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Determination of Δ^9-tetrahydrocannabinol from rabbit plasma by gas chromatography–mass spectrometry using two ionization techniques

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Abstract

The purpose of the study was to develop a gas chromatography–mass spectrometric (GC–MS) method for the identification and quantitation of Δ^9-tetrahydrocannabinol (THC) in rabbit plasma. Two ionization techniques were utilized for GC–MS: electron impact ionization (EI) after i.v. administration and negative chemical ionization (NCI) after sublingual administration. THC was isolated from plasma by solid phase extraction and derivatized by either trimethylsilylation (EI) or trifuoroacetylation (NCI), with deuterated THC as an internal standard. The validity of analytical method was confirmed by investigating selectivity, limit of quantitation, linearity, accuracy, precision, recovery and stability of the analyte. The method proved to be selective, linear, accurate and precise over a range of 10–430 and 0.3–530 ng/ml of THC in plasma for EI and NCI, respectively. The extraction recovery was >81% for each concentration level studied, and the analyte was shown to be stable during storage and sample preparation. The method was applied successfully in analysing THC from rabbit plasma.

Keyword: Δ^9-Tetrahydrocannabinol

1. Introduction

Cannabinoids are a group of compounds that originate exclusively from *Cannabis sativa* L., the plant source of marijuana and hashish. Cannabinoids have been used for thousands of years for both recreational and medicinal purposes. Over the last few years cannabinoids have been reported to be useful in the treatment of various medical conditions such as nausea, AIDS associated wasting, anorexia and glaucoma [1,2]. The main active component in both marijuana and hashish is Δ^9-tetrahydrocannabinol (THC) (Fig. 1). In therapeutic applications, the oral use of THC is limited, due to its substantial first-pass metabolism, which can be overcome, for example, by sublingual administration. In the present study, a sensitive and specific GC–MS method was developed to analyse THC from rabbit plasma. The present method is used in the development of a novel sublingual formulation, in order to improve the delivery and bioavailability of THC.

To analyse THC from biological samples, several analytical methods have been employed including immunoassay [3], high performance liquid chromatography [4] and gas chromatography [5]. HPLC has been used in combination with UV, fluorescence [6] and electrochemical detection [7]. Gas chromatography has been used with both flame-ionization [8] and mass spectrometric detection. GC–MS in the electron impact ionization (EI) mode is one of the most commonly used techniques in drug analysis [9]. GC–MS–EI enables the detection of several substances in one run, and allows the identification of the drug according to decomposition fragments. Chemical ionization (CI) is less energetic towards THC-molecules, which makes it more sensitive [10], and enables the determination of pharmacokinetic

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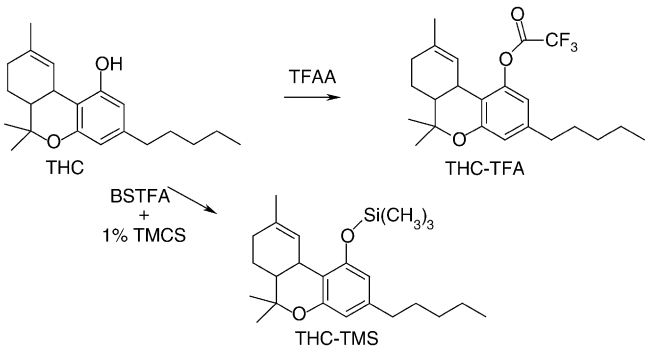

Fig. 1. The chemical structures of THC (M_w 314), and its derivatives, THC–TFA (M_w 410) and THC–TMS (M_w 386), after reactions with TFAA and BSTFA.

parameters for THC in lower plasma concentrations. The two types of CI, positive (PCI) [10] and negative chemical ionization (NCI) [11] have both been utilised in the analysis of THC.

In order to analyse THC from plasma by GC–MS, special attention must be focused on sample purification. The most commonly used methods are liquid–liquid [12] and solid phase extractions [9]. Liquid–liquid extraction may have important disadvantages, such as poor recovery and incomplete outcome in purification. The poor recovery may be related to the binding of THC to plasma proteins, and it might be possible to enhance recovery by competitive protein binding with some other substance, such as urea [13]. Solid phase extraction may provide more alternatives for purification. However, one of the most commonly used phase materials, endcapped C_{18}-phase, is not stable in the basic pH-region and it is also unstable if dried out. Novel phase materials, such as divinylbenzene *N*-vinylpyrrolidone copolymer are more adaptable and may enable a wider choice of applications.

