Jim Gerostamoulos,[1] *B.Sc. (Hons). and*
Olaf H. Drummer,[1] *Ph.D.*

Incidence of Psychoactive Cannabinoids in Drivers Killed in Motor Vehicle Accidents

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ABSTRACT: A high performance liquid chromatography (HPLC) assay was developed for the psychoactive cannabinoids Delta-9-Tetrahydrocannabinol (THC) and 11-Hydroxy-THC (11-OH-THC) using electrochemical detection (ECD). A C8 bonded column was used and the mobile phase consisted of acetonitrile, methanol and 0.01 M sulphuric acid at a flow rate of 1.5 mL/min. The detection limits for both THC and 11-OH-THC were 1.0 ng/mL. Preliminary screening of 193 drivers using an enzyme-multiplied immunoassay technique (EMIT) showed 21 tested positive on either blood, urine or both. Of these subjects 13 were confirmed as positive by the HPLC/ECD method in blood. Blood concentrations for THC ranged from 1.4 ng/mL up to 20 ng/mL and for 11-OH-THC 2.5 ng/mL up to 85 ng/mL.

KEYWORDS: toxicology, HPLC, psychoactive cannabinoids, THC, 11-hydroxy-THC, marijuana

Fatal motor vehicle accidents account for many thousands of deaths per year worldwide. There are many factors that may contribute to these fatalities such as road and weather conditions, state of the motor vehicle, speed, driving skills, etc., as well as the influence of alcohol and other drugs [*1–3*]. Marijuana has been suggested to be a causative factor in 6 to 20% of road traffic fatalities [*3–6*].

While marijuana is composed of more than 60 constituents called cannabinoids, the principle psychoactive agent in cannabis is Δ-9-Tetrahydrocannabinol (THC). THC is metabolized to 11-hydroxy-THC (11-OH-THC) by microsomal hydoxylation [*7*], which is believed to be also pharmacologically active [*8*]. Little THC is excreted in urine, the major urinary metabolite being 11-nor-delta-9-carboxy-THC (THC-COOH) from the subsequent oxidation of 11-OH-THC. THC-COOH has been most often measured to determine the presence and involvement of marijuana in traffic fatalities [*3,9*]. However THC-COOH is not pharmacologically active. The measurement of THC and 11-OH-THC in blood is therefore more likely to be a better indicator of possible impairment of motor coordination than measurement of THC-COOH in blood or urine. However there have been few studies that have measured these two psychoactive chemicals in blood of fatal motor vehicle accidents [*4*] due to difficulties in the measurement of THC and its metabolites with sufficient sensitivity. Early work by Valentine et al. [*10*] using HPLC

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[1]Postgraduate Student in Forensic Medicine, Assistant Director (Scientific Services) and Honorary Senior Lecturer in Forensic Medicine, respectively, Victorian Institute of Forensic Pathology and Department of Forensic Medicine, Monash University, South Melbourne, Victoria, Australia.

with mass spectrometry claimed a practical lower detection limit of 2.5 ng/mL for THC, although this type of work required extensive sample preparation and was not applicable for routine forensic toxicology. Detection limits of 0.1 ng/mL for THC and 11-OH-THC in blood have been achieved using capillary column gas chromatography—negative ion chemical ionization mass spectrometry employing a relatively simple extraction method [*11*]. The use of RIA combined with HPLC to enhance sensitivity has been used for the analysis of samples submitted for forensic work [*9*]. Detection however was restricted to measuring levels of THC-COOH and THC-COOH glucuronide, but not THC.

Recent work by Nakahara and Sekine [*12*] involved the use of electrochemical detection (ECD) with HPLC. Advantages of using ECD are high sensitivity comparable to GC/MS and relatively low cost. Other similar studies using ECD have also reported high sensitivity [*12,14*], but continued work by Nakahara et al. [*15*] has seen improvement in detection limits using solid phase extraction techniques, however published techniques have not used postmortem blood.

We have modified the extraction method by Foltz et al. [*11*] and developed a HPLC method to measure both psychoactive THC and 11-OH-THC in postmortem blood. Drivers killed in motor vehicle accidents in Victoria in a 12 month period were tested with this procedure.

