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## Note

# Confirmation of cannabis use

## III*. Simultaneous quantitation of six metabolites of $\Delta^9$-tetrahydrocannabinol in plasma by high-performance liquid chromatography with electrochemical detection

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$\Delta^9$-Tetrahydrocannabinol (THC), the major psychoactive ingredient of marijuana, is metabolized by a variety of species into a large number of metabolites [1]. In these metabolic pathways of THC, the oxidation at the 11- and 8-positions are main routes, as shown in Fig. 1. Therefore, for in vitro or in vivo metabolic experiments on THC in different species, a sensitive and accurate method is required for the simultaneous determination of the metabolites of THC. However, it might be difficult to separate each metabolite completely by gas chromatography (GC) or high-performance liquid chromatography (HPLC) [2]. Moreover, because THC metabolites tend to bind to proteins strongly, it is very difficult to extract them completely from biological fluids by a simple extraction method [3].

In previous papers [4,5] we reported the sensitive determination of cannabinoids by HPLC with electrochemical detection (ED) and the simultaneous determination of 11-nor-9-carboxy-$\Delta^9$-tetrahydrocannabinol (THC-COOH), 11-hydroxy-$\Delta^9$-tetrahydrocannabinol (11-OH-THC) and THC in urine and plasma by HPLC–ED. This paper deals with the simultaneous, sensitive determination of six main metabolites of THC, THC-COOH, 11-OH-THC, 8$\beta$-hydroxy-$\Delta^9$-THC

*For Part II, see ref. 5.

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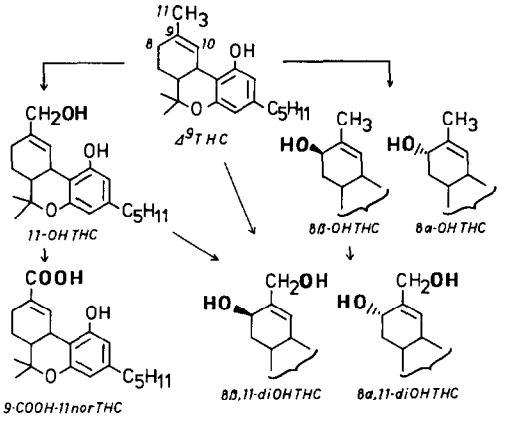


Fig. 1. Main metabolic pathways of THC.

(8$\beta$-OH-THC), 8$\alpha$-hydroxy-$\Delta^9$-THC (8$\alpha$-OH-THC), 8$\beta$,11-dihydroxy-$\Delta^9$-THC (8$\beta$,11-diOH-THC) and 8$\alpha$,11-dihydroxy-$\Delta^9$-THC (8$\alpha$,11-diOH-THC), in plasma by HPLC–ED.

## EXPERIMENTAL

### *Chemicals*

All solvents were redistilled from analytical-reagent grade materials (Nakarai Chemicals, Kyoto, Japan) prior to use. The mobile phase for HPLC was filtered through Millex-FG50 0.2-$\mu$m filters (Millipore, Molsheim, France).

### *Analytical standards*

Six analytical standards, THC-COOH, 11-OH-THC, 8$\alpha$-OH-THC, 8$\beta$-OH-THC, 8$\alpha$,11-diOH-THC and 8$\beta$,11-diOH-THC, were provided by the Research Triangle Institute through the National Institute on Drug Abuse.

### *Apparatus*

A Shimazu Model LC-6A HPLC system equipped with a Rheodyne 7125 injector and a Shimazu LC-6A system controller was used. An Irica E-502 electrochemical detector was used in series with the column and operated at +1.1 V (vs. Ag/AgCl). The chromatograms were obtained on a Shimazu Chromatopac C-R3A data recorder. The column was a 25 cm × 4.6 mm I.D. stainless-steel column packed with Zorbax $C_8$ (DuPont, Wilmington, DE, U.S.A.).

The mobile phase was acetonitrile–methanol–0.01 *M* sulphuric acid (35:15:50, v/v) at a flow-rate of 1.1 ml/min.

### *Standard sample preparation*

Stock solutions were prepared by diluting each sample of the six standards 1000-fold with ethanol to give a concentration of 1 $\mu$g/ml. The stock solutions were used to reinforce the drug-free plasma for calibration standards. Plasma was added to 5.0-, 2.5-, 1.0-, 0.5- and 0.25-ml portions of the stock solutions to yield a

total volume of 50 ml for each standard with concentrations of 100, 50, 20, 10 and 5 ng/ml. The five resulting standards were used for calibration graph calculations. Stock *n*-octyl *p*-hydroxybenzoate in methanol was used as the internal standard (I.S.) at a concentration of 1 μg/ml.