In the analysis of THC by GC–MS, derivatisation of THC is used to improve some of its properties such as volatility, sensitivity and ionisability. In EI-techniques, THC has usually been analysed as the methylated [9] or trimethylsilylated (Fig. 1) derivative [14]. With NCI-techniques, THC has typically been analysed as the trifluoroacetate (Fig. 1) in order to have a large number of leaving electrons on the analyte [15].

The purpose of this study was to develop a GC–MS method in order to analyze THC in rabbit plasma by two ionization techniques: EI was used after i.v. administration and NCI was used after the sublingual administration of THC. A straightforward and efficient solid phase extraction procedure was developed to purify plasma samples. To establish the performance and validity of the two techniques, the following analytical parameters were investigated according to the FDA guideline: selectivity, limit of quantitation, linearity, precision, accuracy, recovery and stability of the analyte [16].

2. Experimental

2.1. Materials

THC was purchased from THC PHARM GmbH (Frankfurt, Germany). Deuterated THC (THC-d_3) was purchased from Cerilliant™ (Austin, Texas, USA) as a methanol solution. Bis(trimethylsilyl)trifluoroacetamide (BSTFA; 1% TMCS), pyridine, hexane and trifluoroacetic anhydride were all of GC-grade, purchased from Sigma–Aldrich (Steinheim, Germany). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was obtained from Wacker Chemie (Burghausen, Germany). Atropine sulphate (Atropin®) was obtained from Leiras (Turku, Finland), fentanyl citrate-fluanisone (Hypnorm®) was obtained from Janssen Pharmaceutica (Beerse, Belgium), and midazolam (Dormicum®) was obtained from Roche (Espoo, Finland). All other reagents were of analytic grade. Oasis® HLB divinylbenzene *N*-vinylpyrrolidone copolymer solid phase extraction cartridges were purchased from Waters Ltd. (Massachusetts, USA).

Human plasma was obtained from healthy volunteers. Rabbits (New Zealand white) were purchased from The National Laboratory Animal Center (Finland). The rabbits were allowed to eat commercial food pellets and drink water ad libitum, except during the first 5 h of each test, when they were under anaesthesia. All procedures with animals were reviewed and approved by the Animal Ethics Committee at the University of Kuopio.

For the calibration and quality control samples, three analogous methanol stock solutions of THC were prepared. Working solutions were diluted in methanol at concentrations 2.6–5300 ng/ml. Deuterated THC (THC-d_3), the internal standard (IS), was diluted in methanol into working solutions of 300 ng/ml for EI and 150 ng/ml for NCI. All solutions were stored at −20 °C.

2.2. Sample preparation

On the day of analysis, plasma samples were thawed to ambient temperature. A total of 50 μl of the IS working solution was added to 500 μl of plasma, vortexed (3 s) and samples then put on crushed ice. To each sample 1 ml of urea solution (8 M) was added, and the samples were again vortexed (1 s). After adding the urea solution, methanol (1 ml) was slowly pipetted into each sample while simultaneously vortexing (5 s).

Prior to extraction Oasis® HLB cartridges were activated with methanol (3 ml) and equilibrated with water (3 ml). The samples were then passed through the cartridges under vacuum. The samples were first washed with an acetic acid–methanol solution (3 ml; 60% (v/v) of methanol and 2% (v/v) of acetic acid), and then with an ammonia–methanol solution (3 ml; 60% (v/v) of methanol and 0.8% (m/v) of ammonia; pH 10.0). After the basic wash, the solid phase material was aspirated to dryness for 30 s. Finally, the samples were eluted with methanol (3 ml). The methanolic elu-

ent was then evaporated under a stream of nitrogen at 40 °C, and the residue was treated with the appropriate derivatising agent. For EI determinations, 150 μl of pyridine and 50 μl of BSTFA were added to the residue. After vortexing (3 s) the tubes were then heated at 60 °C (60 min) and analyzed directly. For NCI, 100 μl of two freshly prepared solutions, A and B, were added to the residue. Solution A was a mixture of pyridine (30 μl), TFAA (100 μl) and hexane (4.87 ml). Solution B was a mixture of BSTFA (50 μl) and hexane (4.95 ml). Samples were vortexed (3 s) after the addition of both A and B, and analyzed directly.

