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Confirmation testing of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in urine with micellar electrokinetic capillary chromatography

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ABSTRACT

The major urinary metabolite of the most commonly abused psychotropic drug, Δ^9-tetrahydrocannabinol, is 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH). With basic hydrolysis, extraction and concentration, this compound can easily be determined using micellar electrokinetic capillary chromatography with on-column multi-wavelength detection. After solid-phase extraction of 5 ml of urine, drug concentrations down to about 10 ng/ml can be unambiguously monitored. Peak assignement is achieved through comparison of the retention time and absorption spectrum of the eluting THC-COOH peak with those of computer-stored model runs. The effectiveness of the approach is demonstrated with data obtained from urine samples from different patients which tested positively for cannabinoids using a fluorescence polarization immunoassay.

INTRODUCTION

The major psychoactive compound in *Cannabis sativa* (marijuana), Δ^9-tetrahydrocannabinol (THC), is highly lipophilic and is therefore accumulated in tissues rich in lipids. Its release from the tissues is slow and its concentration in urine is low. The major metabolite, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), is excreted in urine predominantly in its glucuronated form and can be detected up to about 10 weeks after smoking or oral ingestion of cannabis products [1]. THC-COOH is the target substance employed in screening methods for cannabinoids, such as the enzyme multiplied immunoassay technique (EMIT), fluorescence polarization immunoassay (FPIA) and radioimmunoassays (RIA). Owing to the lack of specificity of these techniques, positive results should be confirmed in order to eliminate any false-positive answer that may have resulted from the initial screening process. For that purpose specimens are typically first hydrolysed under basic conditions and free THC-COOH is then extracted employing bonded-phase technology prior to analysis with a highly specific method, including thin-layer chromatography (TLC) [2,3], high-performance liquid chromatography (HPLC) [4,5] or gas chromatography–mass spectrometry (GC–MS) [6].

Recently, micellar electrokinetic capillary chromatography (MECC), an interface between electrophoresis and chromatography, was found to be an attractive approach for the analysis of urinary barbiturates [7], drugs of abuse and/or their metabolites, including opioids, benzoylecgonine (metabolite of cocaine), amphetamines and methaqualone in human urine [8], and illicit substances, including those in illicit seizure samples [9]. The objectives of the work described in this paper were to investigate different bonded-phase adsorption clean-up procedures for subsequent determination of THC-COOH using MECC with on-column, fast-scanning polychrome UV absorption detection and to

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use this MECC method to confirm the presence of THC-COOH in urine samples which tested FPIA positive for cannabinoids.

EXPERIMENTAL

Chemicals, origin of samples and drug screening

All chemicals were of analytical-reagent or research grade. Deuterated THC-COOH {[5′-$^2H_3$]-9-carboxy-11-nor-Δ^9-tetrahydrocannabinol dissolved in ethanol (100 μg/ml); Research Triangle Institute, Research Triangle Park, NC, USA} served as a standard compound. Urine samples were collected in our routine drug assay laboratory where they were received for drug screening. Our own urine was employed as a blank matrix. The samples were screened for the presence of cannabinoids using FPIA on a TDx analyser (Abbott Laboratories, North Chicago, IL, USA) and stored at 4°C until further analysis. The FPIA test contains THC-COOH for calibration in the range 0–135 ng/ml (six points). Samples which gave a response equal to or higher than 20 ng/ml were interpreted as positive. The detection limit of the immunoassay is 10 ng/ml.

Electrophoretic instrumentation and running conditions

The instrument with multi-wavelength detection employed was described previously [7,8]. Briefly it featured a ca. 90 cm × 75 μl I.D. fused-silica capillary (Product TSP/075/375, Polymicro Technologies, Phoenix, AZ, USA) together with a Model UVIS 206 PHD fast-scanning multi-wavelength detector with a No. 9550-0155 on-column capillary detector cell (both from Linear Instruments, Reno, NV, USA) towards the capillary end. The effective separation distance was 70 cm. A constant voltage of 20 kV was applied using an HCN 14-20000 power supply (FUG Elektronik, Rosenheim, Germany). The cathode was on the detector side.

