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Simultaneous Quantitation of Delta-9-tetrahydrocannabinol (THC) and 11-Nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH) in Serum by GC/MS Using Deuterated Internal Standards and Its Application to a Smoking Study and Forensic Cases

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ABSTRACT: A new procedure for the simultaneous detection of delta-9-tetrahydrocannabinol (THC) and its major metabolite, 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH) in serum has been evaluated. The method combines rapid, efficient, solid-phase extraction and simple derivatization by methylation. Analysis and quantitation is performed by gas chromatography/mass spectrometry (GC/MS) using deuterated cannabinoids as internal standards (IS). Reproducibility and sensitivity of the method are good.

The procedure is applied to serum specimens collected from a smoking study with 24 volunteers and 212 forensic cases. Results are interpreted based upon the current knowledge about THC metabolism and pharmacokinetics.

KEYWORDS: toxicology, cannabinoids, gas chromatography/mass spectrometry

Recently, simple nonradioactive immunoassay procedures for cannabinoid detection in serum have been described [*1–3*]. However, positive immunoassay results must be confirmed in forensic toxicology by an alternate analytical method [*4*]. Gas chromatography/mass spectrometry (GC/MS) confirmation is state of the art. Existing GC/MS methods [*5*] developed for research purposes are less appropriate for normal forensic-science routine. This is a report on an optimized analytical procedure with large sample capacity that works on a low cost GC/MS instrument.

The procedure allows quantitation of THC and THC-COOH in serum. The knowledge of serum cannabinoid levels is required to estimate time of drug ingestion, and to address questions of possible impairment [*6–8*].

Chemicals

The C_{18} bonded-phase adsorption columns were Bakerbond SPE® octadecyl cartridges and were obtained from J. T. Baker. The Vac-Elute vacuum manifold (model SPS 24) was purchased from Analytichem. Dimethylsulfoxide (DMSO) was purchased from Rie-

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del de Haen, methanol, acetone, isooctane, hydrochloric acid, acetic acid and iodomethane from Merck. Deuterated (D_3-)iodomethane was purchased from Aldrich and tetrabutyl-ammoniumhydroxide (TBAH, 55 to 60% in water) from Fluka. TBAH was dissolved in DMSO (2:98 v/v, TBAH/DMSO reagent). THC, THC-COOH, D_3-THC and D_3-THC-COOH were provided by Research Triangle Institute, North Carolina, or NIDA (National Institute on Drug Abuse).

Instrumentation

Immunoassay

Immunoassay [1–3] was performed on SYVA® ETS System with SYVA® EMIT d.a.u. reagents. Methanolic extracts of serum (3:1 v/v methanol:serum) were measured following the method of Gjerde et al. [1]. Drug-free serum and spiked-serum samples (20 ng/mL THC-COOH) were used as negative and positive calibrators.

GC/MS

A Hewlett Packard 5971A mass selective detector was used in combination with a Hewlett Packard 5890/II gaschromatograph for analysis. A Hewlett Packard dimethylpolysiloxane fused silica capillary column (HP-1, 12.0 m * 0.2 mm i.d., 0.33 μm film) was inserted directly into the ion source. Splitless injection with the Hewlett Packard 7673 automatic autosampler system and a valve off time of 2 min was used for injection.

The data system used was a Hewlett Packard Vectra 05/165. Data acquisition and analysis was performed with the Hewlett Packard HP 61030A MS ChemStation (DOS Series) software. The GC/MS was run in the SIM (selected ion monitoring) mode.

The GC operating conditions were as follows: the initial temperature of 100° was held for 3 min, then the temperature was programmed at a rate of 30°/min to 180°, the program was then slowed to 10°/min until 300° was reached. This temperature was maintained for 10 min. Injection port and transfer line temperatures were at 260° and 280° respectively.

Helium was used as carrier gas with a column head pressure of 25 kPa (4 PSI).

The GC/MS was autotuned with perfluorotributylamine and then usertuned for the masses 69, 264, 414. The Electron Multiplier Voltage was set 400 V above Tune value. Two ions were monitored for quantitation of each compound THC: 328/331 (IS); THC-COOH: 313/316 (IS) or 372/375 (IS); at a dwell time of 100 msec.

For qualitative verification ions monitored were: THC: 313, 316 (IS), 328, 331 (IS); THC-COOH: 313, 357, 372 at a dwell time of 75 msec.

Experimental

Extraction and derivatization for GC/MS Analysis

50 μl of the internal standard solution (50 ng/mL D_3-THC, 400 ng/mL D_3-THC-COOH in methanol) was added to 1 mL serum and mixed by sonication. Using the Vac-Elute manifold, the C_{18} column was activated by pretreatment with two column volumes of methanol followed by one column volume of water. Dryness of the C_{18} column should carefully be avoided during pretreatment. The serum was passed through in 5 min. The column was subsequently washed with water, acetic acid (0.25 M), water, 75 μl acetone and then allowed to dry for 15 min under maximum vacuum. THC and THC-COOH were eluted from the column with 1.5 mL acetone (3 * 500 μL aliquots). The eluate was then evaporated to dryness in a heating block at 70° under a stream of nitrogen. The residue was derivatized by adding 150 μL TBAH/DMSO reagent. After 2 min at room

temperature, 50 μL iodomethane was added and the sample was mixed. After 5 min at room temperature the reaction was stopped by 350 μL 0.1 N hydrochloric acid.

The reaction mixture was extracted with 2 mL isooctane (2 * 1 mL). After centrifugation, the top organic layer was transferred to a clean test tube and evaporated to dryness at 70° under a flow of nitrogen. The derivatized residue was dissolved in 30 μL isooctane and 2 μL injected into the GC/MS.