*Optimization of mobile phase for the separation of six metabolites*

In order to determine the optimal conditions for the separation of the six THC metabolites, the capacity factor ($k'$) was measured as a function of the organic modifier content. The ratio of acetonitrile was changed against constant ratio of methanol–0.01 *M* sulphuric acid (15:50).

*Pretreatment of plasma sample*

A plasma sample (2 ml) was mixed with 0.5 ml of I.S. stock solution and 20 ml of acetonitrile–pyridine (10:1), followed by sonication for 10 min. The suspended solution was centrifuged for 10 min at 1800 *g*. The precipitate was washed twice with 10 ml of acetonitrile–pyridine (10:1) by vortex-mixing for 1 min and then removed by centrifugation. Three fractions of the supernatant were combined and concentrated in vacuo. Then 1 ml of 0.1 *M* sulphuric acid and 2 ml of diethyl ether were added to this residue and the mixture was vortex-mixed for 1 min. The organic phase was separated, washed with 0.3 *M* phosphate buffer (pH 9.0) and evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of methanol and a 5-μl volume was injected into HPLC instrument.

The effects of deproteinizing and eluting solvents on the recoveries of the THC metabolites were examined by using acetone, methanol, acetonitrile, acetonitrile–pyridine (10:1), methanol–pyridine (10:1) and 10% trichloroacetic acid.

## RESULTS AND DISCUSSION

*Separation of six metabolites*

Fig. 2 shows the effect of the acetonitrile content with constant methanol–0.01 *M* sulphuric acid (15:50) on the $k'$ values of the six metabolites. Acetonitrile–methanol–0.01 *M* sulphuric acid (35:15:50) gave good separations and appropriate $k'$ values.

Fig. 3 shows a chromatogram of the extract obtained from a plasma sample spiked with 50 ng/ml of the six metabolites. Although the peaks of 8β,11-diOH-THC and 8α,11-diOH-THC are close to each other, the six metabolites and the I.S. could be clearly separated.

*Pretreatment and extraction of THC metabolites in plasma*

*Effects of extraction solvent on recoveries of metabolites in plasma.* Because of the strong binding between THC metabolites and plasma protein, it is known [3] that a poor recovery of the THC metabolites from plasma is obtained by the usual extraction procedure. Therefore, it is essential to precipitate plasma proteins, otherwise poor recoveries of THC metabolites will be obtained. Table I gives the recoveries of THC metabolites from plasma samples obtained with various solvents. The recoveries of 11-OH-THC and THC-COOH with acetone, methanol

TABLE I

RECOVERIES OF THC METABOLITES IN PLASMA AFTER PRECIPITATION WITH VARIOUS SOLVENTS

| Solvent | Recovery (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Supernatant | | 1st wash | | 2nd wash | | Total | |
| | 11-OH-THC | THC-COOH | 11-OH-THC | THC-COOH | 11-OH-THC | THC-COOH | 11-OH-THC | THC-COOH |
| Acetone | 57.5 | 50.3 | 11.0 | 9.7 | 4.5 | 3.1 | 73.0 | 63.1 |
| Methanol | 54.7 | 58.1 | 16.1 | 14.5 | 8.1 | 5.6 | 78.9 | 78.2 |
| Acetonitrile | 65.2 | 52.5 | 13.3 | 10.2 | 4.0 | 5.3 | 82.5 | 68.0 |
| Acetonitrile–pyridine (10:1) | 90.7 | 93.0 | 5.5 | 6.3 | 2.3 | Trace | 98.5 | 99.3 |
| Methanol–pyridine (10:1) | 88.0 | 90.5 | 5.3 | 7.2 | Trace | Trace | 93.3 | 97.7 |
| 10% Trichloroacetic acid | 26.5 | 12.0 | 31.4* | 15.2 | 6.0* | 9.3 | 63.9 | 36.5 |

*Precipitates were washed with acetone.