Materials and Methods

Chemicals and Reagents

THC and 11-OH-THC were provided by the curator of standards at the Australian Government Analytical Laboratories. All solvents used were HPLC grade (Mallinckrodt Inc.). The mobile phase was filtered through a Millipore HV 0.45 μM filter prior to use. EMIT reagents were obtained from Syva Australia.

Chromatography

The chromatography system consisted of a Shimadzu model LC-6AD pump equipped with a Rheodyne 7125 injector (100 μL loop). A 250 by 5.0 mm I.D., Spherisorb C_8 stainless steel column (SGE, Australia) was used with an ESA Coulochem electrochemical detector (ESA Inc., Bedford, MA) equipped with a Model 5010 analytical cell and a Model 5020 guard cell. A Shimadzu chromatopac C-R6A data recorder was used to process the chromatograms.

The mobile phase for the analysis of THC was acetonitrile/methanol/0.01 M H_2SO_4 (45:20:35) and for 11-OH-THC acetonitrile/methanol/0.01 M H_2SO_4 (35:15:50) was used. The flow rate was 1.5 mL/min for both systems and the column temperature was ambient. The working parameters for the electrochemical detector were +0.40 V for detector cell 1, +0.68 V for detector cell 2 and +1.0 V for the guard cell.

Standards

Stock solutions were prepared in ethanol at a concentration of 1 mg/mL. These were stored in a refrigerator at 4°C and used to spike drug-free blood and urine for calibration standards. Blood standards with concentrations of 2, 5, 10, and 15 ng/mL for THC and concentrations of 3, 5, 7, 14, and 21 ng/mL for 11-OH-THC were prepared and extracted with unknown samples.

Emit Analysis

Blood and urine were initially screened for the presence of immunoreactive cannabinoids using the EMIT® d.a.u™ 20 ng Cannabinoid Assay. Urine specimens (200 μl) were

adjusted to a pH range of 5 to 8 by the addition of 1 M HCL or 1 M NaOH before analysis. Urines with high turbidity were centrifuged before use. The urine cut off for the presence of cannabinoids was 20 ng/mL. The cut off for blood was the value given for known blank blood specimens. Any specimen which then gave a greater value was presumed to be positive.

When blood was used, equal volumes of acetonitrile were added to 500 μl of sample (pH 5 to 8). These were vortexed for 1 min and after 10 min of standing were centrifuged at 3500 rpm for 15 min. The supernatant was then transferred to a clean autosample vial and assayed.

An EMIT® drugs-of-abuse screen for amphetamines, barbituates, benzodiazepines and opiates in blood and urine samples of drivers were also performed.

HPLC Analysis

All glassware used was silanized to prevent the adsorption of cannabinoids onto the glassware. This was accomplished by immersing the glassware in a 5% solution of Surfasil® (Pierce Chemical Company) in toluene for 2 h and then rinsing the glassware twice in methanol and then dried before use. The HPLC extraction was based, with modifications, on the procedure of Foltz et al. [*11*].

Sample preparation: To 1 mL of blood/urine in a 10 mL silanized extraction tube, 5 mL of acetonitrile was added and immediately vortexed for 60 s. The solid and liquid phases were separated at 3500 rpm by centrifugation and the supernatant was decanted into a clean tube. The volume of supernatant was reduced to <1 mL by evaporation at 40°C under a stream of dry nitrogen. To the concentrated supernatant was added 1 mL of 0.2 M NaOH and 5 mL of hexane/ethyl acetate (9:1 v/v). The mixture was shaken for 60 min by means of a reciprocating shaker. After centrifugation for 10 min, the organic phase was transferred to a clean culture tube and 2 mL of 0.1 M HCl was added. The tube was shaken for 60 min and the layers separated by centrifugation. The organic layer was transferred to a clean tube and then evaporated to dryness in a sample concentrator (Savant Instruments Inc., NY). The sample was reconstituted in 150 μl mobile phase and a 30 μl volume was injected onto the column.