Samples were applied manually via gravity by lifting the anodic capillary end, dipped into the sample vial *ca.* 34 cm for a specified time interval (typically 5 s). Multi-wavelength data were read, evaluated and stored employing a Mandax AT 286 computer system and running the 206 detector software package version 2.0 (Linear Instruments) with windows 286 version 2.1 (Microsoft, Redmont, WA, USA). Conditioning for each experiment was done by rinsing the capillary with 0.1 *M* NaOH for 3 min and with buffer for 5 min. Throughout this work the Model 206 detector was employed in the high-speed polychrome mode by scanning from 195 to 320 nm at 5-nm intervals (26 wavelengths). A buffer composed of 75 m*M* sodium dodecyl sulphate (SDS), 6 m*M* $Na_2B_4O_7$ and 10 m*M* Na_2HPO_4 (pH *ca.* 9.1) was employed.

Sample pretreatment

Standard solutions of THC-COOH were prepared by evaporation of the ethanol and reconstitution in methanol or running buffer at a concentration of 100 μg/ml. Blank urine was spiked by addition of known aliquots of these solutions to the urine. Glucuronated THC-COOH in samples from patients was hydrolysed under basic conditions (see below) and free THC-COOH was extracted from urine using disposable Bond Elut THC (1211-3044; sorbent amount 500 mg, reservoir volume 10 ml), Bond Elut Certify (1211-3050, 130 mg, 10 ml) and Bond Elut Certify II (1211-3051, 200 mg, 10 ml) solid-phase cartridges from Analytichem International (Harbor City, CA, USA) and also Clean Screen THC (CSTHC101, 100 mg, 1 ml) from Worldwide Monitoring (Horsham, PA, USA). For all types of columns a Vac Elut set-up (Analytichem International) served as a cartridge holder and vacuum manifold for sample extraction. No extended drying periods of the sorbents were applied and no derivatization of eluted THC-COOH was undertaken.

Bond Elut THC. These columns were applied according to the procedures reported by Bourquin and Brenneisen [4], Willson *et al.* [2] and Duc [3]. In all three instances the eluate was evaporated to dryness under a gentle stream of nitrogen at room temperature and the residue was dissolved in 100 μl of running buffer.

Bond Elut Certify. The procedure employed is a modification of the manufacturer's recommendation. For hydrolysis 0.3 ml of 10 *M* KOH was added to 5 ml of urine, vortex-mixed for about 10 s, heated at 50–60°C for 20 min (with constant mixing) and cooled to room temperature. Thereafter 3 ml of 50 m*M* phosphoric acid were added and the pH was adjusted to 3 with concentrated HCl. The Bond Elut Certify cartridges were conditioned immediately prior to use by passing sequentially 2 ml

of methanol and an equal volume of 50 m*M* phosphoric acid through the columns. The vacuum was turned off as soon as the acid solution reached the sorbent bed to prevent column drying. The hydrolysed and vortex-mixed specimen was then applied and drawn slowly (1–2 ml/min) through the cartridge. The column was sequentially rinsed with 9 ml of 50 m*M* phosphoric acid and 3 ml of 50 m*M* phosphoric acid–methanol (80:20). The vacuum (about 10 mmHg for 15–20 s) was turned off as soon as the last drop of the aqueous solution had fully penetrated the column. A rinse with 1 ml of hexane followed (about 5 mmHg for 10–15 s and no complete drying of the sorbent bed) prior to a slow elution (*ca.* 1 ml/min) with 1 ml of hexane–ethyl acetate (80:20) into a test-tube. The eluate was evaporated to dryness under a gentle stream of nitrogen at room temperature and the residue was dissolved in 100 μl running buffer.

