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MEASUREMENT OF Δ^1-TETRAHYDROCANNABINOL IN PLASMA TO THE LOW PICOGRAM RANGE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY USING METASTABLE ION DETECTION

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SUMMARY

A method for the assay of Δ^1-tetrahydrocannabinol (Δ^1-THC) in plasma using combined gas chromatography–mass spectrometry with metastable ion monitoring is described. Δ^1-THC was extracted with hexane and the extracts were methylated with diazomethane to shift the peaks produced by endogenous plasma constituents away from the cannabinoid region. The Δ^1-THC was then converted into its trimethylsilyl derivative and quantitated using the metastable ion at *m/z* 371 formed in in the $M^+ \rightarrow [M - CH_3]^+$ transition with [1″,1″,2″,2″-$^2H_4$]cannabinol as the internal standard. Δ^1-THC could be measured to 5 pg/ml in plasma. This assay is 20–100 times more sensitive than existing assays and has the advantage of not needing the usual extensive purification step.

INTRODUCTION

Plasma levels of Δ^1-tetrahydrocannabinol (Δ^1-THC) during cannabis intoxication are usually in the nanogram range[1] and because of the high lipophilicity of the drug, the levels reached after administration decline very rapidly to the low nanogram or upper picogram region within a few hours of administration. Elimination of the drug from the body is slow but because of the very low plasma levels there have been few direct pharmacokinetic studies. Estimates based on radioactivity measurement suggest a half-life of 1–2 days[2], whereas in the dog a value of 8 days has been found[3]. Current assays of Δ^1-THC in plasma[4,5] do not provide the necessary sensitivity either to obtain accurate pharmacokinetic data for more than a few hours or to confirm the presence of Δ^1-THC for forensic purposes after this time.

Several methods for Δ^1-THC measurement based on gas–liquid chromatography (GLC)[6–10], thin-layer chromatography (TLC)[11,12], gas chromatography–mass spectrometry (GC–MS)[1,13–15] and radioimmunoassay[16–18] have been reported in the recent literature and in two symposia devoted to cannabinoid assays[4,5] and, in addition, combination techniques such as high-performance liquid chromatography (HPLC) coupled with radioimmunoassay[19] or MS[20] have been developed. However, the best of these only provide for measurements to the 100–500 pg/ml range with

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detection limits *ca.* 100–200 pg. Furthermore, except for some radioimmunoassay techniques, considerable clean-up of the extracted drug is required before assay, particularly by GC–MS. Methods based on Sephadex LH-20 chromatography[13,15] or solvent partition[7–9,12,14] are the most common but result in an analysis taking several hours. Radioimmunoassay techniques, although sensitive also detect many cross-reactive THC metabolites. However, an HPLC–radioimmunoassay technique has recently been described[19] which overcomes this problem; this has a reported detection limit of 100 pg/ml.

This paper describes a new assay for Δ^1-THC in plasma, based on GC–MS and using metastable ion monitoring. It is capable of measuring Δ^1-THC in 1 ml of plasma at the 5 pg/ml level and requires no clean-up of the sample before GC–MS. Using this method we have been able to follow the plasma levels of Δ^1-THC in the rabbit for 7 days after a single intravenous dose of 1 mg/kg.

EXPERIMENTAL

Materials

Δ^1-THC was obtained from the National Institute on Drug Abuse (Rockville, MD, U.S.A.) and was found by GC–MS to be 98% pure. [1″,1″,2″,2″-$^2H_4$]cannabinol ([$^2H_4$]CBN) was synthesized from [1″,1″,2″,2″-$^2H_4$]Δ^1-THC by dehydrogenation with chloranil[21,22]; the labelled Δ^1-THC was prepared from the condensation of [1′,1′,2′,2′-$^2H_4$]olivetol with (+)-*trans*-metha-2,8-dien-1-ol as described previously[23]. 3β-Hydroxypregna-5, 16-dien-20-one was obtained from Sigma (London, Great Britain) and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Aldrich (Milwaukee, WI, U.S.A.). An ethereal solution of diazomethane was prepared from Diazald (Aldrich) by distillation. Plasma was obtained from John Radcliffe Infirmary (Oxford, Great Britain).

