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DETERMINATION OF Δ^9-TETRAHYDROCANNABINOL IN PLASMA USING SOLID-PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A method for the determination of nanogram amounts of Δ^9-tetrahydrocannabinol (THC) in plasma and serum is described. THC was quantitatively isolated by solid-phase extraction after addition of an aqueous solution of urea and methanol to the sample. The extracts were analysed by high-performance liquid chromatography with electrochemical detection in the oxidizing mode. The detection limit of THC is *ca.* 100 pg for a signal-to-noise ratio of 3. With this method, levels of 2 ng/ml of THC in plasma can be measured.

INTRODUCTION

The active constituent of *Cannabis sativa* L., Δ^9-tetrahydrocannabinol (THC) (Fig. 1), can impair the performance of complex coordinated psychomotor skills in, *e.g.*, driving a motor vehicle[1]. Consequently, in pharmacology and forensic toxicology the determination of THC in biological matrices (blood, serum or plama) has already received a great deal of attention and many methods for the determination of THC have been described[2,3].

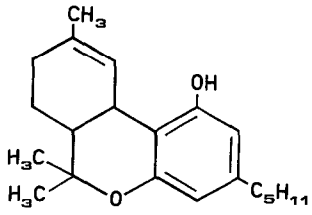

Fig. 1. Structure of Δ^9-tetrahydrocannabinol (THC).

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Very sensitive techniques for the determination of THC are required, on account of the low concentrations of the parent drug usually encountered in body fluids and tissues. Typical concentrations of THC in plasma or blood during intoxication are in the low ng/ml range. For pharmacokinetic studies and screening purposes, fast and cheap methods are preferred and in forensic cases the determination of the inactive metabolite 11-nor-9-carboxy-THC is not sufficient for determining whether a person is actually under the influence of the drug.

Major problems in the determination of THC include extraction from biological matrices with sufficient recovery and obtaining extracts without interfering substances.

Gas chromatographic (GC) methods with electron-capture detection, GC–mass spectrometric (GC–MS) procedures and radioimmunoassay (RIA) have been most frequently[2,3] applied. The GC methods usually require derivatization of the phenolic group of THC; RIA methods do not distinguish completely between THC and its metabolites and degradation products. On the other hand, high-performance liquid chromatographic (HPLC) methods with UV detection are hampered by insufficient sensitivity and selectivity, and fluorescence detection also requires the derivatization of THC[4].

We have developed a simple and sensitive assay for the determination of THC in plasma that can be used in the routine screening of plasma or serum samples and in pharmacokinetic studies. THC is isolated from plasma using solid-phase extraction followed by HPLC with electrochemical detection. The recovery of THC with the described extraction procedure is complete and highly reproducible.

EXPERIMENTAL

Materials

THC (1.00 g mixed with an unknown amount of ethanol in a glass vial) was purchased from Macor (Jerusalem, Israel). The content of the vial was transferred quantitatively into a volumetric flask and diluted to 100 ml with absolute ethanol. The resulting stock solution was stored at −18°C. Standard solutions containing 0.05–10 μg/ml of THC were prepared by diluting portions of the stock solution with methanol and were stored at −18°C until used as standards in chromatography or for spiking plasma. Drug-free citrate plasma was stored at −18°C until used.

Plasma was spiked as follows. The required amount of THC solution in methanol was mixed with the same amount of water in a volumetric flask, then the flask was filled with plasma to the calibration line, mixed and stored for at least 12 h in a refrigerator at 4°C for equilibration before use or storage at −18°C. The volume of the methanolic THC solution mixed with plasma was chosen so that the resulting spiked plasma did not contain more than 1% (v/v) of methanol.

Methanol and diethyl ether were of glass-distilled grade from Rathburn (Walkerburn, U.K.). Tetrahydrofuran (analytical-reagent grade). (Merck, Darmstadt, F.R.G.) was distilled not more than 2 weeks before use and was stored under nitrogen in a brown-glass bottle in a refrigerator. Water was purified using the Milli-Q/Organex-Q system (Millipore, Molsheim, France) and stored in glass containers. Urea was obtained from Sigma (St. Louis, MO, U.S.A.). The urea solution (8 *M* in water) was passed through a Bond-Elut C_{18} column (a 50-ml portion over

a 3-ml activated column containing 500 mg of stationary phase) before use in order to remove organic impurities.