Bond Elut Certify II. Very low recoveries were obtained using the manufacturer's instructions, so the procedure was modified. For hydrolysis 0.3–0.5 ml of 10 *M* KOH was added to 5 ml of urine, vortex-mixed and heated at 50–60°C for 15 min with stirring. A 2-ml volume of 0.1 *M* sodium acetate buffer (pH 7) containing 5% methanol was added and the pH was adjusted to 6.5 with concentrated HCl. The Bond Elut Certify II cartridges were conditioned immediately prior to use by passing sequentially 2 ml of methanol and an equal volume of 0.1 *M* sodium acetate buffer (pH 7) containing 5% methanol. The vacuum was turned off in time to prevent column drying. The hydrolysed and vortex-mixed specimen was then applied and drawn slowly (1–2 ml/min) through the cartridge. The column was rinsed with 10 ml of methanol–water (1:1). The vacuum (about 10 mmHg for 15–20 s) was turned off as soon as the last drop of the aqueous solution had fully penetrated the column. A rinse with 2 ml of ethyl acetate followed by an applied vacuum of about 5 mmHg for 15–20 s (no drying under full vacuum). Elution was effected slowly (1–2 ml/min) with 2 ml of hexane–ethyl acetate (75:25) containing 1% acetic acid into a test-tube. The eluate was then evaporated to drynesss under a gentle stream of nitrogen at room temperature and the residue was dissolved in 100 μl of running buffer.

Clean Screen THC. Hydrolysis and extraction were executed in a slightly modified way as recommended by the manufacturer of the cartridges. For hydrolysis 0.1 ml of 10 *M* KOH was added to 5 ml of urine, vortex-mixed and heated at 50–60°C for 20 min with constant mixing. Thereafter the pH was adjusted to 3 with concentrated HCl. The cartridges were conditioned immediately prior to use by passing sequentially 3 ml of methanol and an equal volume of water through the columns. The vacuum was turned off as soon as the fluid reached the sorbent bed to prevent column drying. The hydrolysed and vortex-mixed specimen was then applied and drawn slowly (1–2 ml/min) through the cartridge. The column was sequentially rinsed with 2 ml of water and 2 ml of 0.1 *M* HCl–acetonitrile (70:30). The vacuum (about 10 mmHg for about 15 s) was turned off as soon as the last drop of the aqueous solution had fully penetrated the column. A rinse with 0.2 ml of hexane followed, prior to a slow elution (*ca.* 1 ml/min) with 2 ml of hexane–ethyl acetate (50:50) into a test-tube. The eluate was then evaporated to dryness under a gentle stream of nitrogen at room temperature and the residue was dissolved in 100 μl of running buffer.

Recovery

The recovery after sample pretreatment was determined by comparing the MECC peak heights after extraction with those obtained by direct injection of equal amounts of THC-COOH dissolved in methanol.

RESULTS AND DISCUSSION

With the experimental conditions used in this work THC-COOH was found to elute after 23.1 ± 0.4 min. Fig. 1 depicts (A) the three-dimensional electropherogram and (B) the absorption spectrum extracted as a normalized spectrum from the gathered data and referred to as the so-called time slice for THC-COOH. For MECC with on-column UV absorption detection, sample concentrations have to be at least on the μg/ml (μ*M*) concentration level [7], which is uncommon for THC-COOH in human urine [1–6]. For direct urine injection, this sensitivity limit is not as good as that of the commonly used immunological screening methods. Therefore, extraction and concentration of THC-COOH are crucial for its confirmation by MECC.