Calibration curve and quality control (QC) samples were analysed with each set of samples. Both calibration standards and QC samples were freshly prepared on the day of analysis by spiking human plasma (450 μl) with a working solution of THC (50 μl). Solutions were then vortexed (3 s), put on crushed ice and treated identically to the plasma samples.

THC concentrations of the calibration standards were selected to cover the linear range: 6–10 standards (EI: 10, 25, 51, 100, 250 and 430 ng/ml; NCI: 0.3, 0.4, 0.7, 3.6, 12, 21, 36, 91, 290 and 530 ng/ml) were prepared, of which at least 5 were used for quantitation. In the case of NCI determinations, separate calibration curves were determined for the low (0.3–21 ng/ml) and high (21–530 ng/ml) concentration ranges. QC samples (n = 3) at three concentrations (EI: 25, 100, 430 ng/ml: NCI: 0.3, 0.7, 250 ng/ml) were used to monitor the sample preparation on each day of analysis.

Reference standards were prepared by mixing 50 μl of the THC working solution and 50 μl of the IS working solution in a test tube. Reference standards were then evaporated, derivatised and analysed. The amounts of THC in the reference standards were equivalent to the amounts of THC in the respective calibration standards (EI: 10, 25, 51, 100 and 250 ng/ml; NCI: 0.3, 0.7, 3.6, 12, 21 and 250 ng/ml).

2.3. Instrumentation

The Agilent GC–MS system consisted of a gas chromatograph 6890N, autosampler 7683 and mass detector 5973N (Palo Alto, California, USA). Data were processed using the Agilent Enhanced ChemStation software (version c.00.01.08). A cross-linked 5% phenyl methyl siloxane capillary column (HP-5MS; 30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Agilent Technologies; Palo Alto, California, USA) was used with helium as the carrier gas, at a constant flow of 2.0 ml/min (column head pressure 16.9 psi). The sample (1 μl) was injected in the pulsed splitless mode, after which a 30 psi inlet pressure was maintained for 1.5 min.

The GC–MS program for EI was as follows: the initial temperature of 75 °C was held for 1 min, after which the temperature was increased by 30 °C/min to 280 °C, where it was maintained for 5 min (total run time 12.83 min). Temperatures of inlet, interface, MS source and quadrupole were 250, 290, 230 and 150 °C, respectively. The ionization energy was 70 eV. MS detection was operated in the SIM mode (50 ms dwell time) with selected ions (THC-d_3 ions in parentheses) of: m/z 386 (389), m/z 371 (374), m/z 315 (318) and m/z 303 (306). The area ratio of 386/389 was used for quantitation, and the other ions were used as qualifiers.

The GC–MS program for NCI was as follows: the initial temperature of 50 °C was held for 1 min, after which temperature was increased by 50 °C/min to 205 °C, then 3 °C/min to 225 °C and finally 60 °C/min to 280 °C where the temperature was maintained for 4 min (total run time 15.68 min). Temperatures of inlet, interface, MS source and quadrupole were 250, 280, 150 and 106 °C, respectively. Methane was used as a reagent gas at a flow rate of 40%. The MS was operated in the SIM mode (50 ms dwell time). The area ratio of selected ions 410 for THC and 413 for THC-d_3 was used for quantitation.

2.4. Selectivity

For selectivity studies, blank plasma samples were analysed for interfering peaks at the retention time of THC. Blank samples were obtained from pooled human plasma and also from each rabbit. Plasma samples were also spiked with anticoagulant, solvents and derivatising reagents, and analysed for interfering peaks.

Possible interference caused by matrix, solvents or reagents were studied by comparing both reference and calibration standard curves: 10–250 and 0.3–21 ng/ml for EI and NCI, respectively. The similarity between reference and calibration standard curves was investigated by comparing the slope and the intercept values of the reference curves to a corresponding 95% confidence interval, calculated from the calibration curves (and vice versa) (Microsoft® Excel 2002 software).

2.5. Linearity, lower limits of quantitation, precision, accuracy and recovery

Linearity was defined by 6–10 calibration standards (EI: 10, 25, 51, 100, 250 and 430 ng/ml; NCI: 0.3, 0.4, 0.7, 3.6, 12, 21, 36, 91, 290 and 530 ng/ml), of which at least 5 were used for the calibration curve. In the case of EI, the calibration curve consisted of six data points. In the case of NCI, separate calibration curves were determined for the low (0.3–21 ng/ml) and high (21–530 ng/ml) concentration ranges.

