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Liquid chromatographic–mass spectrometric quantitation of Δ^9-tetrahydrocannabinol and two metabolites in pharmacokinetic study plasma samples

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Abstract

A sensitive method for the determination of Δ^9-tetrahydrocannabinol and its metabolites, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid and 11-hydroxy-Δ^9-tetrahydrocannabinol, in rat and guinea pig plasma was developed using high-performance liquid chromatographic separation with electrospray ionization mass spectrometry detection and a simple liquid–liquid extraction technique. The mean recoveries for Δ^9-tetrahydrocannabinol, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid, and 11-hydroxy-Δ^9-tetrahydrocannabinol were 96, 92, and 85%, respectively. The lower limit of quantification (LLOQ) for all three compounds was 5 ng/ml and the limit of detection (LOD) was 2 ng/ml. This assay method utilizes the increased sensitivity and selectivity of mass spectrometric (MS) detection and a simple extraction step for the determination of Δ^9-tetrahydrocannabinol and its metabolites in plasma, and thus yields a more efficient pharmacokinetic analysis method than has previously been described.

Keywords: Δ^9-Tetrahydrocannabinol; 11-Nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid; 11-Hydroxy-Δ^9-tetrahydrocannabinol

1. Introduction

Δ^9-Tetrahydrocannabinol (THC) is the principle psychoactive constituent of marijuana [1], and is prescribed in oral capsule form (Marinol) to relieve the nausea and vomiting side effects associated with cancer chemotherapy, as well as to increase appetite and weight gain in AIDS patients [2]. Other types of dosage forms for THC are currently under development, including a transdermal patch [3]. A sensitive and simple analytical method is necessary for the pharmacokinetic analysis of THC and its two major active metabolites, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) and 11-hydroxy-Δ^9-tetrahydrocannabinol (THC-OH) in plasma samples from small animal models, where the volume of plasma is very low (<200 μl). There are several reported methods for the estimation of THC or its metabolites in plasma by GC/mass spectrometry (MS) after liquid/liquid or solid-phase extraction (SPE) and derivatization [4–7]. The major disadvantage of this method is the elaborate sample preparation and the need to use various derivatization techniques for non-volatile and thermolabile compounds. Other chromatographic methods reported were thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) with ultraviolet or electrochemical detection (UV, ED), and gas chromatography (GC) with electron capture, flame ionization or nitrogen–phosphorus detection (ECD, FID, NPD). Generally these methods lack either specificity or sensitivity. Recently, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) with LC/MS or LC/MS/MS were found to be suitable for the detection of drugs of abuse like opiates, amphetamines, cocaine-metabolites [8,9], and for THC-COOH and THC-COOH-β-glucuronide [10–15]. Two methods have been reported on the simultaneous determination of THC and its two metabolites [4,7] by GC/MS; both of these methods required derivatization of samples and 1 ml plasma sample volumes. Additionally, two LC–MS–MS methods have been reported for the analysis of THC and its two metabolites, THC-OH and THC-COOH, in blood and urine samples with detection limits of 1 ng/ml for an ion trap

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instrument equipped with an APCI source (positive mode MS–MS) [13], and 0.25 ng/ml with a triple quadrupole APCI instrument [14]. Tai and Welch [15] determined only THC-COOH levels with HPLC–ESI–MS (negative mode) in urine with SPE. The purpose of this manuscript is to describe a new LC–MS method for pharmacokinetic determination of THC and its metabolites in small volumes of plasma (without derivatization) using a simple liquid–liquid extraction technique. This method was developed specifically for the estimation of THC and its metabolites in rat and guinea pig plasma after intravenous THC doses and topical applications of THC in a transdermal patch.

2. Experimental

2.1. Materials and chemicals

Δ^9-Tetrahydrocannabinol was obtained from the National Institute on Drug Abuse (NIDA Drug Supply, Research Triangle Park, NC). 11-Nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid and 11-hydroxy-Δ^9-tetrahydrocannabinol were obtained from Sigma Chemical Company (St. Louis, MO). Ammonium acetate, ethyl acetate, and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fairlawn, NJ). Water was purified by Millipore Elix 5 reverse osmosis and a Milli-Q® (Millipore) Gradient A10 polishing system (Millipore, Bedford, MA, USA).