Calibration Curves

Calibration standards were prepared from drug free serum at concentrations of 1, 2, 4, 6, 10, 15 ng/mL THC respectively, the THC-COOH concentrations were 3, 5, 10, 20, 40, 60 ng/mL respectively. Internal standard (IS) concentrations were 2.5 ng/mL D_3-THC and 20 ng/mL D_3-THC-COOH.

Reproducibility of the Quantitative Analysis

Serum samples with IS and concentrations of THC: 2 and 6 ng/mL and THC-COOH: 20 and 60 ng/mL were each extracted 8 times, derivatized and analyzed as mentioned above.

Recovery Experiments

Calibration standards without IS were extracted on the C_{18} solid phase. After eluting with acetone, the IS was added to the eluent.

Recovery rate was calculated as the difference of slope of this calibration curve and the normal calibration curve.

Results and Discussion

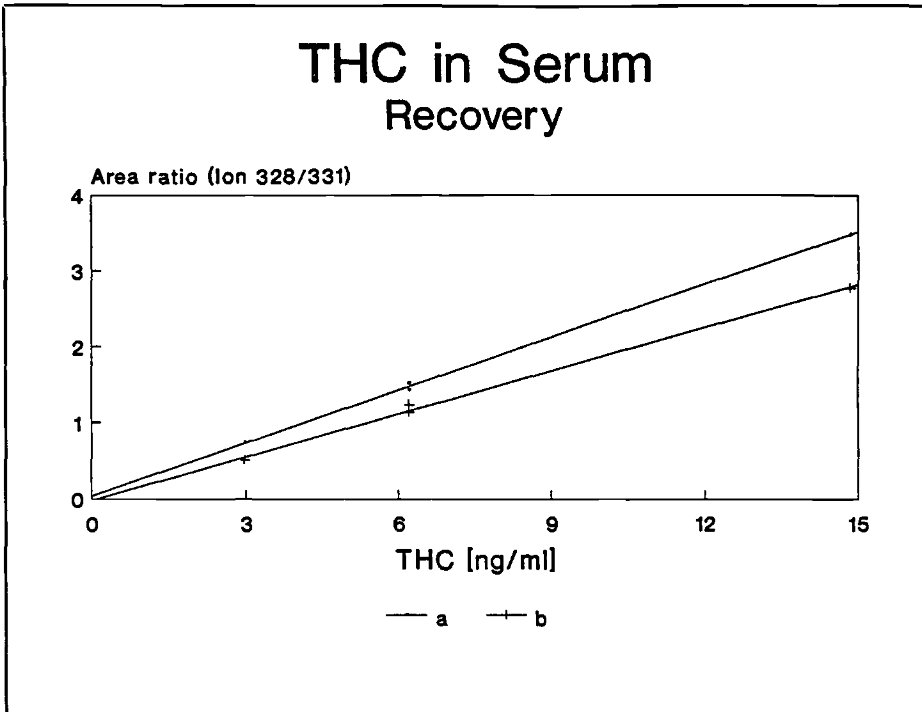
Recovery experiments for the solid phase extraction were done (a) by adding the internal standards before extraction to serum samples of known cannabinoid amount and (b) by adding the internal standards after the extraction of serum samples of known cannabinoid amount to the acetone eluent. Recovery was calculated by the difference in slope of the calibration curve a to b (Fig. 1). Serum samples are extracted with 100% recovery for THC-COOH and 80% for THC on the solid C_{18} phase. Deproteinizing extraction produces cleaner extracts in GC/MS, but is more time consuming [*9*]. Hydrolysis, while necessary for urine, was omitted because the concentrations of conjugated metabolites in serum are reported to be low after smoking marijuana [*10,11*], which is the most common form of marijuana use. However, if oral ingestion of marijuana is suspected, hydrolysis is advisable. Then THC-COOH glucuronide constitutes the major metabolite in serum similar to the urinary excretion pattern [*10,12*].

The derivatization procedure is similar to that of Whiting and Manders [*13*], which is often applied to detect THC-COOH in urine samples [*14–18*] and also adapted to THC analysis in serum samples [*19,20*]. The advantage of methylation is that the products are less sensitive to moisture than those of other derivatizing procedures such as silylation [*21,22*], trifluoroacetylation [*23*], pentafluorobenzylation [*24*] and pentafluoropropionylation [*10,25–27*].

Compared to HPLC/ECD methods [*9,11,28–31*] GC/MS is superior in selectivity and of more common use in forensic science laboratories.

The GC/MS SIM chromatogram of a serum extract (Fig. 2) shows minimal background of impurities and interfering peaks.