Lower limits of quantitation (LLOQs) were established by determining the precision of six calibration standards.

Precision and accuracy were determined by analysing QC samples (n = 3) at three separate concentrations: 25, 100 and 430 ng/ml for EI, and 0.3, 0.7, 250 ng/ml for NCI. Freshly prepared samples and an independent calibration curve were analysed on three separate days to obtain both within day and between day precisions.

The extraction recovery was defined as the per cent relation of analyte peak areas of calibration and reference standards. For recovery, calibration samples (n = 3) from six different concentrations (0.3, 0.7 and 230 ng/ml (NCI);

25, 100 and 250 ng/ml (EI)) were analysed together with appropriate reference standards.

2.6. Stability

Stability of the analyte was studied in plasma throughout the entire analytical method: over 4 h at room temperature (+24 °C), 24 h at +4 °C, during 3 weeks deep-freeze storage (−80 °C) and after three freeze–thaw cycles. The stability of the analyte was also monitored in stock solutions and in the autosampler. Calibration samples from three concentrations (0.4, 20 and 500 ng/ml; $n = 3$) were analysed by NCI after each condition, together with an independent calibration curve, and the calculated concentrations were compared to the mean of corresponding results obtained prior to the stability experiment.

2.7. In vivo sublingual absorption of THC

The rabbits ($n = 6$) were anaesthetized using atropine sulphate (0.2 mg/kg), fentanyl citrate (0.1 mg/kg), fluanisone (3 mg/kg) and midazolam (2 mg/kg). THC was given either intravenously (500 μg/kg) as a 0.7 mg/ml HP-β-CD-solution (20% m/v HP-β-CD) or sublingually (250 μg/kg) as a 12 mg/ml ethanol solution. Blood samples were taken either from a central artery or marginal vein of the ear prior to THC administration (blank sample) and 2–300 min after administration. Blood samples were centrifuged within 30 min at 4 °C (3700 × *g* for 10 min) (Megafuge 1.0R centrifuge; Heraeus Instruments, Osterode, Germany) and the plasma was immediately frozen and stored at −80 °C until analysis. All samples were analysed within 3 weeks.