Bond-Elut C_{18} solid-phase extraction columns (1 ml, containing 200 mg of stationary phase) were purchased from Analytichem International (Harbor City, CA, U.S.A.). To these columns a 1-ml polypropylene sample reservoir was attached. Polypropylene tubes (10 ml) were obtained from Greiner (Alphen aan den Rijn, The Netherlands).

Glass centrifuge tubes (10 ml) were silanized by allowing them to dry after rinsing with a 2% (w/v) solution of dimethyldichlorosilane in 1,1,1-trichloroethane (LKB, Bromma, Sweden) and rinsing then with methanol. The tubes were capped with polyethylene cap when in use.

Apparatus

The chromatographic system consisted of a U6K injector and a Model 510 solvent delivery system (both from Waters Assoc., Milford, MA, U.S.A.). A 300 × 4.6 mm I.D. column packed with 5-μm silica was placed between the pump and injector in order to damp pressure pulses. The analytical column was a stainless-steel (100 × 4.6 mm I.D.) Chromsep high-resolution cartridge packed with reversed-phase C_{18}-modified silica of 3 μm particle size (Chrompack, Middelburg, The Netherlands). A standard Chromsep guard column (10 × 2.1 mm I.D.) filled with C_{18}-coated 40-μm pellicular silica (Chrompack) preceded the analytical column.

Isocratic elution was employed with a mobile phase consisting of tetrahydrofuran–methanol–0.005 *M* sodium citrate buffer, pH 7.0 (7.5 : 68 : 24.5, v/v), prepared 24 h before use and degased by sonication for 10 min. Chromatography was performed at ambient temperature at a flow-rate of 1.0 ml/min.

The electrochemical detector used was described by Holthuis[5]. The working electrode was made of glassy carbon (Metrohm, Herisau, Switzerland) with a diameter of 3 mm, and a silver–silver chloride (3 *M* potassium chloride) electrode was used as a reference electrode. The auxiliary electrode was made of stainless steel. The electrochemical cell was connected to a Metrohm 641 VA potentiostat. The glassy carbon electrode was polished daily for 1 min with 0.3 μm aluminium oxide powder (Metrohm, EA 1086).

To speed up the stabilization, the working electrode was polarized in the detector cell for 20 min at +960 mV. The potential was then decreased to the working potential of +760 mV. Chromatograms were recorded with a flat bed BD-40 recorder (Kipp & Zonen, Delft, The Netherlands). Injections were made with a Hamilton (Bonaduz, Switzerland) microlitre syringe.

Sample preparation

Sample preparation was carried out with a Supelco (Supelchem, Leusden, The Netherlands) vacuum manifold. The solid-phase extraction column was fitted to a 10-ml polypropylene sample reservoir. The column was activated by rinsing it with 2 ml of methanol and 2 ml of water.

Plasma (1.00 ml) was transferred into a polypropylene tube and 2.0 ml of 8 *M* urea solution were added. After vortexing for 5 s, 2.0 ml of methanol were added and after vortexing again for 5 s the mixture was transferred into the sample reservoir of the solid-phase extraction column. The fluid passed through the column within 2–3

min. The polypropylene tube was rinsed with 2 ml of water–methanol–8 *M* urea solution (1:2:2) and this fluid was also transferred to the solid-phase column. The reservoir was removed and the column was rinsed subsequently with 2 ml of methanol–water (1:1, w/w), 1 ml 0.2 *M* hydrochloric acid, 1 ml of methanol–water (1:1, w/w), 1 ml 0.01 *M* sodium hydroxide solution and 3 ml methanol–water (1:1, w/w). The column was centrifuged for about 10 min in order to remove remaining fluid. THC was eluted with 0.5 ml of diethyl ether. The eluate was evaporated to dryness at 40°C in a 10 ml silanized tube and the residue was dissolved in 100.0 μl of methanol and vortexed for 5 s. A 10.0-μl volume was injected into the chromatographic system.

Calibration was performed by injecting extracts of plasma samples spiked with THC at concentrations ranging from 0 to 100.0 ng/ml. Peak heights were measured.

The mean extraction yield and inter- and intra-assay variability were determined by spiking 50 ml of blank plasma with THC at a concentration of 20 ng/ml and determining the THC concentration in five 1-ml portions each day on four different days. Peak heights of THC obtained after injection of 10 μl of the plasma extracts were compared with those obtained by injecting 10.0 μl of methanol containing 2.00 ng of THC.