Quantitative THC-COOH determination could be performed either by monitoring the



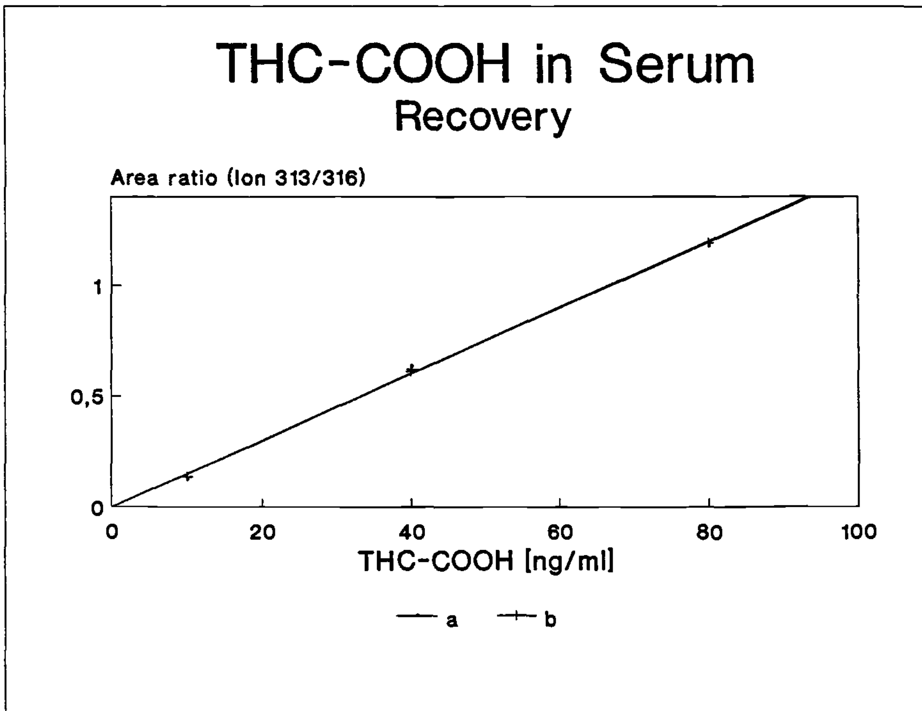


FIG. 1—*Recovery of the solid phase extraction calibration curve* (a) *IS added before extraction calibration curve* (b) *IS added after extraction.*

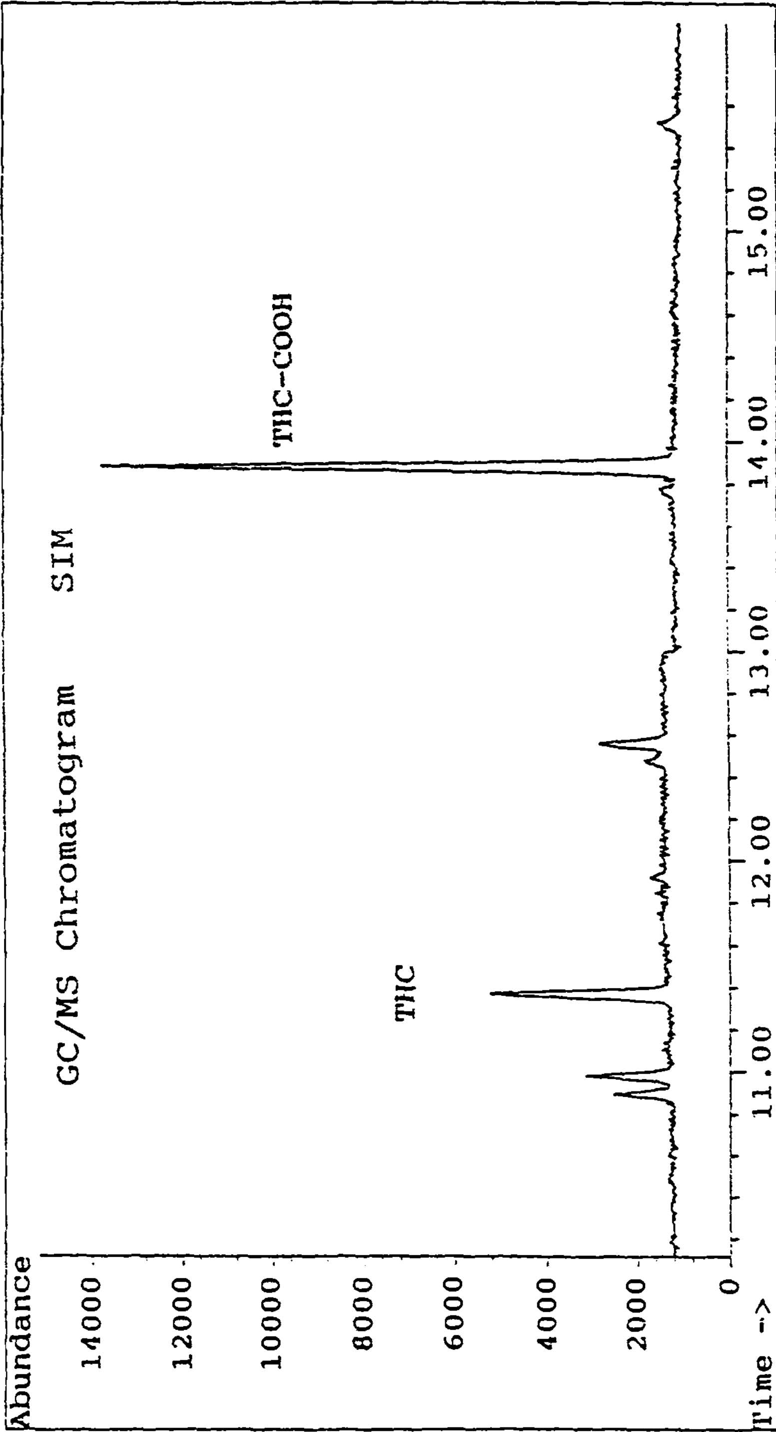


FIG. 2—*GC/MS-SIM chromatogram of serum extract (m/z = 328 and 331 until 13 min., m/z = 372 and 375 from 13 min.).*

molecular ion peak (m/z 372) or base peak (m/z 313) with three fold higher intensity. For THC, the molecular ion peak was used for quantitation (Fig. 3). The THC base peak (m/z 313) was not apt because of possible interferences.