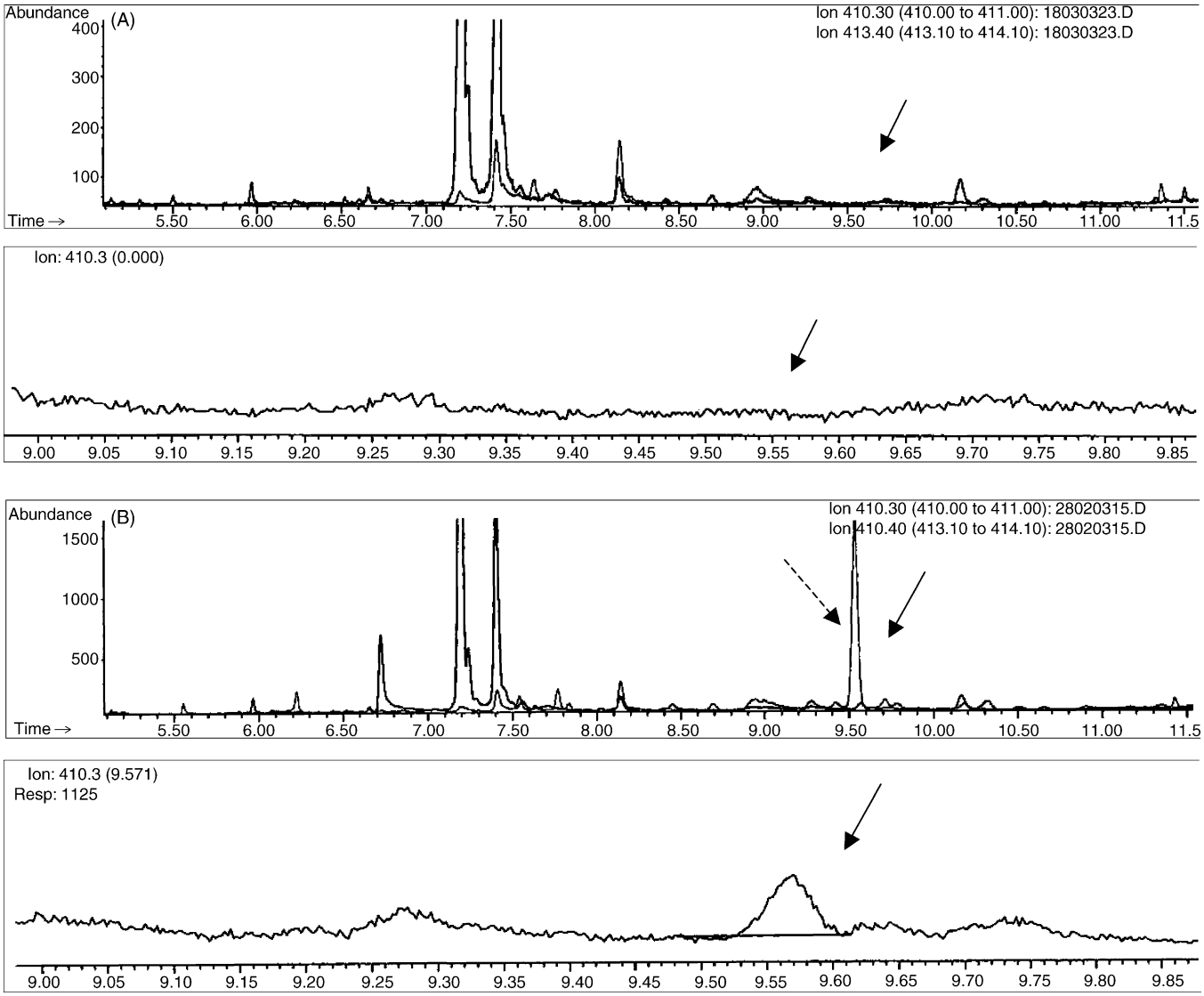

Fig. 2. NCI chromatograms from a blank plasma sample (A) and a 0.4 ng/ml calibration standard (B). The upper chromatograms show both *m/z* 410.3 (THC–TFA) and *m/z* 413.4 (THC-d3-TFA). On the lower chromatograms *m/z* 410.3 has been detailed. The blank plasma sample has no trace of THC–TFA (black arrow) whereas 0.4 ng/ml calibration standard has peaks for both THC–TFA and THC-d3-TFA (dashed arrow).

Table 1
Linearity of the method

Ionisation technique	Linear range (ng/ml)[a]	Calibration points	Calibration curve[a]	Correlation coefficient (r)	LLOQ[b] (ng/ml (%R.S.D.))
EI	10–433	6	$y = 0.788X + 0.266$	0.993	10 (20)
NCI	0.3–21	6	$y = 1.147X + 0.002$	0.999	0.3 (12)
	21–530	5	$y = 1.103X + 0.147$	0.999	

[a] $n = 3$.
[b] $n = 6$.

3. Results and discussion

3.1. Selectivity

Selectivity of the method was studied using blank human and rabbit plasma samples. Pooled human plasma was free of co-eluting peaks at the retention time of THC (EI: 8.6 min; NCI: 9.6 min). Also blank samples collected from rabbits and samples spiked with anticoagulant, solvents or derivatising reagents showed no trace of interfering substances. A representative chromatogram of blank rabbit plasma sample analysed by NCI is presented in Fig. 2.

Reference and calibration curves were compared to detect any potential interference caused by the matrix. As all of the slope and intercept values were within the corresponding confidence intervals, it was concluded, that matrix interference does not occur.

A temperature gradient was designed for EI, and this technique was found to be selective. With NCI, the same temperature gradient was first applied. However, an interfering peak was spotted at the retention time of THCs trifluoroacetate-derivative. This interfering peak was successfully separated from the analyte peak by modifying the temperature gradient.

3.2. Linearity, lower limits of quantitation, precision, accuracy and recovery

A linear correlation was found between the relation of peak areas and the relation of concentrations over the range of 0.3–530 and 10–430 ng/ml for NCI and EI, respectively (Table 1). The calibration curve was linear over the entire studied range for both EI and NCI. However, because the linear range of NCI was so large, the calibration curve was divided in two concentration ranges: 0.3–21 and 21–530 ng/ml. Another reason for using two calibration curves was the fact that the plasma concentrations in sublingual studies remained very low, and only a calibration curve with the lower concentration was needed.