RESULTS AND DISCUSSION

Chromatography and detection

Several stationary phases were tested for the chromatography of THC and plasma extracts, *viz.*, C_2 (5 μm) (250 × 4.6 mm I.D.), cyanopropyl (5 μm) (100 × 3.0 mm I.D.), diol(5 μm) (100 × 3.0 mm I.D.) and C_{18} (5 μm) (100 × 3.0 mm I.D.) bonded phases. However, none of these was suitable for separating THC from plasma components. Methanol as the modifier of first choice resulted in a poor peak shape of THC in some instances. When using a C_2 column this could be improved by using modifiers of lower viscosity, *e.g.*, acetone or acetonitrile. However, in these instances the detector sensitivity decreased rapidly, causing unacceptable baseline drift. With other columns we could not separate THC from plasma components adequately. In order to obtain a greater separation power we tried smaller particle sizes of the stationary phase. C_{18} column material of particle size 3 μm was selected, with which methanol as modifier gave the best detector stability and sensitivity. Tetrahydrofuran gave poor stability and sensitivity but a substantial improvement of chromatographic resolution in an equi-eluotropic concentration with respect to THC. The mobile phase that we used [THF–methanol–buffer (7.5:68:24.5)] was a compromis giving sufficient chromatographic separation and acceptable detector stability and sensitivity.

In order to find the optimal detection potential we constructed a hydrodynamic voltammogram for THC using a THC standard in methanol. Fig. 2 shows the hydrodynamic voltammogram of THC. A plateau is reached at +820 mV, indicating the optimal potential for the detection of THC. A small peak in the chromatogram of some plasma extracts (equivalent to about 2 ng/ml of THC) was not completely separated from the peak of THC. This small peak could be completely eliminated by choosing +760 mV as the detection potential, resulting in a slight loss of sensitivity.

The detection limit (signal-to-noise ratio = 3) is approximately 100 pg, as can be seen in Fig. 3a. The detector signal was linear from 0.5 to 100 ng ($r = 0.9999$), provided that the amounts were injected in the same volume of methanol (*e.g.* 10 μl). Fig.

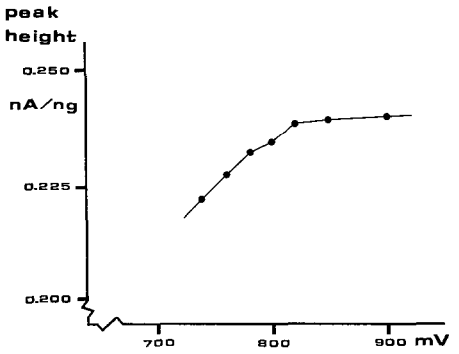

Fig 2. Hydrodynamic voltammogram obtained after repeated injection of 5 ng of THC in 10 μl of methanol.

3 shows chromatograms of 1 ng of THC, extracts of drug-free plasma, plasma spiked with 20 ng/ml of THC and an extract of the serum from a victim of involuntary ingestion of cannabis resin.

Isolation of THC from plasma

Numerous methods for extracting THC from body fluids have been publised, although few of them give exact extraction yields[6–13] (Table I). Most of them give

TABLE I
PUBLISHED EXTRACTION RECOVERIES OF THC

Ref.	*Year*	*Method**	*Sensitivity (ng/ml)*	*Matrix*	*Extraction solvent*	*Recovery (%)*
6	1977	HPLC + GC–ECD**	0.2	Plasma	Heptane–isoamyl alcohol (98.5:1.5)	90.8 ± 2.6
7	1980	TLC–MS	<0.5	Plasma	Extrelut column with diethyl ether or ethyl acetate	81.5–97
8	1981	HPLC + vis.***	50	Plasma, brain tissue	DCM§§–hexane (2:5) after addition of methanol	76.2 ± 9.2
9	1983	GC–MS	0.2	Plasma	Acetonitrile	86
10	1983	GC–MS	5	Serum, blood	Hexane	40
11	1984	TLC + fluor.§	1	Plasma	Methanol, 3% isoamyl alcohol in hexane	95–98
12	1986	GC–MS	0.8	Plasma	Heptane–isoamyl alcohol (98.5:1.5)	64.9
13	1986	GC–ECD, GC–MS	0.3	Plasma	XAD-2 resin after adding 15% (v/v) of acetonitrile	Max. 95%§§§

* ECD = electron-capture detection; TLC = thin-layer chromatography.
** Fractionation by HPLC and determination of THC by GC with ECD after derivatization.
*** Derivatization of THC to a coloured product and determination by HPLC with visible light absorbance detection.
§ Extraction followed by labelling with a fluorescent label and fluorimetry performed on the isolated TLC spot.
§§ DCM = dichloromethane.
§§§ Dependent on contact time between resin and plasma.

