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Note

Confirmation of cannabis abuse by the determination of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in urine with high-performance liquid chromatography and electrochemical detection

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Immunological assays are widely used in the field of sociomedical and forensic urine drug screening. The most common methods used to detect 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), the major urinary metabolite of the psychoactive cannabis constituent Δ^9-tetrahydrocannabinol (THC) [1–3], are enzyme-multiplied immunoassay techniques (EMIT) [4,5] and radioimmunoassays (RIA) [5–7]. As these tests are unspecific, positive results must be confirmed by a second independent method, e.g. thin-layer chromatography (TLC) [5,8–10], gas chromatography (GC) [11–13], gas chromatography–mass spectrometry (GC–MS) [5,13–18] or high-performance liquid chromatography (HPLC) [13,19–21].

This paper describes a procedure using a simple bonded-phase adsorption clean-up and HPLC with electrochemical detection (ED). The sensitive method allows to measure THC-COOH in urine samples down to the low ng/ml level.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of an Altex 420 controller/programmer (Kontron, Zurich, Switzerland), two Altex 110A pumps, a pulse-dampener, a Rheodyne 71-25 injection valve with a 20-μl loop, a thermostatted Metrohm 656/VA 641 electrochemical detector (Metrohm, Herisau, Switzerland) and a Shimadzu Chromatopac C-R1A recording data processor (Kontron). The glassy carbon working electrode was set at 1.2 V versus an Ag/AgCl reference electrode; the sensitivity was 5 nA full scale. Separation was performed on a 150×4.6 mm I.D.

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column, packed with Spherisorb 3-μm ODS-2 using a slurry technique [22]. The mobile phase was methanol–5% aqueous acetic acid (76:24) at a flow-rate of 1.5 ml/min.

Mass spectra were run on a 5993 GC–MS system (Hewlett-Packard, Waldbronn, F.R.G.), equipped with a 25 m×0.2 mm I.D. fused-silica cross-linked 5% phenyl methyl silicone (HP-5) column, operated at 250°C. The carrier gas was helium at a flow-rate of 0.6 ml/min. The mass spectrometer was operated in the selected-ion monitoring (SIM) mode with an EM voltage of 2800.

Chemicals and reagents

All chemicals were of analytical or HPLC grade, purchased from Fluka (Buchs, Switzerland). Spherisorb 3 ODS-2 (PhaseSep) was obtained from Ercatech (Berne, Switzerland), Bond-Elut®-THC columns (500 mg, bonded phase silica gel) from Analytichem International (Harbor City, CA, U.S.A.) through ICT (Basle, Switzerland). THC-COOH was provided by Research Triangle Institute (Research Triangle Park, NC, U.S.A.), cannabinol (CBN) by UN Narcotic Laboratory (Vienna, Austria; commercially available at Supelco, Gland, Switzerland). The silylation reagents N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) and *tert.*-butyldimethylsilyl chloride (TBDMS-Cl) were obtained from Pierce (Rockford, IL, U.S.A.) through Kontron.

Method

Most of the urine samples were obtained from pharmacies or health and rehabilitation centres that participate in a sociomedical programme against drug abuse. The urines were screened for the presence of cannabinoids by the EMIT®-st cannabinoid urine assay (Syva, Palo Alto, CA, U.S.A.; Merck, Zurich, Switzerland). The cutoff calibrator of this test contains 100 ng/ml 11-nor-Δ^8-THC-9-carboxylic acid (Δ^8-THC-COOH). Samples that gave a UV response equal or higher than the calibrator's response were interpreted as positive and confirmed by the HPLC method.

To 10 ml of urine (blank, spiked or EMIT-st positive), 10 μl of a 90 μg/ml methanolic solution of CBN (internal standard; I.S.) and 2 ml of 10 *M* potassium hydroxide were added. After hydrolysis with stirring at 50°C for 20 min, the urine was then adjusted to pH 5–6 with concentrated hydrochloric acid. The sample clean-up was performed on a Bond-Elut-THC column according to ElSohly et al. [19] and the manufacturer's directions, but with the following modifications: before eluting with two aliquots of 750 μl of acetonitrile the column was dried under vacuum for 5 min. The first aliquot was allowed to percolate through the column without vacuum, the second was aspirated slowly under vacuum. Aliquots of 7 μl of the combined eluates were injected into the HPLC system. Quantitation was done by measuring the peak heights of THC-COOH and the I.S.

For the GC–MS analysis, aliquots of 750 μl of the urine extract were evaporated under a stream of nitrogen, and the residue was dissolved in 25 μl of acetonitrile and 25 μl of MTBSTFA with 1% TBDMS-Cl. The mixture was heated at 60°C for 1 h and 5 μl of the derivatized extract were injected splitless into the GC–MS