LLOQ of the EI technique was 10 ng/ml, which was sufficient to monitor THC in plasma after i.v. administration (Table 1). However, more sensitivity was required for monitoring the plasma concentrations of THC after sublingual administration. The LLOQ of the NCI technique was 0.3 ng/ml (Table 1), which was suitable for monitoring the plasma concentrations of THC after sublingual administration. A chromatogram of a typical calibration standard (0.4 ng/ml) is presented in Fig. 2.

The data on precision and accuracy are presented in Table 2. The precision of the determinations was acceptable—less than 15% (%R.S.D.) in every concentration. The accuracy of the determinations was also acceptable; all samples were within ±15% of the nominal value.

The extraction recovery for THC varied between 81 and 106% (Table 2). THC, like many other lipophilic molecules, binds to plasma proteins [17]. Consequently, good recovery may be partly related to the release of the protein-bound THC. Plasma proteins are usually precipitated in the sample preparation process, which might result in the elimination of some THC. The addition of urea into samples prevents the precipitation of proteins. It does break the quaternary structure of protein, but the denatured form remains water-soluble [13]. The addition of urea also competes with THC from lipophilic binding sites on proteins, and methanol was added to samples in order to make THC more soluble and, thus, more available to bind with the solid phase. The addition of methanol did not lead to precipitation of the plasma proteins in the presence

Table 2
Summary of validation parameters: precision, accuracy and recovery ($n = 3$)

Ionisation technique	Concentration (ng/ml)	Within-day precision[a]	Between-day precision[a]	Accuracy[b]	Recovery[c]
EI	25	8.0	11.6	89.9 (3.5)	106.2 (6.4)
	100	2.6	6.8	108.0 (4.6)	106.3 (4.3)
	250	–	–	–	98.0 (3.6)
	430	2.2	4.2	97.4 (4.5)	–
NCI	0.3	2.1	11.2	98.3 (2.1)	104.0 (32.1)
	0.7	8.1	6.5	106.9 (8.1)	99.0 (8.8)
	250	0.5	3.5	99.4 (0.5)	81.3 (6.8)

[a] Percent relative standard deviation (%R.S.D.).
[b] Percent of the nominal concentration (with %R.S.D. in parenthesis).
[c] Percent relation of analyte peak areas of calibration and reference standards (with %R.S.D. in parenthesis).

Table 3
Stability of THC in plasma during storage and sample preparation (n = 3)

The nominal plasma concentration of THC (ng/ml)	4 h at room temperature (24 °C)[a]	24 h at + 4 °C[a]	3 weeks at −80 °C[a]	Three freeze–thaw cycles[a]
0.5	119.4 ± 5.8	116.7 ± 8.8	103.6 ± 7.0	103.6 ± 7.0
20	101.1 ± 0.5	104.0 ± 3.4	111.6 ± 3.9	111.6 ± 3.9
520	97.1 ± 3.1	92.4 ± 3.3	95.6 ± 1.5	95.6 ± 1.5

[a] Accuracy (%) ± S.D.

of urea. In the preliminary studies, addition of urea increased recovery of THC to over 40% of the maximum theoretical value (Fig. 3). The addition of methanol further increased recovery to the theoretical maximum.

The extraction recovery of THC was good, especially when the volume of organic solvent (methanol) in washing solution is considered. Different concentrations of methanol in washing solutions, and also the effect of different phase materials, were compared in preliminary studies (data not shown). The Oasis® HLB column, along with an ammonia wash (60% methanol) gave 100% recovery, while a C_{18} column with the same wash gave only 80% recovery. When combined with an acetic acid wash, the Oasis® HLB and C_{18} columns were comparable up to 60% of methanol, with recoveries over 90% for both. With an 80% methanol-containing wash, Oasis® HLB proved best with 70% recovery, compared to less than 30% recovery with the C_{18} column. Based on previous results, the Oasis® HLB column and a 60% methanol-containing washing solution were chosen as the final method.

The sample preparation described in this paper is straightforward and effective. It involves solid phase extraction with only two washes: a basic wash to remove acidic impurities, and acidic wash to remove basic impurities. After elution, samples were evaporated and derivatised in the same test tubes, and finally pipetted into sample vials for analysis. Considering the simplicity of these techniques, one person can easily process up to 50 samples in one working day.