All solvents were distilled twice before use and all glassware was silanized with a 5% solution of dichlorodimethylsilane followed by methanol.

Preparation of standard Δ^1-THC samples

Δ^1-THC (100 μg) was converted into its trimethylsilyl (TMS) derivative by reacting it with BSTFA (100 μl) for 10 min at 60°C. Serial dilutions with BSTFA were made to give solutions containing 100 ng, 10 ng, 1 ng, 100 pg, 10 pg and 1 pg/ml.

Solutions containing, 2, 5, 10, 20, 30 and 50 pg of Δ^1-THC with 10 ng of 3β-hydroxypregna-5,16-dien-20-one, and 100, 200, 500 and 1000 pg of Δ^1-THC with 100 ng of the steroid were prepared in BSTFA (100 μl per sample).

For experiments with [$^2H_4$]CBN as the internal standard, 100 μl solutions of Δ^1-THC and [$^2H_4$]CBN were prepared containing 2, 5, 10, 20 and 30 pg of Δ^1-THC with 20 pg of [$^2H_4$]CBN; 50, 100 and 200 pg of Δ^1-THC with 200 pg of [$^2H_4$]CBN; and 500 and 1000 pg of Δ^1-THC with 2000 pg of [$^2H_4$]CBN.

Determination of detection limits and instrumental precision

Samples (1 μl) of derivatized Δ^1-THC were injected at increasing dilution until the peak could no longer be detected. The internal standards were then evaluated in a similar way. Concentration *versus* peak ratio curves were drawn using peak height measurements, and two samples containing 7 pg of Δ^1-THC with the appropriate amounts of each internal standard were injected several times to check the precision of

the peak height measurements in the presence of background noise and as a check on instrumental reproducibility.

Quantitation of Δ^1-THC in plasma

Samples were run in triplicate and extractions were performed in 10-ml screw-cap sample vials. For the calibration curve, Δ^1-THC and [$^2H_4$]CBN in ethanol (up to 20 μl) were added to plasma in the concentrations shown in Table I and left to equilibrate with plasma proteins for 30–60 min. Sodium bicarbonate (*ca.* 1 g) was added and the drugs were extracted with 2 ml of hexane, each sample being agitated for 2 min on a vortex mixer. The samples were then centrifuged for *ca.* 10 min at 1500 *g* to break the emulsion. The extraction was repeated twice and the combined hexane extracts were blown to dryness with a nitrogen stream in a 0.3 ml conical vial. The residue was taken up in methanol (50 μl) and an excess of ethereal diazomethane was added. After 2 min the solution was again blown to dryness with a nitrogen stream and 20 μl of BSTFA were added. The mixture was then heated at 60°C for 10 min. Aliquots (0.1–5 μl) were examined by GC–MS.

TABLE I

CALIBRATION OF Δ^1-THC IN PLASMA

Volume of plasma (ml)	*Δ^1-THC/ml*	*[$^2H_4$]CBN/ml*	*Steroid/ml*
1	1 μg	2 μg	—
1	100 ng	200 ng	—
1	50 ng, 10 ng, 5 ng	20 ng	500 ng
1	1 ng, 500 pg	2 ng	50 ng
1	100 pg, 50 pg, 40 pg	200 pg	10 ng
4	20 pg	20 pg	—
5	10 pg	20 pg	—

Recovery

For measurements of recovery, 3β-hydroxypregna-5,16-dien-20-one was added to the above samples before extraction in the concentrations shown in Table I.