The use of four different disposable solid-phase

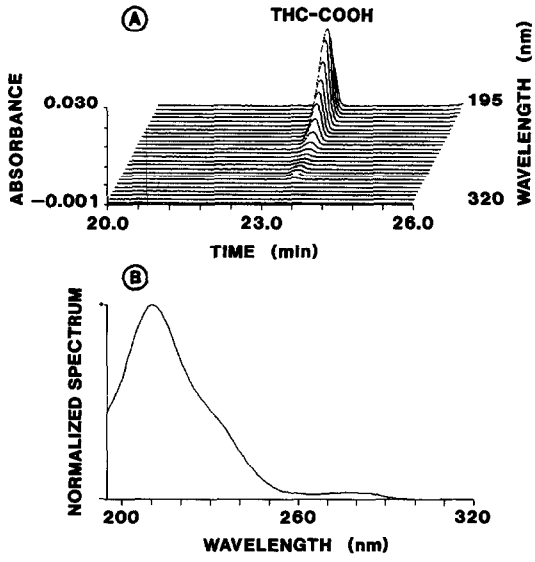

Fig. 1. (A) Three-dimensional MECC electropherogram of THC-COOH and (B) extracted, normalized absorption spectrum at 23.4 min. The sample was 100 μg/ml of THC-COOH dissolved in running buffer. The applied voltage was a constant 20 kV and the current was *ca.* 80 μA.

extraction columns was investigated. First Bond Elut THC cartridges containing a bonded-phase silica gel were employed according to three procedures reported in the literature [2–4]. The MECC analysis of the extracts obtained with blank urine spiked with 200 ng/ml of THC-COOH provided very simple and clean electropherograms (Fig. 2A). The compound of interest was recovered, but the extraction efficiencies were found to be of the order of only 10%. No work was undertaken to try to optimize the use of this column type because the recovery was much improved with the Clean Screen THC columns which contain a proprietary bonded silica sorbent with mixed-mode properties, a hydrophobic and ion-exchange copolymer [10]. With this approach (5 ml of urine and final reconstitution in 100 μl of SDS buffer) the extraction efficiency was always at the 80 ± 10% level, allowing the determination of THC-COOH concentrations in urine as low as 30 ng/ml. Although the electropherograms were found to be more complex than with Bond Elut THC (Fig. 2A and B) THC-COOH eluted free from interferences. Fig. 2C and D depict single-wavelength data obtained after Bond Elut Certify and Bond Elut Certify II extraction, respectively, of blank urine spiked with 400 ng/ml of THC-COOH. With the Bond Elut Certify column, which also contains a hydrophobic and ion-exchange copolymer, THC-COOH was found to be extracted at the same level as with the Clean Screen THC