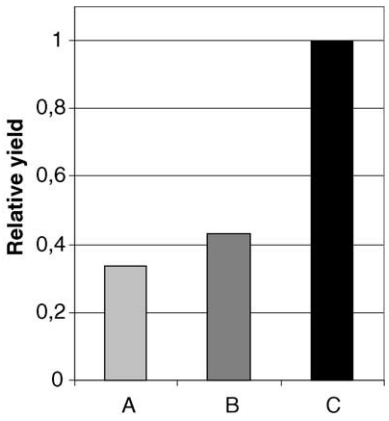

Fig. 3. The effects of urea and methanol on the relative yield of THC: (A) nothing added to plasma sample; (B) 1 ml urea (8 M) added to plasma sample; (C) 1 ml urea (8 M) and 1 ml methanol added to plasma sample.

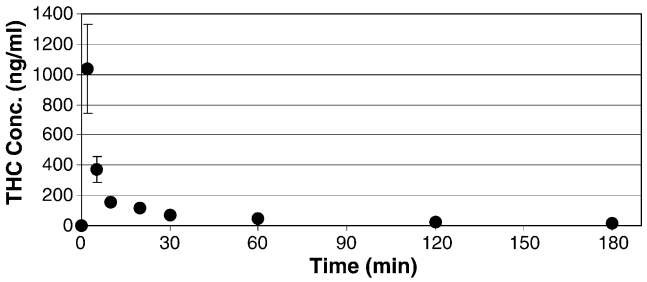

Fig. 4. THC concentration in plasma after i.v. administration (500 μg/kg), determined by EI (n = 3; mean ± standard error of mean).

3.3. Stability

The stability studies examined the degradation of THC during sample preparation and storage. The stability of THC in plasma samples was demonstrated at three concentration levels during storage and sample preparation (Table 3). Analyte concentration remained within ±20% of the target concentration under all conditions. THC was also found to be stable in stock solution as well as in the autosampler (data not shown). These results indicate that THC is stable during storage and sample preparation.

3.4. In vivo sublingual absorption of THC

The in vivo studies were performed as a part of a larger research project, of which the aim is to increase the bioavailability of THC by sublingual administration. In this study, THC was administered both sublingually and i.v. to rabbits, and pharmacokinetics were followed.

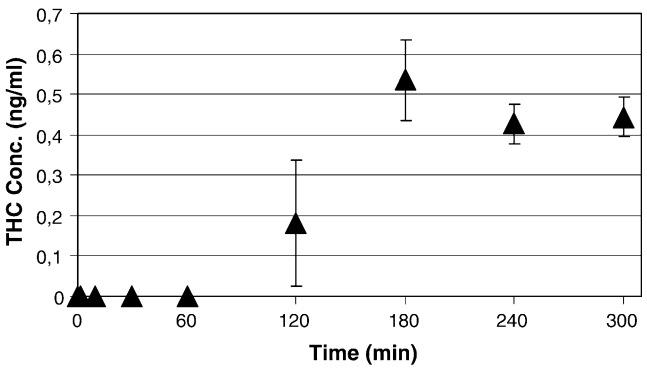

Fig. 5. THC concentration in plasma after sublingual administration (250 μg/kg), determined by NCI (n = 3; mean ± standard error of mean).