2.2. Extraction procedure

Concentrated THC and metabolite standard solutions were made in acetonitrile and used immediately to spike plasma. Calibration standards were prepared in plasma by spiking with concentrated standards to obtain 5–200 ng/ml of THC and its metabolites. Fifty microliters of plasma sample was placed into a siliconized microcentrifuge tube and extracted with 500 μl of acetonitrile: ethyl acetate (1:1, v/v). The mixture was vortexed for 30 s and centrifuged at 10,000 × *g* for 20 min. The supernatant was pipetted into a silanized 3 ml glass test tube and evaporated at 37 °C under nitrogen. The residue was reconstituted with 200 μl of acetonitrile and sonicated for 15 min. The samples were transferred into autosampler vials containing silanized low volume inserts and 20 μl was injected onto the HPLC column.

2.3. Liquid chromatography

Chromatography was performed on a Waters Symmetry® C_{18} (2.1 × 150 mm, 5 μm) column at 35 °C with a mobile phase consisting of ammonium acetate(2 mM):acetonitrile (30:70, v/v) at a flow-rate of 0.25 ml/min. A Waters Symmetry® C_{18} (2.1 × 10 mm, 3.5 μm) guard column was used.

2.4. Mass spectrometry

The LC/MS system consisted of a Waters Alliance 2690 HPLC pump (Waters, Milford, MA, USA), a Waters Alliance 2690 autosampler, and a Micromass ZQ detector (Waters, Milford, MA, USA) using electrospray ionization (ESI) for ion production. Selected ion monitoring (SIM) was performed in negative mode for ions *m/z* 313 [THC-H]- (dwell time 0.30 s), *m/z* 329 [THC-OH-H]-, and *m/z* 343 [THC-COOH-H]- (dwell time 0.30 s). Capillary voltage was 3.0 kV and cone voltage was 30 V. The source block and desolvation temperatures were 120 and 250 °C, respectively. Nitrogen was used as a nebulization and drying gas at flow rates of 50 and 450 l/h, respectively. The retention times for THC, THC-OH and THC-COOH were 20.14 ± 0.18, 5.85 ± 0.10, and 3.11 ± 0.12 min, respectively. Calibration graphs were constructed using a linear regression of the drug peak-area of the product ions versus nominal drug concentrations.

2.5. Validation

For the determination of intra-day and inter-day accuracy and precision of the assay, aliquots of 50 μl of plasma were spiked with various quantities of THC, THC-OH and THC-COOH to yield 10, 50 and 100 ng/ml. Accuracy was expressed as the mean% [(mean measured concentration)/(expected concentration)] × 100. Precision was calculated as inter and intra-day coefficient of variation [%CV = (S.D./mean) × 100].

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was investigated by extracting “blank” normal plasma and reconstituting with acetonitrile containing a known amount of the analytes, analyzing the reconstituted extracts, and then comparing the peak areas of the analytes with that of analytes in acetonitrile.

Absolute recoveries of the analytes were determined in triplicate in normal plasma by extracting drug-free plasma samples spiked with THC and its metabolites. Recoveries were calculated by comparison of the analyte peak areas of the extracted samples with those of the unextracted samples (analytes in acetonitrile).