Measurement of plasma Δ^1-THC levels in the rabbit

One female New Zealand white rabbit (2.21 kg) was treated intraveneously (marginal ear vein) with a suspension (2.21 ml) of Δ^1-THC in Tween-80 and physiological saline at a dose of 1 mg/kg. Blood samples were collected into heparinized glass tubes from the marginal ear vein of the other ear periodically for 7 days under procaine local anaesthesia. The plasma was separated by centrifugation and stored at −20°C until required. Samples (1 ml) were used for analysis of the early fractions; 2–3-ml samples were used for later time periods. Δ^1-THC was extracted as described above using [$^2H_4$]CBN as the internal standard; 200 ng of [$^2H_4$]CBN were added to samples taken to 20 min, 20 ng to samples taken to 4 h, 2 ng to samples taken to 12 h, and 200 pg to samples taken to 7 days.

Gas chromatography–mass spectrometry

GC–MS measurements were made with a V.G. Micromass 70/70 F mass spectrometer interfaced via a glass jet separator to a Varian 2400 GLC instrument.

The chromatograph was fitted with a 2 m × 2 mm I.D. glass column packed with 1% SE-30 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) and operated at 220°C. The injector and separator temperatures were 300°C and 280°C, respectively. Helium at 30 ml/min was used as the carrier gas. The mass spectrometer was operated at 70 eV with a trap current of 1 mA and an ion source temperature of 260°C. Tuning of the metastable peak was achieved as follows: the instrument was tuned to *m/z* 371 with the accelerating voltage at 4 kV and the collector slit was opened to give a flat-top peak (resolution *ca.* 700). The accelerating voltage was then raised to 4.16 kV to bring the metastable ion into focus, and the source focusing controls were adjusted to give maximum sensitivity.

RESULTS AND DISCUSSION

The major factor limiting the sensitivity of most reported assays for Δ^1-THC is interference by endogenous compounds having similar extractive and analytical properties to Δ^1-THC. This results in limits of detection of the drug in plasma being several orders of magnitude higher than can be achieved with the pure compound. Using GC–MS for example, we have observed a 5-pg limit for the TMS derivative of pure Δ^1-THC but find that this is increased to *ca.* 1 ng when the drug is assayed in plasma. It would appear, therefore, that the best way to improve sensitivity would be to reduce the interference from endogenous compounds. Most assays have attempted this by the use of an extensive purification step between the extraction and assay stages. However, by using a low polarity solvent such as hexane[15] to reduce the amount of endogenous material extracted and by improving the selectivity of the GC–MS stage by the use of metastable ion monitoring, we have been able to dispense with the purification stage and to achieve an almost clean background from a blank plasma. Against this, Δ^1-THC can be assayed to the detection limit of the instrument.

Metastable ion monitoring[24] on a Nier–Johnson-type double focusing mass spectrometer involves raising the accelerating voltage to the point where daughter ions formed in a metastable transition in the first field free region acquire sufficient energy to be transmitted through the electrostatic and magnetic analysers. Other fragment ions, most of which arise from the endogenous contaminants by different fragmentation mechanisms, are trapped by the electrostatic analyser leaving an almost clean background.

For the present assay the metastable ion at *m/z* 371 from the $M^+ \rightarrow [M-CH_3]^+$ transition in the spectrum of the TMS derivative was monitored; previous work has indicated that this derivative gives the best GC–MS characteristics[25]. The detection limit for the TMS derivative of pure Δ^1-THC was determined by injecting the compound into the instrument at increasing dilutions. The concentration *versus* peak height ratio was plotted and the response was found to be linear from 1 μg to the detection limit of 500 fg (3:1 signal-to-noise ratio).

The best type of standard for the assay was considered to be one giving a common ion which could be monitored by single ion recording. This method has been reported to give greater precision than the use of deuterated standards with multiple ion monitoring[26] and in any case multiple ion monitoring would involve independent variation of accelerating and electrostatic analyser voltages. An available steroid, 3β-hydroxypregna-5,16-dien-20-one, whose TMS derivative also contained

an ion at *m/z* 371 formed in a metastable transition from *m/z* 386 was evaluated as a suitable standard. It had a suitable retention time (6 min 15 sec on SE-30 compared with 2 min 20 sec for Δ^1-THC) but the calibration curve was linear only for samples containing the same amount of the steroid standard injected onto the column. This was attributed to the unequal responses of the compounds and the different slopes of their concentration response lines. Even so, with samples giving a linear calibration from 1 to 50 pg, 7 pg of Δ^1-THC could be measured fairly accurately (found 7.1 pg, coefficient of variation (C.V.) 4.07% ($n = 3$); and 7.1 pg, C.V. = 12.06%, $n = 3$).