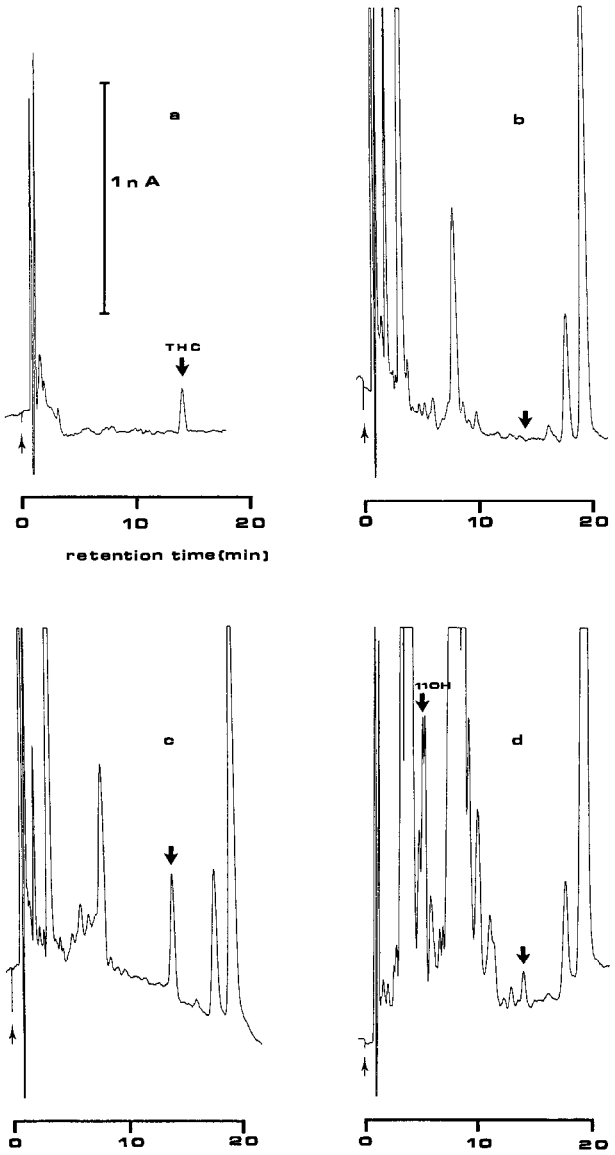

Fig. 3. (a) Chromatogram obtained after injection of 1.0 ng of THC in 10.0 μl of methanol. (b) Chromatogram of blank plasma. (c) Chromatogram of an extract of plasma spiked with 20.0 ng/ml of THC. (d) Chromatogram of an extract of serum from a victim of involutary ingestion of cannabis resin, which appeared to contain 5 ng/ml of THC. The peak marked 11-OH coincides with 11-hydroxy-THC.

incomplete extraction recoveries and poor reproducibility. When studying the solid-phase extraction behaviour of THC from plasma and from spiked aqueous phosphate buffers (0.1 *M*, pH 7.4), we found that the recovery was poor. Retention on and elution from the bonded-phase column could not be the problem, because when THC was placed directly on the packing of the activated bonded-phase column in

a very small volume of methanol (10 μl) the THC could be eluted quantitatively with 0.75 ml of methanol even after washing with 5 ml of methanol–water (1:1, v/v). The recovery from spiked buffers could be improved by adding the same volume of methanol to these aqueous solutions. In this way the solubility of the very hydrophobic THC was increased sufficiently to prevent adsorption on the walls of the vessel in which the solutions were prepared. When plasma is spiked with THC, the THC is solubilized by binding to proteins and lipoproteins. When spiked plasma was placed on a bonded-phase column the recovery was poor (about 40%). Obviously the diffusion of THC from the binding sites to the C_{18} layer through the unmodified aqueous phase has to be facilitated. However, this could not be applied to plasma samples because addition of methanol resulted in precipitation of proteins and with this significant coprecipitation of THC occurred, resulting in clogging of the bonded-phase column, low extraction yields and poor reproducibility.