3. Results and discussion

The ESI mass spectra of THC, THC-OH and THC-COOH at cone voltages of 30, 40, 50 and 60 V are shown in Figs. 1–3. At lower cone voltages (30 V), the quasi-molecular ion $[THC\text{-}H]^-$, *m/z* 313 was observed (Fig. 1). However, at higher cone voltages (40 and 50 V) two major fragments (*m/z* 281, *m/z* 255) were observed along with the quasi-molecular ion (*m/z* 313). These fragments could be due to the loss of CH_4O and the side chain (C_4H_{10}), respectively. The mass spectrum for THC-OH (Fig. 2) at cone

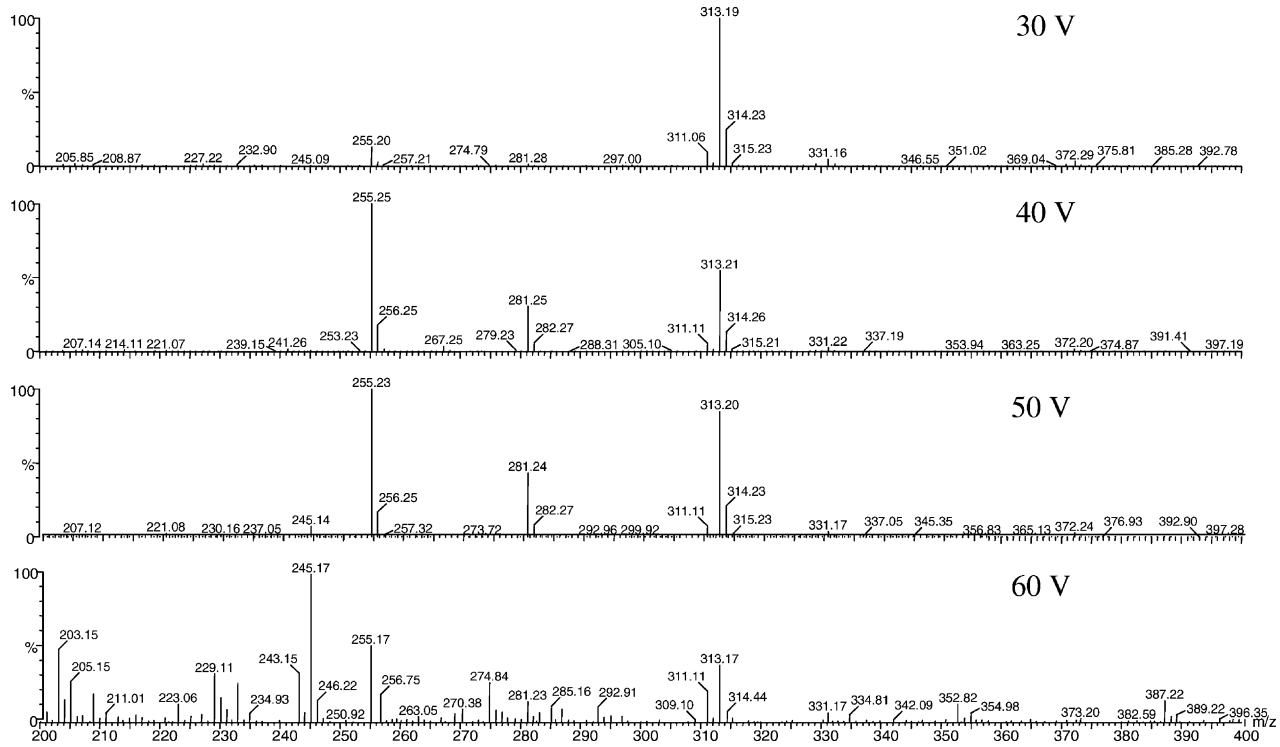

Fig. 1. Full scan mass spectrum of THC at different cone voltages.