The best standard would be another cannabinoid whose spectrum contained a metastable ion at *m/z* 371 formed from *m/z* 386 as it should exhibit similar extractive and GC–MS properties to Δ^1-THC. Isomeric THCs (Δ^6- and Δ^7-THC) were investigated, but they did not separate from Δ^1-THC under GLC conditions appropriate for the assay. Consequently, CBN labelled with four deuterium atoms in the side-chain was synthesized from the available [1″,1″,2″,2″-$^2H_4$]Δ^1-THC[23]; the deuterium label increased the molecular weight of its TMS derivative to 386 and the spectrum contained a prominent $[M-CH_3]^+$ ion at *m/z* 371. It separated from Δ^1-THC on 1% SE-30 (retention time Δ^1-THC = 2 min 20 sec; retention time [$^2H_4$]CBN = 3.0 min) and gave a similar metastable response. Fig. 1 shows the trace recorded for the TMS derivatives of 10 pg of Δ^1-THC and 20 pg of [$^2H_4$]CBN injected onto the column. Using this standard, a linear calibration was obtained from 1 ng to 2 pg; 7 pg was measured as follows: 7.0 pg, C.V. = 3.9% ($n = 7$) and 7.05 pg, C.V. = 4.4% ($n = 4$).

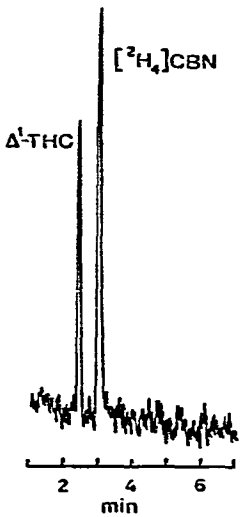

Fig. 1. Metastable ion chromatogram (*m/z* 386 → *m/z* 371) of 10 pg of Δ^1-THC and 20 pg of [1″, 1″, 2″, 2″-$^2H_4$]CBN separated as their TMS derivatives on a 1% SE-30 packed column at 220°C.

Δ^1-THC and [$^2H_4$]CBN were extracted from plasma with hexane in the presence of sodium bicarbonate. When the samples as their TMS derivatives were examined by GC–MS, additional peaks were present (Fig. 2) which interfered with the measurement of Δ^1-THC. These endogenous compounds were also extracted from

Fig. 2. Metastable ion chromatogram (m/z 386 → m/z 371) of 10 pg of Δ^1-THC and the [$^2H_4$]CBN standard extracted from plasma and separated as their TMS derivatives (1% SE-30, 220°C).

blank plasma and could not be separated from Δ^1-THC on either SE-30 or OV-17 columns. From their GLC characteristics they appeared to be fatty acids. The plasma extracts were therefore methylated with diazomethane before TMS ether formation in an attempt to achieve selective methylation of the endogenous compounds. Under the conditions used no reaction was observed for Δ^1-THC but the endogenous compounds were completely methylated and disappeared from the chromatogram. Chromatograms from blank plasma samples then contained no peaks in the cannabinoid region and against this Δ^1-THC could be measured to 5 pg/ml. In addition, no differences were found either in the background or the extractive properties of Δ^1-THC and [$^2H_4$]CBN from rabbit, guinea-pig or human plasma.