Obviously protein binding of THC is still a problem after precipitation of the proteins. Therefore, we searched for means of denaturing plasma proteins without precipitation even when methanol was to be added to the solution. It is well known that urea is capable of denaturing proteins by influencing their tertiary structure. The protein molecules are deconvoluted and in this way binding sites for smaller molecules are lost, although sometimes new ones are created[14]. The denaturation by urea is believed to be based on the following two effects[15]. First, the interaction between water molecules is altered in concentrated urea solution, facilitating the dissolution of hydrophobic parts of the protein. This, of course, will also have an effect on the dissolution of other hydrophobic compounds, *e.g.*, THC. Second, urea interacts with the peptide groups of the protein, resulting in the loss of the tertiary structure.

The above mechanisms of protein denaturation might give the impression that urea alone is capable of increasing THC recoveries, but it was evident that the addition of methanol resulting in a final methanol concentration of at least 25–30% (v/v) was also necessary to give maximum extraction yields in bonded-phase extraction. This was in good agreement with the results of Rosenfeld *et al.*[13], who found that extraction of THC with XAD-2 resin was more complete and effected in a shorter time when 15% (v/v) of acetonitrile was added to the plasma sample.

Of course, after reconstitution of the dried extract with 100 μl of methanol evaporation should be avoided and measurement of the injected volume must be

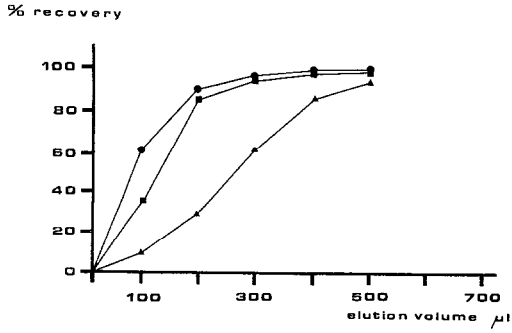

Fig. 4. Cumulative recoveries of 1 μg of THC placed on a solid-phase extraction column and eluted with 100-μl portions of different solvents: ●, diethyl ether; ■, methanol; ▲, hexane.

TABLE II
MEAN EXTRACTION RECOVERY FOR THC AT A CONCENTRATION LEVEL OF 20 ng/ml DETERMINED FIVE TIMES ON FOUR DIFFERENT DAYS

Day No.	*Recovery (%)*	*S.D. (%) (n=5)*
1	99.0	1.79
2	99.7	1.74
3	99.5	1.77
4	99.4	2.05
Mean	99.4	1.84

accurate. When stored well capped at −18°C the extracts were stable for at least 4 days.

THC was eluted from the bonded-phase column with diethyl ether, 0.5 ml of wich was enough for complete elution. The ether fraction dried in about 10 min. Using methanol a larger volume (0.75 ml) was needed and also about 40 min were required for evaporation, the chromatograms being essentially the same. Fig. 4 shows the elution profile of THC from the column with different solvents.

The calibration line measured using samples spiked with concentrations from 6.25 to 50 ng/ml was linear ($r=0.9999$, $n=8$).

To validate this relatively uncommon procedure without the use of an internal standard we determined the intra- and inter-assay variability as described. There was no significant difference between the within-day recovery and the standard deviation of these recoveries and the between-day values at the 20 ng/ml concentration level, as can be seen from Table II. Owing to the complete recovery of THC and the high reproducibility, no internal standard was necessary.

The chromatogram for plasma from a victim of cannabis resin intoxication (Fig. 3d) shows more peaks than that for spiked plasma. This could be due to other cannabinoids present in the cannabis resin and metabolites of THC. We noticed that the peak in Fig. 3d marked 11-OH coincides with the peak of the 11-hydroxy metabolite of THC.

CONCLUSIONS

The recovery of THC from plasma with bonded-phase extraction columns is complete if the transfer of THC from binding sites (proteins, lipids) to the bonded phase is facilitated. In the method described this is achieved by the addition of urea and methanol. The extraction method combined with HPLC and electrochemical detection results in a sensitive method for measuring THC levels in plasma at concentrations of pharmacological interest. Investigations on the applicability of the procedure to the determination of THC metabolites in plasma, serum and urine are in progress.

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