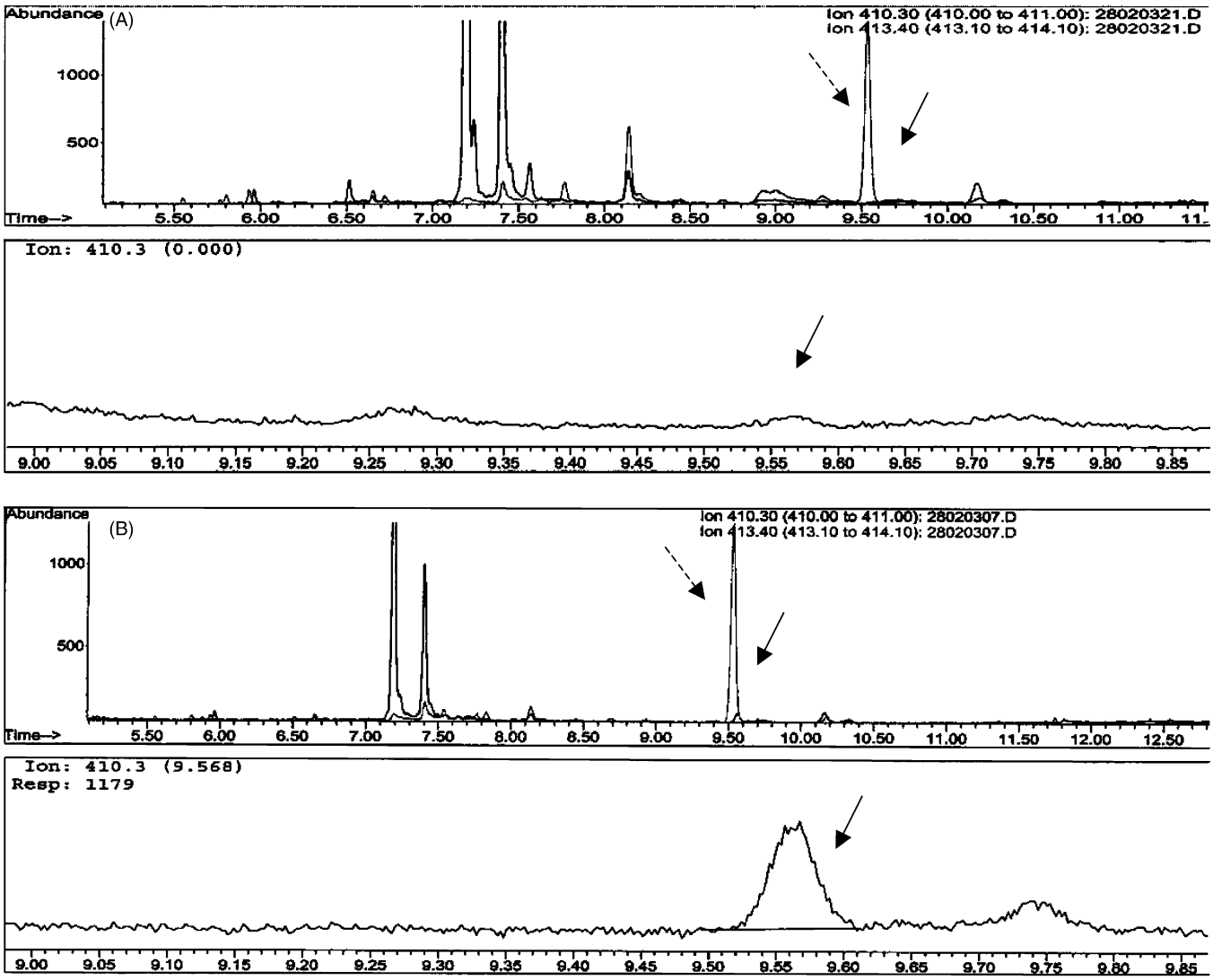

Fig. 6. NCI chromatograms from in vivo plasma samples before (A) and 120 min after (B) sublingual administration of THC to rabbit. The upper chromatogram shows both *m/z* 410.3 (THC–TFA) and *m/z* 413.4 (THC-d3-TFA). On the lower chromatograms, *m/z* 410.3 has been detailed. The plasma sample before administration has no trace of THC–TFA (black arrow), yet a clear peak exists for THC-d3-TFA (dashed arrow). On the 120 min sample, however, peaks for both THC–TFA and THC-d3-TFA are clearly visible.

Fig. 4 shows the mean plasma concentrations of THC after i.v. administration. These results show that the maximum plasma concentration of THC was over 1000 ng/ml. The concentration fell rapidly, but after 3 h was still measurable by EI. Fig. 5 shows that the plasma concentration of THC after sublingual administration was below 1 ng/ml at each data point. By using NCI, the monitoring of pharmacokinetics was possible: THC could be quantitated even up to 24 h after administration (data not shown). Typical NCI–chromatograms before and after sublingual administration of THC are presented in Fig. 6.

4. Conclusions

This paper describes the development of a novel GC–MS method with two ionization techniques. The method was validated and proved to be selective, linear, accurate and precise with good recovery. The method was applied successfully in analysing THC from rabbit plasma.

The EI technique was used for identifying THC from chromatogram by its decomposition fragments and for monitoring THC in plasma after i.v. administration, and the NCI technique was used for monitoring the pharmacokinetics of THC after sublingual administration.

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