Quantitation was achieved by comparing the area ratios of the respective ions (cannabinoid:IS) and plotting the ratio as a function of concentration (Fig. 4). Good linearity was obtained over the concentration range studied (Fig. 1). Correlation coefficient of calibration curves were excellent (0.999) for all diagnostic ion pairs.

At higher cannabinoid (THC > 30 ng/mL, THC-COOH > 100 ng/mL) concentrations, calibration curves deviate from linearity. This non linearity could be due to isotope spillover, which could be mathematically corrected for [14]; or nonlinearity of the GC/MS electron multiplier, which can't measure low and high signals simultaneously with the same precision. Increasing IS concentration and factorizing leads to linear curve fitting; or saturation of the GC/MS electron multiplier, which could be avoided by diminishing electron multiplier voltage.

In the reproducibility test spiked serum samples of different cannabinoid concentrations were each analyzed eight times. Concentrations and coefficients of variation (CV) are listed in Table 1. CVs are comparable to other methods [*11*] and derivatizing procedures [*10,32*].

At optimal system performance, the detection limit (signal-to-noise ratio = 3) is about 0.3 ng/mL THC and 3 ng/mL THC-COOH.

The method can also be applied to detect cannabinoids in hair [*33*].

Smoking Study

The present method was used to study the time course of THC and THC-COOH decline in serum of volunteers who had smoked marijuana. A total of 24 experienced users of cannabis, 12 male and 12 female, smoked marijuana cigarettes containing 300 μg THC/kg body weight. Blood specimens were taken before, and 40, 100, 160, 220 minutes after smoking. Each sample was analyzed twice. Mean deviation of the middle value was ±6.5% for THC and ±6.9% for THC-COOH. Serum concentrations of THC and THC-COOH are reported in Table 2.

The concentrations vary considerably among the subjects due to their different smoking techniques. The THC concentrations fall rapidly after smoking of the drug. Mean half life of THC (Fig. 5) is 55 min (approximated as simple monoexponential kinetics), estimated over the time range of 30–220 min. This value corresponds to half lives of the rapid disposition phase after intravenous dosing of THC of 0.4 to 0.6 h [*34*]. The major metabolite THC-COOH has already built up after 40 min and shows a much slower decrease in concentration with time as THC (Fig. 6: Mean serum cannabinoid levels after smoking). Time period observed is too short to allow precise calculations of the half life of THC-COOH. The described features of cannabinoid concentration course in serum agree well with the known characteristics of THC metabolism and kinetics after smoking [*10,20,34–41*].

Forensic Serum Samples

The GC/MS assay described was also applied to the analysis of 212 serum samples of forensic cases that were cannabinoid positive in the EMIT screening test.

According to the German practice in all forensic-science cases, serum was available. In few cases of hemolyzed blood, the detection of THC-COOH was possible, but that of THC was sometimes hindered because of impurities. These specimens were omitted. For the forensic-science samples the internal standard quantities were raised (5 ng/mL D_3-THC, 40 ng/mL D_3-THC-COOH) to cover a broader concentration range of can-