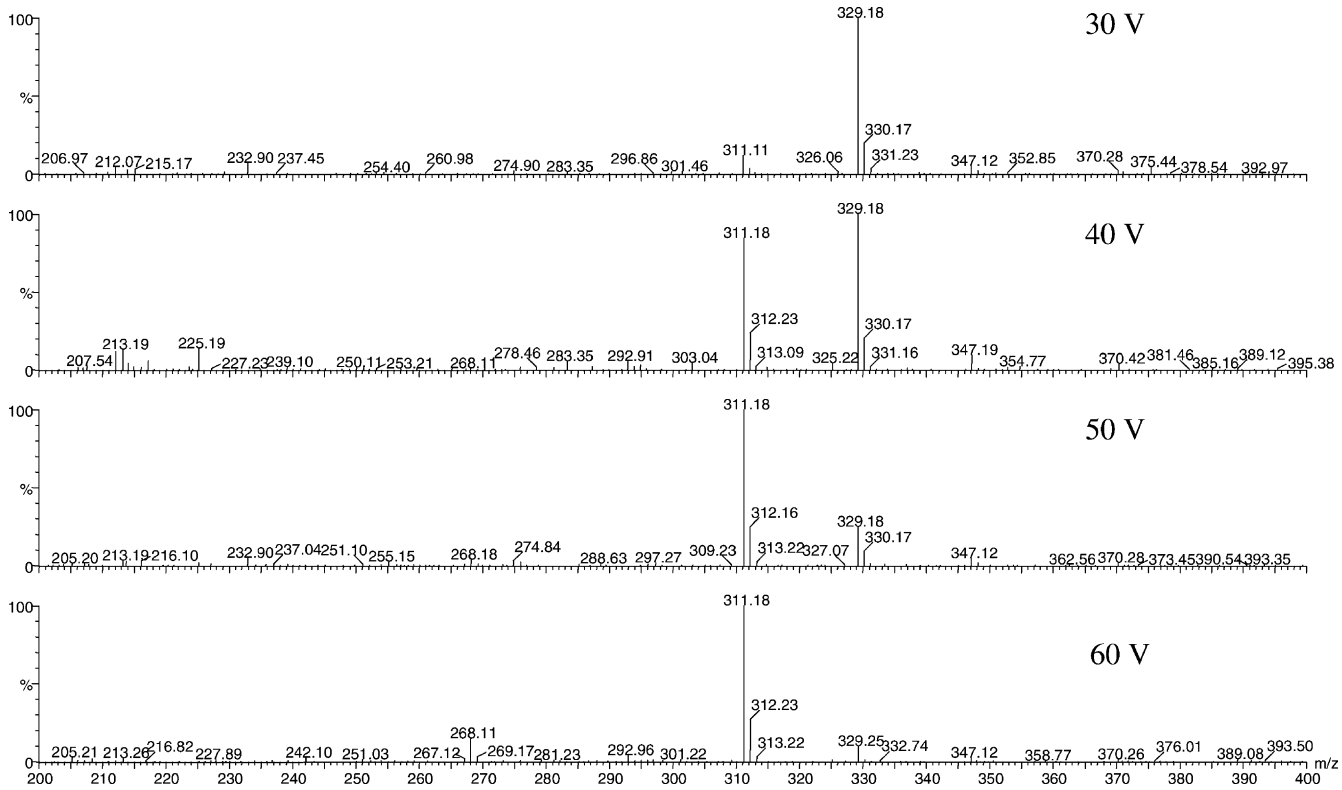

Fig. 2. Full scan mass spectrum of THC-OH at different cone voltages.