Recovery of Δ^1-THC and [$^2H_4$]CBN was measured using 3β-hydroxypregna-5,16-dien-20-one as the internal standard and were in the range 70–80% for all concentrations tested (Table I). It was essential that all glassware was silanized before use to achieve high recovery. It was then well washed with redistilled ethyl acetate before use. The calibration curve using [$^2H_4$]CBN as the internal standard was linear over the range tested (1 μg to 10 pg/ml) and is shown in Fig. 3. The chromatogram for one of the 10 pg/ml samples is shown in Fig. 4. Accuracy and precision measurements are shown in Table II. The average time for a complete assay was *ca.* 1 h. Care was taken to ensure that, during repetitive injections onto the GLC column, the cannabinoid peaks did not co-elute with cholesterol from a previous injection (retention time = 19 min). This compound appeared to be the most concentrated residual plasma component and its spectrum contained a weak metastable ion at m/z 371. When co-elution did occur the metastable ion abundance from the cannabinoid was increased considerably thus upsetting the assay. No other interactions of this type were observed. In no case did we find sufficient CBN levels in the plasma, either with the calibration samples or in subsequent experiments involving treatment of animals, to interfere with the peak height of the [$^2H_4$]CBN standard; earlier reports that CBN is a major intermediary metabolite of Δ^1-THC[27] appear to have been discounted[28].

To evaluate the assay under experimental conditions, a female New Zealand white rabbit was treated intravenously with Δ^1-THC at a dose of 1 mg/kg and plasma

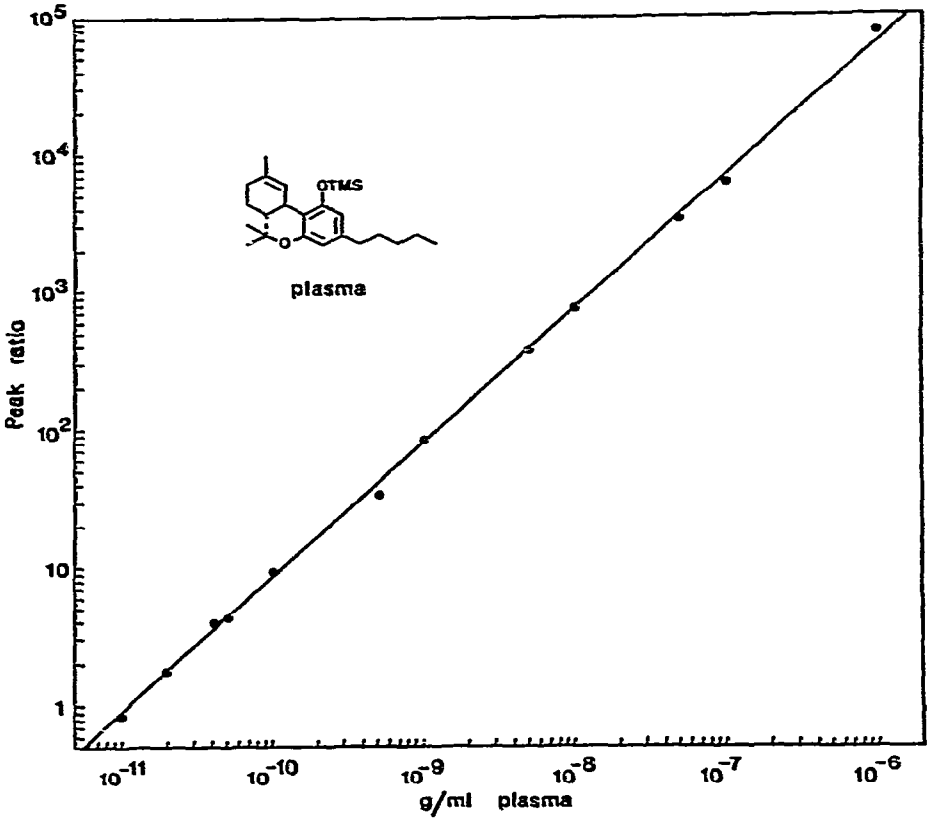

Fig. 3. Calibration curve for Δ^1-THC and [$^2H_4$]CBN extracted from plasma.

