

Internal standard (20 ng/mL) was added in urine sample before its preparation. First alkaline hydrolysis was performed using 3 mL of urine and mixing with KOH in concentration of 10 mol/L and incubating the mixture for 30 min at 60 °C.

After cooling to the room temperature and

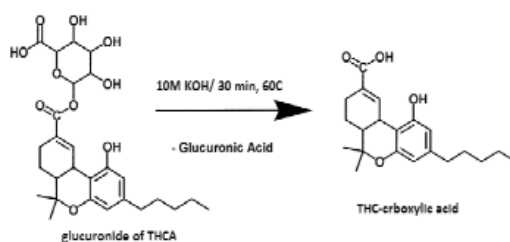


Figure 1. Scheme of urine sample preparation - hydrolysis of THCA-glucuronide

adjusting pH to the value of 3 with HCl in concentration of 6 mol/L, THCA and IS were extracted with mixture of n-hexan : ethyl-acetate (7:1; V:V). Organic layer was separated from urine sample and evaporated in stream of air. Dry extract was reconstituted in 1 mL of methanol and analyzed by LC-MS method.

The calibration curve solutions were prepared by adding of THCA standard solution in blank urine and treated like urine samples. Calibration curve was linear in the concentrations range of 10, 20, 25, 50, 75 and 100 ng/mL.

RESULTS

The calibration curve for THCA in urine has shown in Table 2.

Table 2. Calibration curve for THCA

| concentration (ng/mL) | AUC (THCA) | AUC (IS) | AUC(THCA)/AUC(IS) |
|-----------------------|------------|----------|-------------------|
| 10 | 7362 | 13689 | 0,537804 |
| 20 | 11345 | 9117 | 1,244379 |
| 25 | 16268 | 10997 | 1,479313 |
| 50 | 28380 | 10947 | 2,592491 |
| 75 | 44380 | 11374 | 3,901881 |
| 100 | 62030 | 12169 | 5,097379 |

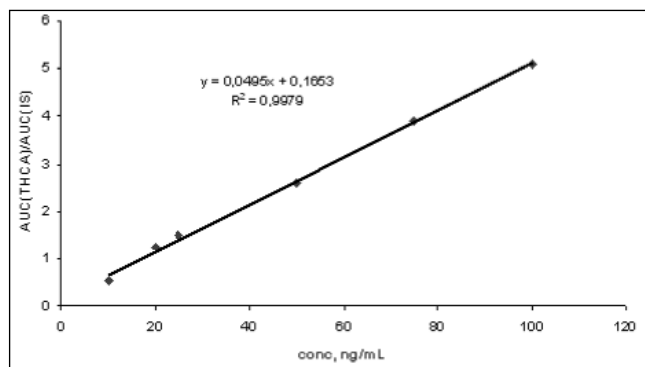


Figure 2. Calibration curve of urine spiked with THCA

Retention times of THCA and IS were 21.54 min and 21.52 min respectively. Chromatogram of spiked urine is shown on Figure 3.

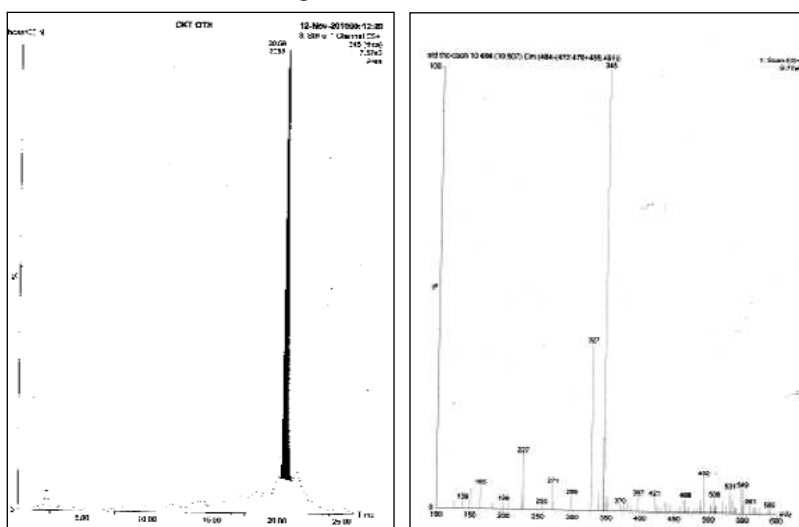


Figure 3. Chromatogram of urine spiked with THCA and mass spectrum of THCA

Analytical recovery for THCA is shown in Table 2.

Table 2. Analytical recovery for THCA

| concentration of THCA (mg/L) | Recovery (%) |
|------------------------------|--------------|
| 10 | 102,31 |
| 20 | 81,38 |
| 25 | 84,82 |
| 50 | 89,02 |
| 75 | 86,62 |
| 100 | 85,37 |

Limit of detection (LoD) was defined as the concentration at which the signal to noise is equal to or greater than three, and the limit of quantitation (LoQ) was defined as the concentration at which the signal to noise is equal to or greater than ten (Table 3).