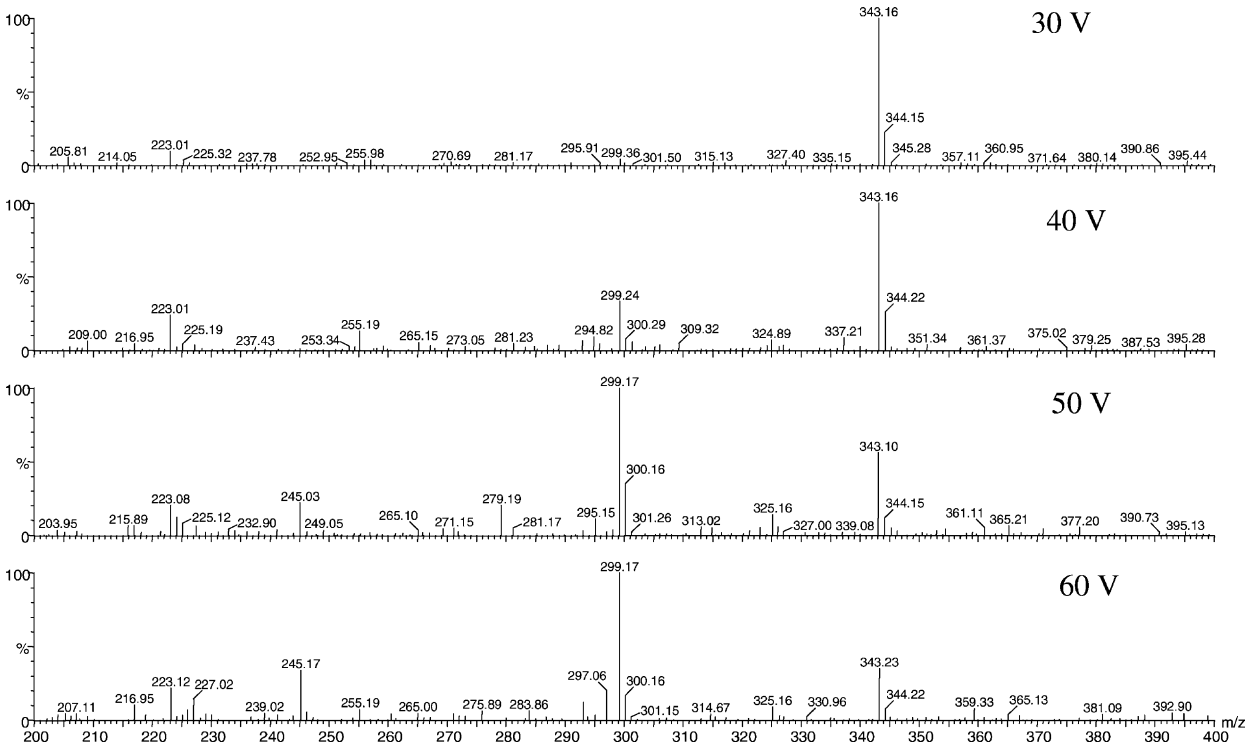

Fig. 3. Full scan mass spectrum of THC-COOH at different cone voltages.

voltage 30 V shows a quasi-molecular ion (THC-OH-H, *m*/*z* 329) and a minor fragment ion (*m*/*z* 311). However, increasing the cone voltage (40–60 V) produced a major fragment at *m*/*z* 311 (loss of water). Fig. 3 shows the fragmentation pattern for THC-COOH at different cone voltages. At the lower cone voltage (30 V), a quasi-molecular ion (THC-COOH-H, *m*/*z* 343) was observed. However, at higher cone voltages, one major fragment (*m*/*z* 299) and one minor fragment (*m*/*z* 325) were observed along with the quasi molecular ion (*m*/*z* 343). The fragmentation pattern observed here could be explained by the loss of the CO_2 (*m*/*z* 299) and water (*m*/*z* 325), respectively.

Typical ion chromatograms of 50 ng/ml THC, THC-OH, and THC-COOH in plasma obtained from the pharmacokinetic study are shown in Fig. 4. Retention times of THC, THC-OH, and THC-COOH were 20.14 ± 0.18, 5.85 ± 0.10 and 3.11 ± 0.12 min, respectively. The total run time for each sample was about 30 min. There were no interfering peaks with the drug peaks, and only two additional peaks were observed at 9.91 and 26.28 min, which were well separated from the THC drug peak. Standard curves prepared for THC, THC-OH, and THC-COOH in plasma were linear over a range of 5–200 ng/ml. The mean ($n = 3$) calibration curves for THC, THC-OH, and THC-COOH were $y = 523.92x - 23.648$, $R^2 = 0.998$; $y = 1006x - 263.6$, $R^2 = 0.999$; $y = 71.23x - 121.14$, $R^2 = 0.999$, respectively, where y and x are the peak area and concentration (ng/ml), respectively.