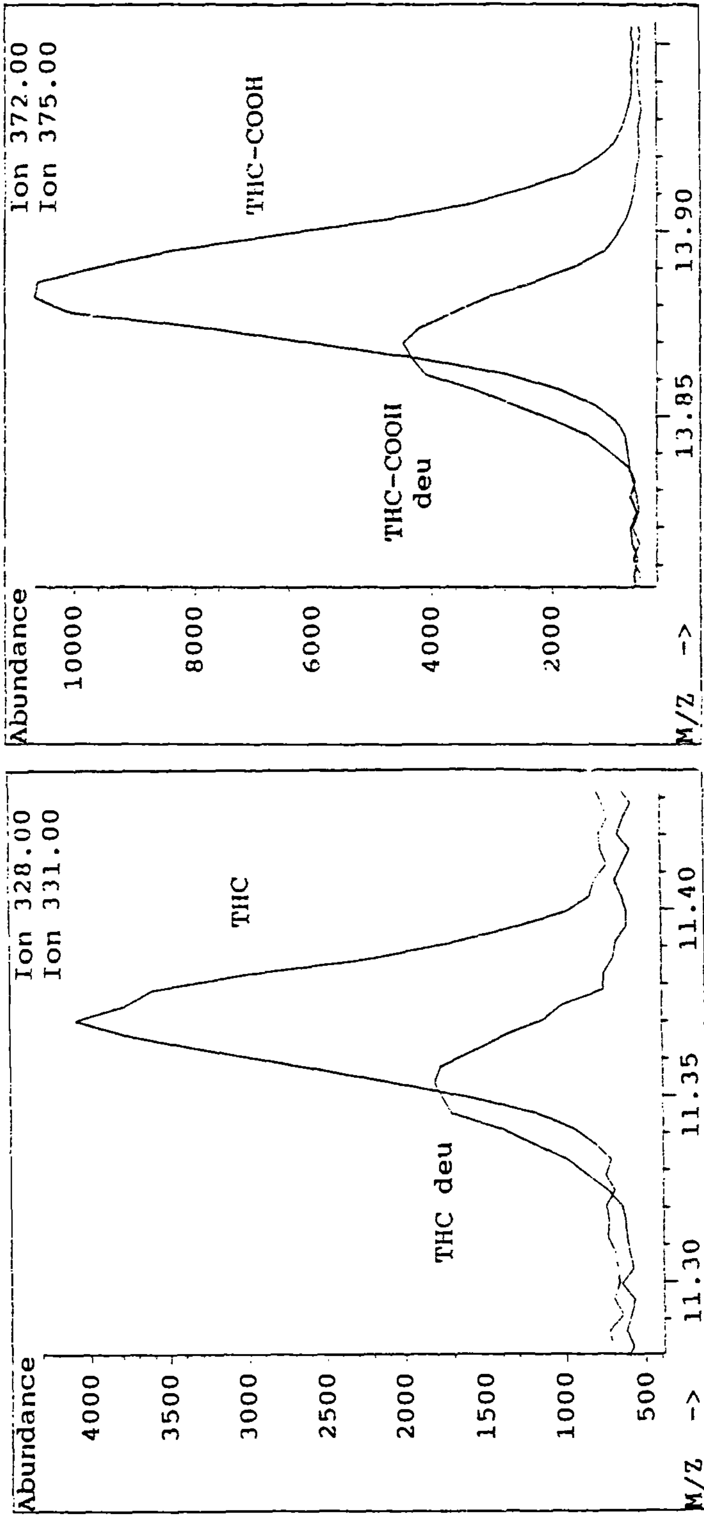
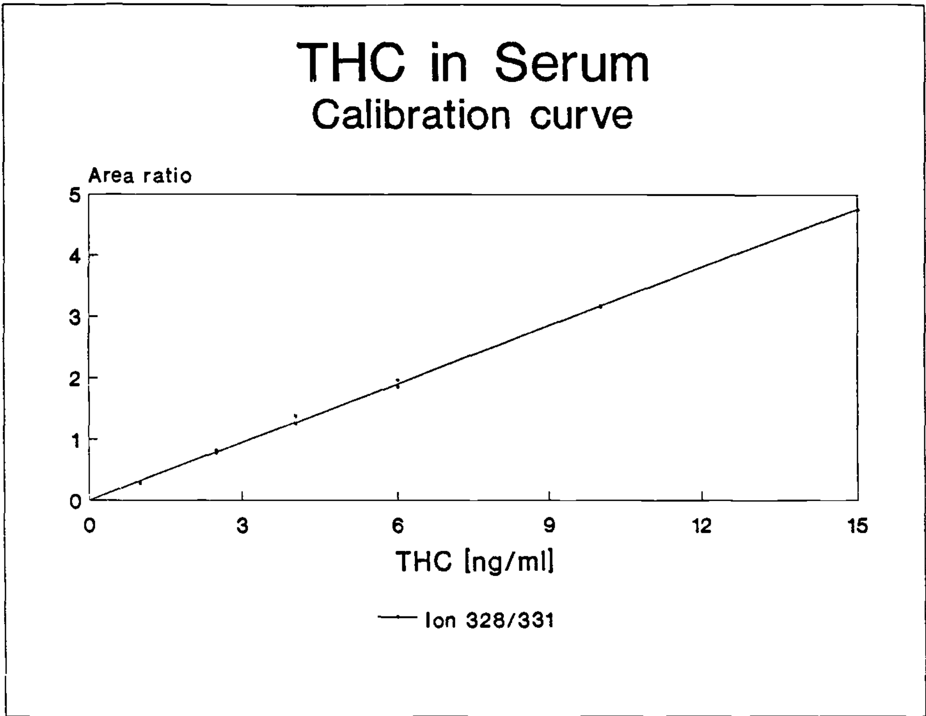


FIG. 3—*Quantitative signals of THC and THC-COOH.*



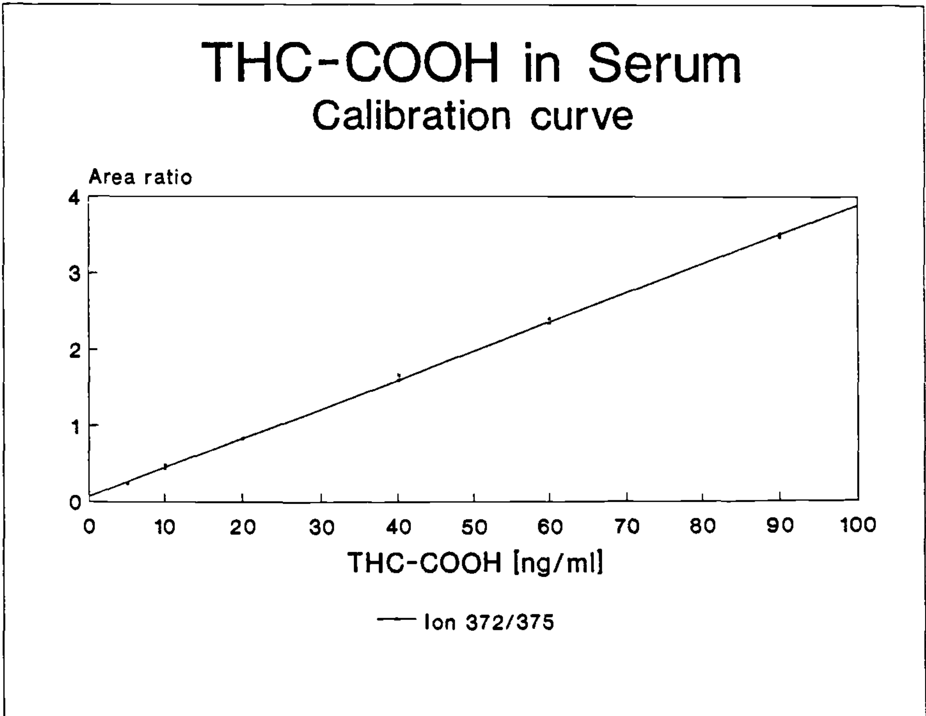


FIG. 4—*Calibration curves.*

TABLE 1—*Reproducibility of the quantitative analysis (n = 8).*

Analyte	Concentration (ng/mL)	CV (%)
THC	2	12.6
THC	6	4.6
THC-COOH	20	3.8
THC-COOH	60	3.2