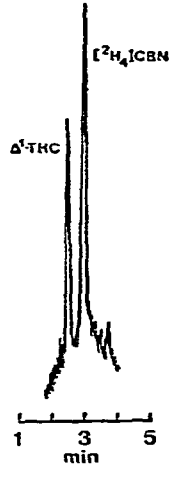

Fig. 4. Metastable ion chromatogram (*m/z* 386 → *m/z* 371) of 10 pg of Δ^1-THC and the [$^2H_4$]CBN standard separated on a 1% SE-30 packed column at 220°C following methylation and trimethylsilylation of the extract.

TABLE II

ACCURACY AND PRECISION FOR THE DETERMINATION OF Δ^1-THC IN PLASMA

Actual concn.	*Observed concn.*	*C.V. (%)*	*n*
30 pg	26.5 pg	12.1	5
70 pg	74.0 pg	10.3	5
300 pg	258 pg	0.74	3
3 ng	3.01 ng	9.1	3
30 ng	28.7 ng	3.2	3
300 ng	293 ng	6.4	2

levels were followed to the detection limit of the assay. Δ^1-THC could be measured for 7 days to a level of 10 pg/ml (Fig. 5). A chromatogram from the sample taken on day 7 is shown in Fig. 6.

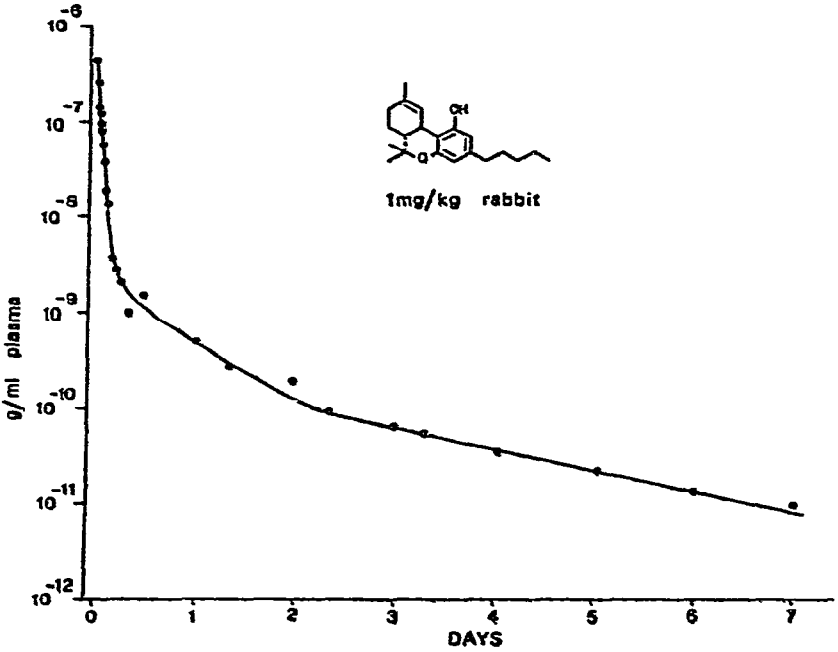

Fig. 5. Plasma levels of Δ^1-THC in the rabbit following a 1 mg/kg intravenous injection.

CONCLUSION

This assay, based on metastable ion monitoring, is more sensitive than existing assays and can detect Δ^1-THC to 5 pg/ml in plasma using 1-ml plasma samples. In addition it requires no purification step between extraction and assay stages; the time taken for a single assay is thus reduced to *ca.* 1 h. Using the method it is possible to follow Δ^1-THC elimination in plasma for times in excess of one week and it should thus be possible to obtain accurate pharmacokinetic data. As metastable transitions

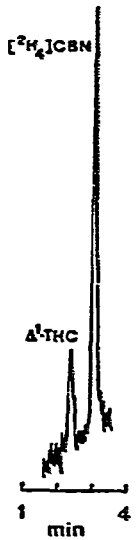

Fig. 6. Metastable ion chromatogram of Δ^1-THC and the [$^2H_4$]CBN standard from the sample taken on day 7 from the rabbit treated with Δ^1-THC.

can be promoted by the use of a collision cell[29,30] it is hoped that in the near future the sensitivity of this assay can be improved still further.

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