Table 3. Limits of detection and quantification for THCA

| Compound | LoD (ng/mL) | LoQ (ng/mL) |
|---|-------------|-------------|
| 11-nor- Δ^9 -THC-9-carboxylic acid | 2,65 | 8,82 |

Intra day precision of the method was done by preparing of ten THCA standard samples concentration of 10 ng/mL and determining by LC-MS method. Coefficient of variation was 7.62%. Data on inter-day precision are shown in Table 4.

| Added concentration (mg/L) | Obtained concentration (mg/L) 1st day | mean | SD | CV (%) | Obtained concentration (mg/L) 2nd day | mean | SD | CV (%) |
|----------------------------|---------------------------------------|--------|------|--------|---------------------------------------|--------|------|--------|
| 10.0 | 10.15 | 10.16 | 0.79 | 7.86 | 10.14 | 9.59 | 0.90 | 9.42 |
| | 10.97 | | | | 8.55 | | | |
| | 9.37 | | | | 10.09 | | | |
| 50.0 | 49.23 | 50.14 | 2.09 | 4.16 | 49.432 | 49.84 | 1.96 | 3.94 |
| | 48.66 | | | | 48.11 | | | |
| | 52.53 | | | | 51.98 | | | |
| 100.0 | 101.23 | 100.88 | 2.72 | 2.70 | 100.98 | 100.35 | 2.92 | 2.91 |
| | 103.42 | | | | 102.90 | | | |
| | 98.01 | | | | 97.16 | | | |

Table 4. Inter day coefficient of variation (CV) for determination of THCA by LC-MS method

DISCUSSION

Different type of immuno-tests are using for identification of cannabinoids abuse. One of the fastest and the most sensitive is immunochromatographic test, which can detect 50 ng/mL of THC metabolites in urine sample. However, because of non-specificity, each positive result has to be confirmed by mass spectrometric method.

Gas chromatography with mass spectrometry is the usually method for determination of THCA (2-5). Recently, LC-MS method is using for its determination because it does not need derivatization of cannabinoid ingredients i.e. analytes (6-16).

For the purpose of method development, ion spray ionization mass spectra of THCA and IS were acquired at different cone voltages. We decided to use cone voltage of 30 V, because higher voltage leads to more intensive fragmen-

tation and for final identification of THCA we used ions m/z: 345 (molecular ion), 327 and 299. For detection of cannabis abuse different biological samples can be used. The most important specimen is urine, because of non-invasive sampling and relative high volume which can be taken. Taking into account that concentration of THCA as the main metabolite of THC in urine is small, high volume allows concentration of sample and possibility of its detection.

THCA is eliminated via kidney in form of glucuronide, so the first step in sample preparation is hydrolysis of glucuronides in urine. For hydrolysis of THCA-glucuronide alkaline (2-3, 5-7) and enzymatic hydrolysis (8-9) are used. Ester bonding is unstable to both enzymatic and alkaline hydrolysis (9). We used KOH in concentration of 10 mol/L, because it is less cost than enzyme β -glucuronidase which also could be used for hydrolysis. After alkaline hydrolysis it was important to adjust pH between 3 and 4 in order to suppress dissociation of carboxylic group and enable extraction of THCA with organic solvent with good analytical recovery.

Extraction of THCA can be done by liquid-liquid extraction (LLE) with organic solvent (10) or solid-phase extraction (SPE) (11). SPE is the most used sample preparation. It requires smaller volume of organic solvent and less time in comparison to liquid-liquid extraction. We performed LLE with mixture n-hexane : ethylacetate (V:V) and SPE on Waters MCX cartridges. LLE gave cleaner extracts with similar analytical recovery.

Nestić et al performed molecularly imprinted solid phase extraction with analytical recovery of 75.2% (3).

Recovery after SPE was 85.7% (8). Our results were similar (mean analytical recovery was 88.25% (Table 2)).

Teixeira et al. presented LC-MS method for determination of THCA with intra- and inter-day variation of 6.8% and 6.2% respectively (16). Our results were similar to literature.

Limit of quantitation of our method was 8.2 ng/mL. It was higher than LC-MS-MS method, but satisfactory considering that LC-MS method was used for confirmation of cannabinoids abusing. Described method was performed in confirmation of positive results after immunochromatographic tests. Our results have shown that there were not false positive results.

CONCLUSION

We developed precise, accurate, reproducible and reliable LC-MS method for determination of THCA in urine as an extra proof of cannabis abuse, after first confirmation with immuno-tests. Presented LC-MS method is also simple and sensitive. It can be applied as routine analysis for confirmation of positive results after immunoassay. Beside

method, we also adjusted sample preparation: alkaline hydrolysis and liquid-liquid extraction which also do not require expensive reagents in comparison to other methods described in literature. It should be emphasised that use of deuterized internal standard allowed more precise determination of THCA in urine which is important especially in interpretation of results after intake of cannabinoids by passive inhalation.

Sažetak

Detekcija najčešće zloupotrebjavane psihoaktivne supstance – marihuane u urinu vrši se imunohromatografskim testom. Međutim, svaki pozitivan rezultat mora da bude potvrđen maseno-spektrometrijskom metodom nakon analize uzorka nekom od hromatografskih tehnika. Mi smo razvili metodu tečne hromatografije sa masenom spektrometrijom za određivanje 11-nor- Δ^9 -tetrahidrokanabinol-9-karboksilne kiseline (THCA) kao glavnog metabolita THC u urinu. Zbog malih koncentracija slobodne THCA u urinu, potrebno je uraditi hidrolizu glukuronida. Hirdoliza glukuronida iz uzorka urina vršena je pomoću KOH na 60°C. THCA i interni standard THCA-D3 su izolovani iz urina pomoću smeše n-heksan-etilacetat (7:1) posle podešavanja pH na 3. THCA je određivana LC-MS metodom u SIM modu sa jonskim masama m/z 345, 327 i 299 za THCA i 348, 330 i 302 za IS. Linearnost je postignuta u opsegu koncentracija od 10-100 ng/mL. Retenciona vremena za THCA i IS su bila 21,54 i 21,52 min. Limit detekcije i kvantifikacije za THCA bili su 2,65 i 8,82 ng/mL. Koeficijent varijacije je iznosio 7,62%. Prikazana LC-MS metoda za određivanje THCA u urinu je precizna, tačna, ponovljiva i pouzdana metoda za potvrdu zloupotrebe kanabisa.

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