TABLE 2—*Smoking study. Cannabinoid concentrations in plasma.*

TIME[min] [ng/mL]	0		40		100		160		220	
	THC	THC-AC[a]	THC	THC-AC	THC	THC-AC	THC	THC-AC	THC	THC-AC
Subject										
1	0.0	0.0	4.6	10.4	1.3	9.6	0.6	7.4	0.4	5.5
2	0.0	0.0	9.9	4.9	3.5	4.6	1.3	3.0	1.1	1.6
3	0.0	0.0	19.3	11.0	3.0	11.5	1.0	7.5	0.2	5.5
4	0.0	0.0	17.2	39.9	5.9	26.8	3.0	15.8	1.0	10.1
5	—	—	11.2	19.0	4.0	14.6	1.5	17.3	0.7	13.6
6	0.0	0.0	18.2	3.3	6.8	2.9	3.2	3.2	1.8	2.2
7	0.0	0.0	20.4	25.6	5.9	19.6	2.5	17.9	0.7	13.0
8	0.0	0.0	8.1	17.9	3.2	13.0	1.0	8.0	0.5	4.3
9	0.0	0.0	3.9	8.0	0.5	13.9	0.3	0.5	0.0	0.0
10	3.0	63.0	35.5	96.4	9.9	72.4	6.8	63.5	5.1	67.0
11	0.0	0.0	15.7	20.9	6.1	18.7	3.1	18.3	1.4	13.5
12	0.0	0.0	6.8	13.6	2.6	13.9	1.3	12.4	0.8	10.7
13	0.0	0.0	1.2	0.0	0.5	0.0	0.0	0.0	0.0	0.0
14	0.0	0.0	6.7	28.8	3.0	25.9	1.2	22.3	0.8	13.0
15	0.0	0.0	4.5	8.7	1.6	6.8	—	—	0.6	6.0
16	0.0	0.0	9.5	17.3	4.2	16.8	2.1	13.6	1.3	11.1
17	0.0	0.0	3.3	6.3	1.1	4.3	0.6	4.8	0.5	4.3
18	0.0	3.7	5.4	12.0	2.4	10.4	1.1	9.5	0.8	8.3
19	1.2	23.7	13.3	61.9	4.5	47.3	2.3	43.8	1.6	37.5
20	0.0	0.0	18.8	21.6	5.8	19.2	2.7	14.7	1.3	13.0
21	0.0	0.0	19.7	44.6	5.7	36.6	2.8	29.0	1.7	17.6
22	0.0	0.0	45.5	32.4	15.2	31.0	5.6	31.1	3.6	23.3
23	0.0	0.0	5.9	5.0	1.4	4.9	0.9	5.1	0.6	5.8
24	0.0	0.0	9.8	12.9	2.5	12.8	1.4	9.5	1.3	8.0

[a]THC-AC = THC-COOH.

nabinoids. In each series measured three standards were included—a blank, standard 1 (2 ng/mL THC, 20 ng/mL THC-COOH) and standard 2 (10 ng/mL THC, 100 ng/mL THC-COOH). Coefficients of variation (CV) of the standards over a 5 month period for $n = 12$ was 9% (2 ng/mL THC), 5.5% (20 ng/mL THC-COOH), 12% (10 ng/mL THC) and 10% (100 ng/mL THC-COOH).

Distribution of cannabinoid concentrations in 212 forensic serum specimens are displayed in Fig. 7. Presence of THC and THC-COOH could only be confirmed by GC/MS in about 70% of serum samples qualified as positive in screening test with immunoassay. It is well documented in literature that EMIT does not correlate effectively with GC/MS results [*42*]. Cannabinoid concentrations of samples found EMIT positive are sometimes below the level of sensitivity for GC/MS verification and EMIT values are up to seven times higher than GC/MS measurements [*10,15,27,43,44*]. Urinary EMIT test also yields false negatives [*45*]. EMIT tests are specified for the primary metabolite THC-COOH, but also cross react with secondary THC metabolites to some extent. Therefore, this difference in results can be attributed to other metabolites, differences