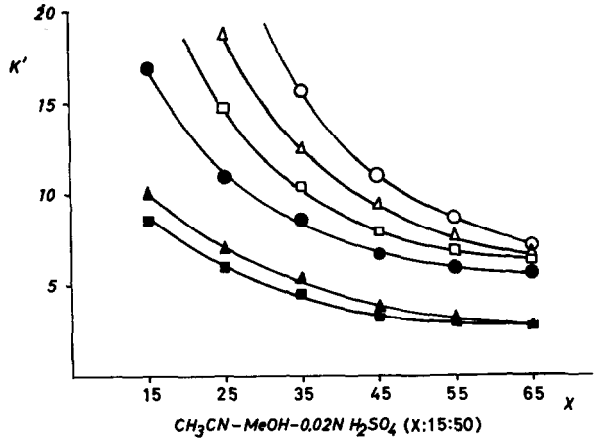


Fig. 2. Plot of capacity factors ($k'$) of THC metabolites versus ratio of acetonitrile to methanol–0.01 *M* sulphuric acid (15:50). Column, Zorbax ODS $C_8$ (25 cm×4.6 mm I.D.); flow-rate, 1.1. ml/min. ■, 8$\beta$,11-diOH-THC; ▲, 8$\alpha$,11-diOH-THC; ●, 8$\beta$-OH-THC; □, 8$\alpha$-OH-THC; △,11-OH-THC; ○, THC-COOH.

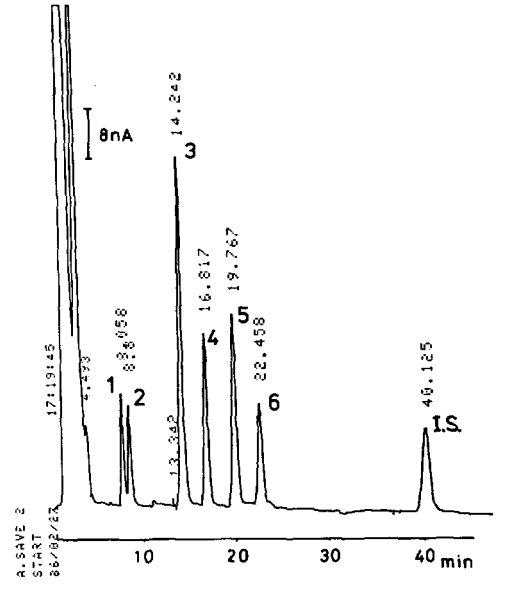


Fig. 3. Chromatogram of extract from plasma spiked with six THC metabolites at concentrations of 50 ng/ml. Peaks: 1=8$\beta$,11-diOH-THC; 2=8$\alpha$,11-diOH-THC; 3=8$\beta$-OH-THC; 4=8$\alpha$-OH-THC; 5=11-OH-THC; 6=THC-COOH; I.S.=*n*-octyl *p*-hydroxybenzoate.

or acetonitrile were at most 63–82% after second washing. Addition of pyridine to acetonitrile or methanol gave quantitative recoveries. The use of trichloroacetic acid gave comparatively low recoveries, especially THC-COOH (36.5%). Because the extract obtained with acetonitrile–pyridine (10:1) contained fewer interfering substances than methanol–pyridine (10:1), the former was used for this study.

TABLE II

REPRODUCIBILITY OF HPLC-ED DETERMINATION OF THC METABOLITES IN PLASMA ($n=10$)

| Analyte | Known concentration (ng/ml) | Observed concentration (mean ± S.D.) (ng/ml) | C.V. (%) |
|---|---|---|---|
| 8β,11-diOH-THC | 50 | 52.3 ± 3.07 | 5.87 |
| | 20 | 23.6 ± 4.46 | 18.9 |
| 8α,11-diOH-THC | 50 | 51.8 ± 2.86 | 5.52 |
| | 20 | 24.2 ± 5.18 | 21.4 |
| 8β-OH-THC | 30 | 30.7 ± 0.87 | 2.83 |
| | 10 | 12.4 ± 1.40 | 11.3 |
| 8α-OH-THC | 30 | 30.2 ± 1.32 | 4.37 |
| | 10 | 13.3 ± 2.06 | 15.5 |
| 11-OH-THC | 20 | 21.1 ± 0.89 | 4.20 |
| | 10 | 11.9 ± 1.15 | 9.66 |
| 9-COOH-THC | 10 | 9.6 ± 0.31 | 3.23 |
| | 5 | 4.5 ± 0.39 | 8.67 |

*Reproducibility of HPLC-ED.* The standard deviations (S.D.) and coefficients of variation (C.V.) were determined at concentrations of 5–50 ng/ml of THC metabolites. Reasonable reproducibility was obtained, although the C.V.s of the α- and β-isomers of 8-OH-THC and 8,11-diOH-THC were over 10% at lower concentrations, as shown in Table II.

## CONCLUSION

The method effectively separated the six THC metabolites and gave good recoveries from plasma. Moreover, the described method can be used for the sensitive, selective and accurate determination of THC metabolites in plasma without derivatization. The calibration graphs for the six THC metabolites were linear between 5 and 100 ng/ml of plasma. The limits of detection (signal-to-noise ratio $>3$) was around 1 ng/ml of metabolites in plasma.

## ACKNOWLEDGEMENT

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