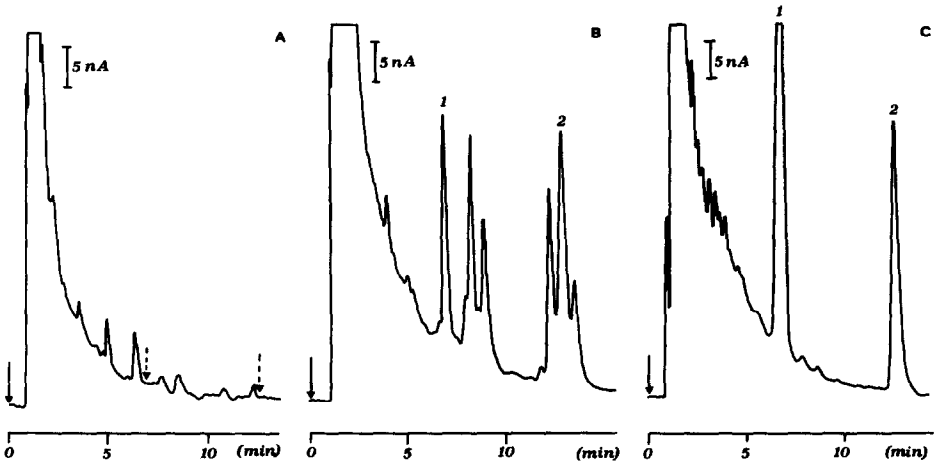

Fig. 1. Chromatograms of (A) blank urine, (B) blank urine spiked with 100 ng/ml THC-COOH and 90 ng/ml CBN and (C) EMIT-st positive urine (168 ng/ml THC-COOH). Peaks: 1 = THC-COOH, 2 = CBN (I.S.).

system. The TBDMS derivative of THC-COOH was identified on the base of the diagnostic ions 572, 557, 515 and 413.

RESULTS AND DISCUSSION

Because 65–100% of urinary THC-COOH is excreted as O-glucuronide conjugates [20], urine samples have to be hydrolysed to get the free, unconjugated THC-COOH. The internal standard CBN is a naturally occurring cannabinoid, formed by degradation of THC during storage or smoking of cannabis products [23]. However, it is mainly excreted in the faeces, with only 8% being found in the urine, almost entirely as acidic metabolites [24]. No detectable amounts of unchanged CBN could be observed in EMIT-st positive urines, which were used without adding I.S. to evaluate the chromatographic system. A simple and rapid solid-phase extraction eliminates most of the endogenous urine compounds. The characteristic chromatogram of a blank urine is shown in Fig. 1A. THC-COOH and the I.S. have the same recovery of $90 \pm 5\%$, despite the structural difference of one carboxyl group. The clean-up procedure is also applicable to urine volumes smaller than 10 ml. If, for instance, only a 5-ml sample is available, 5 μl instead of 10 μl of I.S. solution are added and 14 μl instead of 7 μl injected into the HPLC system. The urine extracts are stable over several months when stored at $-20\,^{\circ}$C.

The same extracts can also be used for alternative confirmation methods, e.g. TLC or GC–MS. GC–MS analysis was performed with some urine extracts derivatized with the new silylation reagent MTBSTFA and TBDMS-Cl as catalyst. The resulting TBDMS derivatives are more stable, formed with a better yield and show therefore a greater sensitivity than trimethylsilyl (TMS) derivatives [25]. The mass spectrum of the TBDMS derivative of THC-COOH contains the typical and intense $(M-57)^+$ ion, corresponding to the loss of a *tert.*-butyl frag-

ment. Other characteristic ions are 572 (M^+), 557 [$(M-15)^+$] and 413. With SIM it was possible to confirme urine samples down to the detection limit of the proposed HPLC method.

Among the C_{18} reversed-phase materials tested only Spherisorb 3-μm ODS-2 (12% C, fully capped) was sufficiently efficient to separate even complex urine extracts. About 5% of the urine samples showed an uncommon endogenous peak pattern (see Fig. 1B). However, these peaks did not interfere with the quantitation of THC-COOH. The same holds true for urine specimens containing other drugs (opiates, barbiturates, cocaine, etc.).

The chromatographic system showed excellent stability with little drift in retention times over the course of a working day. Nevertheless, to avoid possible false THC-COOH peak identification the separation factor α (relative retention, selectivity) was calculated after each run using the capacity factors (k') of the I.S. and THC-COOH. For a positive identification the α value had to be 2.02 ± 0.002. The nominal α value was determined by analysing ten times an urine spiked with the I.S. and THC-COOH (Fig. 1B).

Under the described chromatographic conditions, an applied potential of +1.2 V was considered to offer the best sensitivity and similar detector response for THC-COOH and the I.S. At 5 nA the limit of detection for THC-COOH was 5 ng/ml of urine with a signal-to-noise-ratio of 5:1. In this case, injecting 7 μl of urine extract corresponds to an absolute amount of 230 pg of THC-COOH.

The standard curve was obtained by using blank urine spiked with 25–300 ng/ml THC-COOH and 90 ng/ml I.S. The samples were analysed by the procedure described under Experimental. A linear relationship ($r=0.999$) was found between the peak-height ratio of THC-COOH versus I.S. and the concentration of THC-COOH. The reproducibility of the method was measured by the analysis of five replicates of two blank urine samples spiked with 100 and 25 ng/ml. The between-day coefficients of variation for THC-COOH were 2.2 and 3.3%, respectively.

With the HPLC method, all of 50 EMIT-st positive urine specimens could be confirmed as THC-COOH positive. The chromatogram of an EMIT-st positive urine is shown in Fig. 1C. The THC-COOH content varied between 30 and 280 ng/ml of urine. THC-COOH contents far below the cutoff calibrator concentration of 100 ng/ml Δ^8-THC-COOH can be explained by the fact that an EMIT immunoassay may react with endogenous compounds and other THC urine metabolites [11]. The high sensitivity of the ED even allows the confirmation of EMIT-d.a.u. (cutoff 20 ng/ml) positive urines.

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