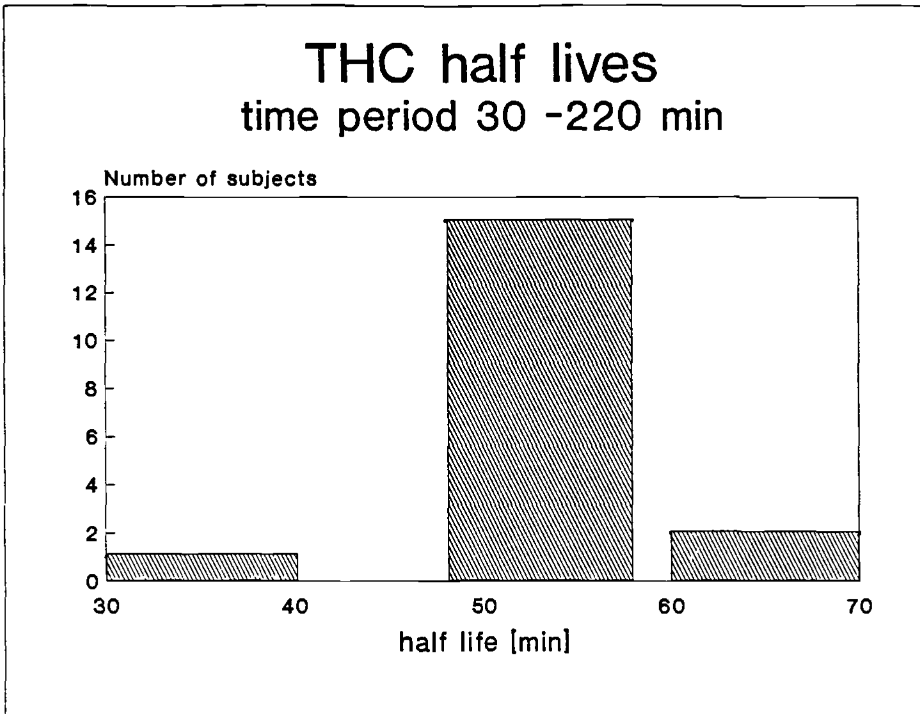


FIG. 5—*Half lives of THC in the time range 30–220 min after smoking (from 18 subjects).*

in cross reactivity, or affinity of the various metabolites. In order to lower the number of false positives in EMIT technique, the positive calibrator concentration can be raised [*1*].

However, a GC/MS confirmation of cannabinoids in serum indicate a more recent cannabis consumption with greater reliability than urinary cannabinoid levels. In serum, the elimination half life for THC-COOH after smoking is 25–55 hrs [*34*], and in urine a half life of 1 to 10 days is reported in literature [*44*]. Urinary excretion of THC-COOH lasts up to 77 days with mean excretion time of 27 days [*46*].

The same holds true for oral ingestion. Cannabinoids could be detected in blood for up to 5 days and in urine for up to 12 days following a single oral dose of THC [*12*].

In 80% of positive THC serum specimens, concentration ranged between 0.5 to 3 ng/mL (Fig. 6). This THC serum concentration is usually reached 4 to 5 h after smoking [*10*] and then persists for a long time with an elimination half life of 3 to 5 days [*47*]. The higher THC concentrations in 19% of positive samples can be related to more recent smoking (that is, within 6 h) [*10*].

Elevated THC-COOH concentrations as found in two serum samples (>600 ng/mL) are known to occur after oral ingestion of cannabis [*10,34*].

In 17% of positive serum samples THC-COOH levels exceed 60 ng/mL. Compared to the metabolite concentration in the smoking study (Table 2), this may lead to the assumption of multiple successive marijuana use. But these intermediate THC-COOH levels cannot be interpreted clearly because only few and single studies examine temporal behavior and elimination of the main metabolite in serum and even less is known about metabolite concentration ranges after repeated smoking and oral consumption of marijuana.

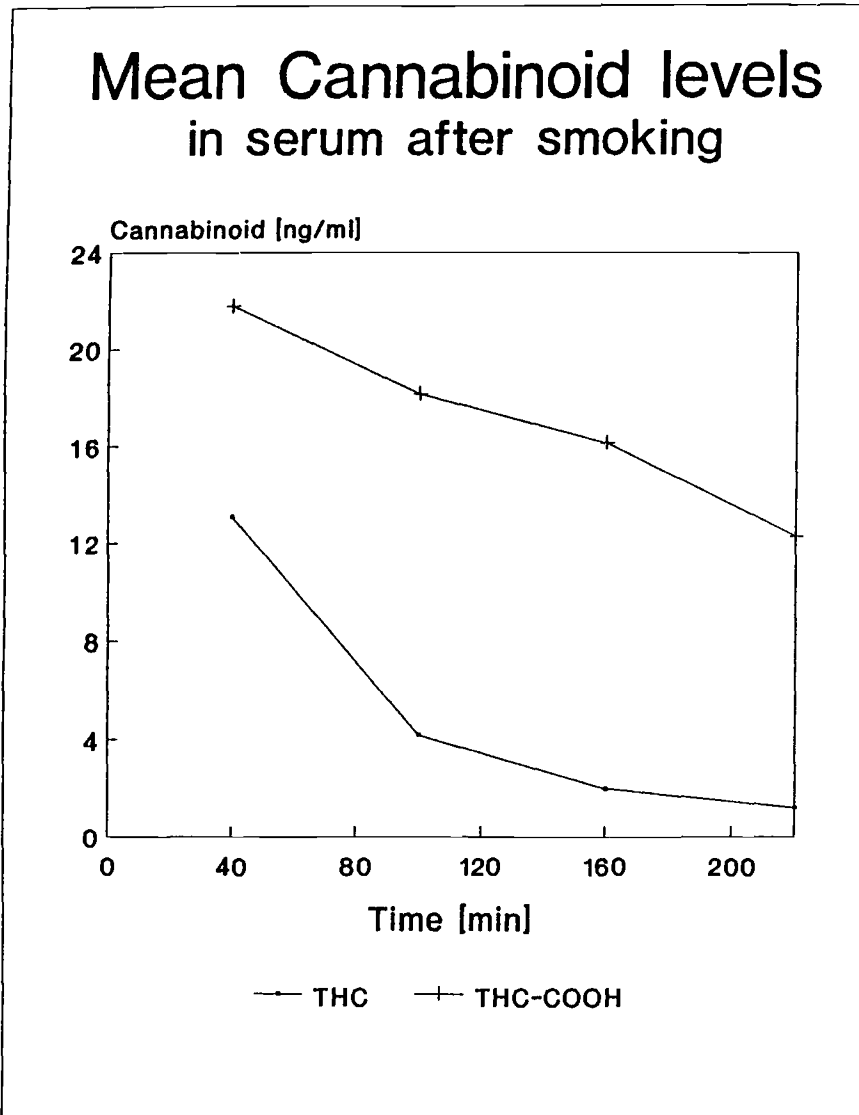
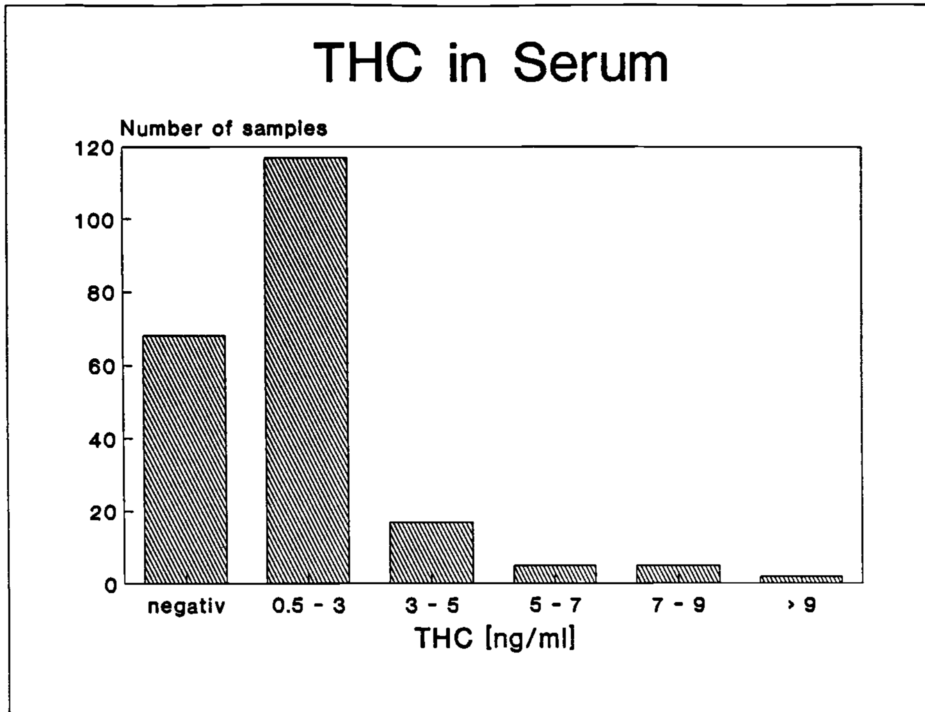