Selection Criteria of Cases

All drivers in the study were chosen from a list of 247 motor vehicle/motor cycle deaths that occurred in Victoria from the period of June 1989 to June 1990. Drivers (193) were selected on the basis of sample availability at the Victorian Institute of Forensic Pathology. Police reports, circumstances and all other relevant information pertaining to these road deaths were obtained from the State Coroner's Office.

Results

Recoveries of cannabinoids in blood samples were calculated by measuring the peak heights of a known unextracted amount of THC and 11-OH-THC and comparing this value to the peak heights obtained from a sample containing known concentrations of THC and 11-OH-THC extracted from blood. The extraction recovery for THC (at 2.0 ng/mL, $n = 4$) was 40%, and for 11-OH-THC 38% (at 3.5 ng/mL, $n = 4$). Coefficients of variation (CV) for intra-assay and inter-assay reproducibility for THC and 11-OH-THC are shown in Table 1. Intra-assay variability show CVs generally less than 15%, whilst interassay CVs gave similar values except at the lowest concentration. The accuracy of the assay was also acceptable for both analytes. Deviations calculated from true concentrations were all less than 20%. The detection limits for both THC and 11-OH-THC was calculated at 1.0 ng/mL. This was determined to be at a signal three times baseline noise. The calibration curves were linear. Linear regression analysis gave the

TABLE 1—*Reproducibility of measurement of THC and 11-OH-THC from postmortem blood.*

	THC			
	Concentration (ng/mL)			
THC (n = 4)	2.0	5.0	10.0	15.0
Intra-assay CV %	12	12	7.1	10
Inter-assay CV %	22	16	7.8	14

	11-OH-THC			
	Concentration (ng/mL)			
11-OH-THC (n = 4)	3.5	7.0	14.0	21.0
Intra-assay CV %	9.3	1.6	14	8.0
Inter-assay CV %	39	8.9	15	12

following fits for typical examples: THC, $y = -0.9758 + 2.7775x$, $r^2 = 0.988$; 11-OH-THC, $y = -1.3190 + 1.8153x$, $r^2 = 0.993$.

Typical chromatograms are shown in Figs. 1 and 2 for THC and 11-OH-THC, respectively. While endogenous peaks were detected in the chromatograms there was no interference in the elution times of THC and 11-OH-THC. Extraction of a number of postmortem specimens that did not show a positive immunoreactivity to cannabinoids did not show peaks eluting near THC and 11-OH-THC that may have interfered with the analyses.

A total of 193 drivers (149 samples with blood only and 44 cases with both blood and urine samples) were screened for cannabinoids using EMIT analysis. Males represented 156 cases (81%), of whom 13% were motorcyclists, and females represented 37 cases

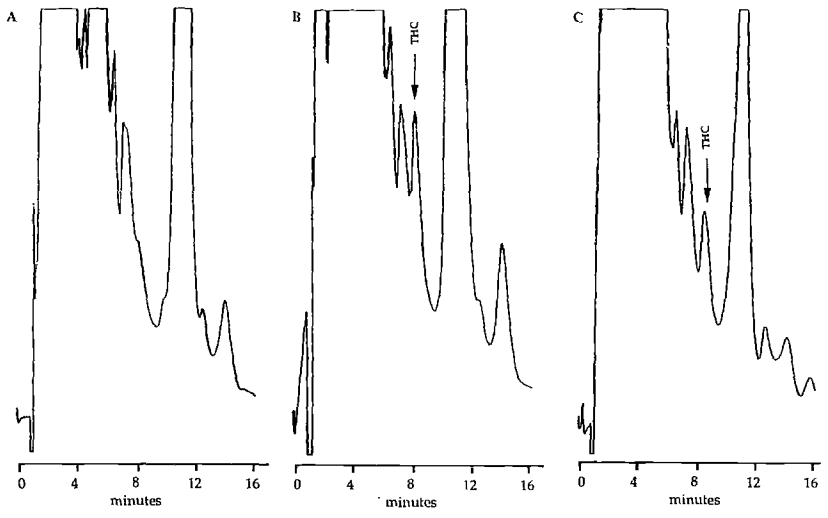

FIG. 1—*(A) Chromatogram of extracted blank blood using HPLC conditions designed to detect THC, (B) chromatogram of extracted standard of THC in blood (15 ng/mL) and (C) chromatogram of positive case (10.4 ng/mL, THC).*