The mean absolute recoveries of THC, THC-OH, and THC-COOH determined in triplicate in the concentration range of 5–200 ng/ml were 96% (%CV 9), 92% (%CV 8), and 85% (%CV 8), respectively. No significant matrix effect was observed for the analytes in the plasma samples. The peak areas of the reconstituted samples had a coefficient of

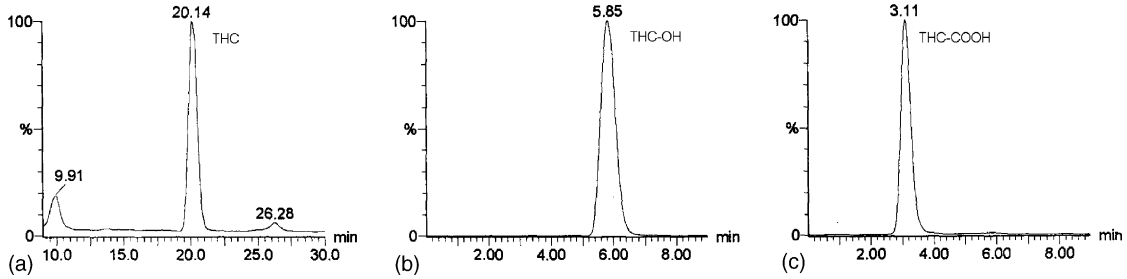

Fig. 4. Typical HPLC/MS ion chromatograms for 50 ng/ml THC and its metabolites in plasma: (a) THC (20.14 min), (b) THC-OH (5.85 min), (c) THC-COOH (3.11 min).

Table 1
Intra-day and inter-day quality control results of THC

Intra-day variation				Inter-day variation			
Concentration (ng/ml)	Mean concentration found (ng/ml)	%CV[a]	Accuracy (%)	Concentration (ng/ml)	Mean concentration found (ng/ml)	%CV[a]	Accuracy (%)
10	9.98	4.26	99.8	10	9.97	5.60	99.70
50	48.75	4.86	97.50	50	48.69	4.88	97.38
100	100.0	3.65	100.00	100	99.65	1.69	99.65

[a] %CV: coefficient of variation.

Table 2
Intra-day and inter-day quality control results of THC-OH

Intra-day variation				Inter-day variation			
Concentration (ng/ml)	Mean concentration found (ng/ml)	%CV[a]	Accuracy (%)	Concentration (ng/ml)	Mean concentration found (ng/ml)	%CV[a]	Accuracy (%)
10	9.92	5.32	99.2	10	9.95	5.26	99.50
50	50.01	5.62	100.2	50	49.29	6.01	98.58
100	99.06	3.2	99.06	100	99.25	4.36	99.25

[a] %CV: coefficient of variation.

variation of 4%, indicating that the extracts were "clean" with no co-eluting compounds influencing the ionization of the analytes.

Initially a LC–MS method was developed using 1% formic acid and acetonitrile (30:70) at a flow rate of 0.25 ml/min, but the metabolites (THC-OH and THC-COOH) were not separated. The mobile phase containing 2 mM ammonium acetate and acetonitrile (30:70, v/v) provided good resolution of the metabolites. In both cases the run time was 30 min, but the ammonium acetate provided the best peak resolution. The much higher selectivity of MS detection allowed the development of a very specific and rapid method for the determination of THC and its metabolites in plasma.

The LLOQ, defined as that concentration of THC and its metabolites which can still be determined with acceptable precision (%CV < 10) and accuracy was found to be 5 ng/ml and the LOD for THC and its metabolites was 2 ng/ml. Results of the intra-day and inter-day validation assays presented in Tables 1–3 indicated that the accuracy of the assay was >90% and CV did not exceed 10%. On-instrument stability was inferred from stability of samples which were prepared and included in the validation batch. No significant degradation was detected in the samples left in the autosampler at 12 °C for at least 48 h. Due to the high selectivity of MS detection, no interfering peaks were found when blank plasma extracts were analyzed.