FIG. 6—*Mean serum cannabinoid levels after smoking.*

In 10% of positive samples, both cannabinoids are present simultaneously at elevated concentrations, THC > 3 ng/mL and THC-COOH > 60 ng/mL.

Of course it is of forensic-science interest to connect cannabinoid serum concentrations, time of ingestion and drug effects, especially in cases of motor fatalities, homicides and impaired motorists [*48–50*]. It is well known that no direct correlation as that for alcohol exists. The "high" resulting from smoking or oral ingestion of marijuana does not coincide in time with maximal serum concentration of the psychoactive THC, but it seems to correlate better with maximal concentration of THC-COOH [*6,7,34–41*]. Some authors propose ratio of THC: THC-COOH for estimating time of smoking [*20,48*] or detection of a more early occuring metabolite [*10*].



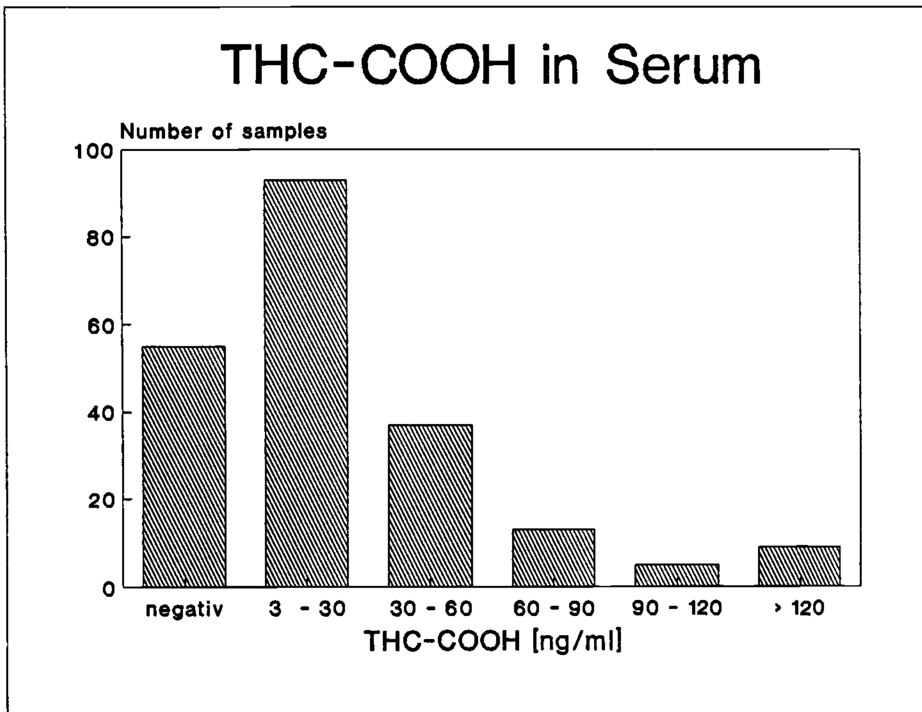


FIG. 7—*Distribution of cannabinoid levels in 212 forensic serum samples.*

In general, serum cannabinoid analysis is indispensable if recent marijuana use must be proved and this analysis also supplies reliable evidence of marijuana consumption even in stored serum or blood samples [*22,51*].

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