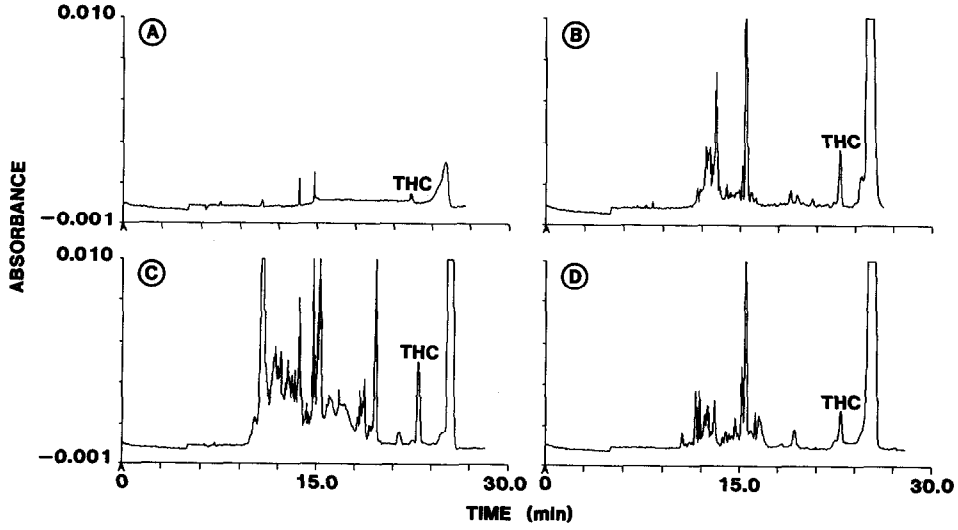

Fig. 2. Single-wavelength electropherograms (210 nm) of the MECC of THC-COOH extracted from spiked blank urine using (A) Bond Elut THC with 200 ng/ml THC-COOH, (B) Clean Screen THC (200 ng/ml), (C) Bond Elut Certify (400 ng/ml) and (D) Bond Elut Certify II (400 ng/ml). THC indicates the THC-COOH peaks. Other conditions as in Fig. 1.

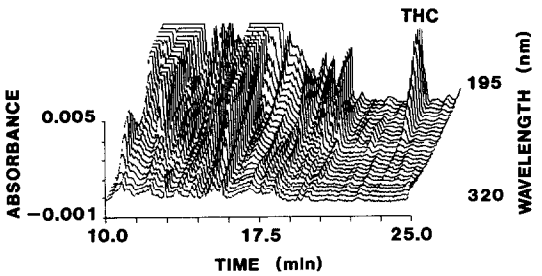

Fig. 3. Analysis of a cannabinoid-positive urine sample from a patient after extraction with Bond Elut Certify. Other conditions as in Figs. 1 and 2.

cartridge, but providing a more complex electropherogram (Fig. 2B and C). The urine matrix becomes more simplified with Bond Elut Certify II (Fig. 2D) than with Bond Elut Certify, presumably

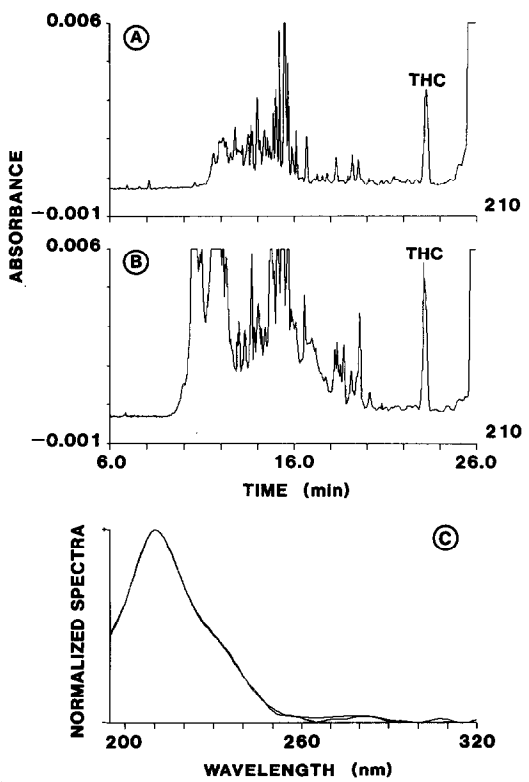

Fig. 4. (A and B) Single-wavelength electropherograms (210 nm) and (C) normalized absorption spectra of a markedly cannabinoid-positive urine sample from a patient employing (A and C) the Clean Screen THC and (B) the Bond Elut Certify extraction procedures. Other conditions as in Figs. 1 and 2.

because the former modified silica gel material exhibits three types of interactions, hydrophobic, polar and ion exchange [5,6]. The recovery, however, was not as good (about 25%), in contrast to the results reported by Dixit and Dixit [6]. With acidification of the urine specimens to pH 4.8 or 3.0 the yields with the Certify II columns became even lower than 25%.