Several extraction procedures were tested which included protein precipitation, solid-phase, and liquid–liquid extraction methods. A liquid–liquid extraction procedure proved to be the most successful with high recovery rates. THC gave a much higher (10-fold) response with negative electrospray ionization (ESI) than positive ESI. THC-OH and THC-COOH gave a 30 and 10 fold higher response with negative ESI than positive ESI, respectively. It was decided to work without an internal standard, because the external standard assay had an accuracy of >90% and the CV did not exceed 10%. The extraction efficiencies were high and consistently reproducible. The assay measures three different compounds with different ionization potential, and adding three internal standards to correspond with the ionization chemistry of each compound seemed impractical. The ionization response monitored by injecting a system performance verification standard at the beginning and at the end of each batch indicated that the system response remained stable.

The described method was applied to a pharmacokinetic study of THC in a guinea pig and a rat. All animal studies were approved by the University of Kentucky IACUC. Representative plasma concentration time curves after an

Table 3
Intra-day and inter-day quality control results of THC-COOH

Intra-day variation				Inter-day variation			
Concentration (ng/ml)	Mean concentration found (ng/ml)	%CV[a]	Accuracy (%)	Concentration (ng/ml)	Mean concentration found (ng/ml)	%CV[a]	Accuracy (%)
10	9.81	4.9	98.10	10	9.95	4.1	99.50
50	50.23	5.26	100.46	50	49.05	4.9	98.10
100	100.40	1.65	100.40	100	99.85	2.8	99.85

[a] %CV: coefficient of variation.

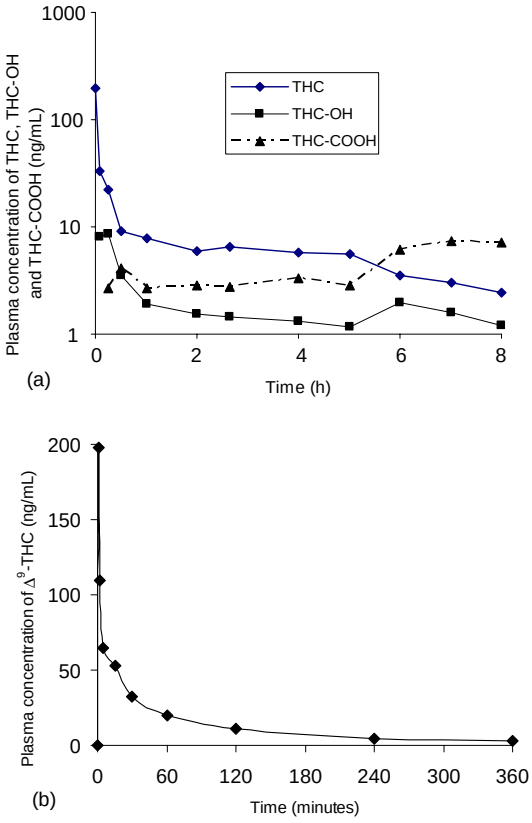

Fig. 5. (a) Representative THC, THC-OH, and THC-COOH rat plasma concentration vs. time profiles as obtained after intravenous administration (1 mg/kg). (b) Representative THC guinea pig plasma concentration vs. time profile as obtained after intravenous administration (1 mg/kg); no metabolites were detected in 6 h.

intravenous bolus dose of THC are shown in Fig. 5. The maximum THC plasma concentration obtained after intravenous administration of 1 mg/kg in guinea pigs was 197.5 and 194 ng/ml in the rat. No metabolites were detected in the guinea pig plasma samples taken after the intravenous dose over this short time course, however, significant metabolite levels were identified by this method in the rat pharmacokinetic study. The guinea pig has a different THC metabolic profile than many mammals [16,17], but the rat metabolizes THC rapidly to similar metabolites observed in humans [18].

4. Conclusion

A sensitive and selective method for the pharmacokinetic determination of THC and its metabolites in small volumes of plasma was developed, using high-performance liquid chromatographic separation with mass spectrometry detection. With an LLOQ of 5 ng/ml, pharmacokinetic profiles of the drug could be constructed for up to 8 h after a single intravenous bolus administration of 1 mg/kg of THC. This method is more efficient than previously described pharmacokinetic analytical methods because it requires relatively simple sample preparation for simultaneous quantitation of three analytes.

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