Based on our investigations discussed above, both the Clean Screen THC and Bond Elut Certify procedures were used for confirmation of urine samples from patients which tested FPIA positive. MECC data for a urine specimen which was found to be markedly positive for cannabinoids (> 135 ng/ml), and also positive for cocaine (> 300 ng/ml) and negative for methadone and opiates (< 300 ng/ml each) employing EMIT assays, are depicted in Figs. 3 and 4. These data clearly illustrate that with the Clean Screen THC method a much cleaner extract is obtained than with the Bond Elut Certify approach, and that with both extraction techniques well established THC-COOH peaks were monitored (Fig. 4A and B). Further, having data between 195 and 320 nm, in addition to reference spectra, permitted a rapid and reliable confirmation of the presence of THC-COOH in this urine. The excellent agreement between the time slices with those of computer-stored standards is shown by the graphs in Fig. 4C. With simple calibration graphs based on peak heights, the THC-COOH concentration was calculated to be *ca.* 300 ng/ml. It is interesting that benzoylecgonine could not be assigned to one of the peaks detected, particularly those with a retention time of about 12 min [8].

The second example presented concerned a urine sample tested FPIA positive for cannabinoids (67 ng/ml) and EMIT negative for cocaine and opiates (< 300 ng/ml each). This urine sample was also treated with the Bond Elut Certify and Clean Screen THC procedures. MECC data are shown in Fig. 5. In both instances, small THC-COOH peaks were obtained which could be unambiguously assigned by comparison of spectra (Fig. 5C). The concentration of this compound in the urine was determined to be about 40 ng/ml, which compares favorably with the 67 ng/ml obtained with FPIA, a method which responds to different THC metabolites simultaneously. In the experiments performed THC-COOH was extracted from 5 ml of urine and

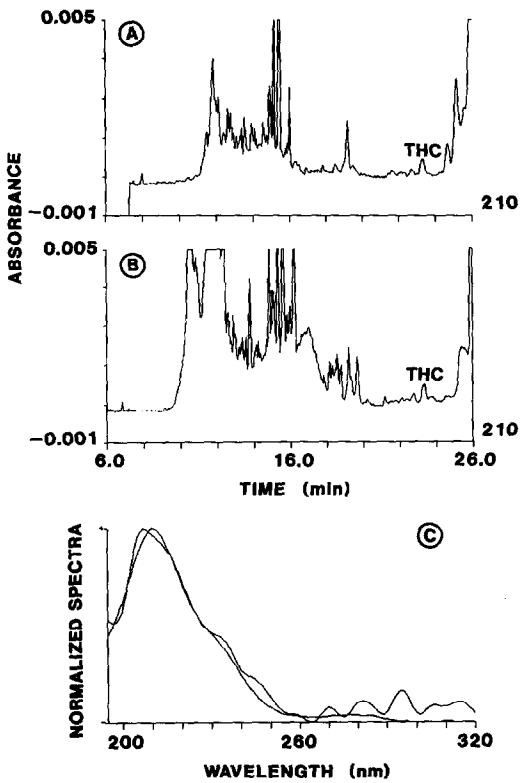

Fig. 5. Similar data to Fig. 4, but for a urine specimen with a much lower THC-COOH content, depicting the limit of the MECC approach with multi-wavelength detection.

reconstituted into 100 μl of SDS buffer, providing THC-COOH peaks which are close to a detection limit of about 30 ng/ml. Detection limits of 10 ng/ml or lower can be attained by employing a larger urine volume and/or final reconstitution in less than 100 μl of SDS buffer.

It is interesting that after investigating urine samples from more than fifteen patients no false-positive specimen was found. The presence of THC-COOH was confirmed in all seven positively tested specimens. Further, THC-COOH could not be found in six urines which gave no clear result with FPIA, cases in which the FPIA instrument provided a warning because of much too high background signal, a possible consequence of sample adulteration [1].

In conclusion, solid-phase extraction of THC-COOH using copolymeric sorbents has been shown to provide extracts that are sufficiently clean and concentrated for the MECC determination of THC-COOH with on-column multi-wavelength UV absorption detection and, without complete optimization, providing a detection limit of about 10 ng/ml. Thus MECC represents an attractive confirmation method for urine specimens that tested positive for cannabinoids by an immunological screening method and therefore represents an interesting alternative to existing chromatographic methods.

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