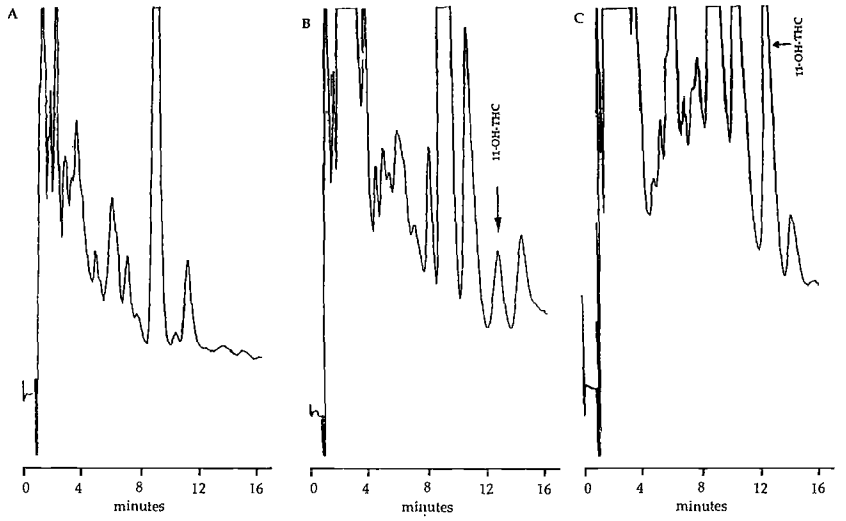

FIG. 2—*(A) Chromatogram of extracted blank blood using HPLC conditions designed to detect 11-OH-THC, (B) chromatogram of extracted standard of 11-OH-THC in blood (14 ng/mL) and (C) chromatogram of positive case (61 ng/mL, 11-OH-THC).*

(19%). Ages of drivers ranged from 15 to 83 years. The number of drivers who initially tested positive to cannabinoids was 21 (18 males, 3 females).

Confirmatory analysis by HPLC/ECD using the method described, detected the presence of THC in 8 of these 21 cases. In 10 subjects 11-OH-THC was detected and in six cases both psychoactive cannabinoids were detected. In total, 13 of these initial EMIT® positives were confirmed positive for psychoactive cannabinoids (Table 2). Age of positive subjects ranged from 18 to 66 years (11 males, 2 females). The overall mean concentration in blood for THC and 11-OH-THC were 5.4 ng/mL and 18 ng/mL, respectively. Five cases also had significant amounts of alcohol. The alcohol concentrations of these five drivers ranged from 0.10 g/100 mL to 0.31 g/100 mL. The mean concentration in blood

TABLE 2—*Concentrations of THC and 11-OH-THC in cases confirmed positive for cannabinoids.*

Case no.	Sex/Age	Accident type	[THC] ng/mL	[11-OH-THC] ng/mL	Alcohol g/100 mL	Other drugs
1	M 30	S	1.4	2.5	0.31	ND
2	M 29	S	4.9	2.9	ND	ND
3	M 60	M	1.7	3.9	ND	Benzodiazepines
4	M 31	S	5.6	2.9	0.29	ND
5	M 35	M	ND	61	ND	ND
6	M 41	S	10.4	34	0.22	ND
7	F 18	M	5.4	ND	ND	ND
8	M 28	M	8.1	ND	0.18	Amphetamines
9	M 25	M	ND	3.3	trace	ND
10	M 47	M	ND	20.0	ND	ND
11	M 61	S	ND	5.5	ND	ND
12	F 20	S	20.0	85.0	0.10	ND
13	M 18	M	ND	3.5	ND	ND

ND = not detected, S = single vehicle accident, M = multi-vehicle accident.

for THC and 11-OH-THC in these cases also involving alcohol was 9 ng/mL and 26 ng/mL, respectively, compared to 1.5 ng/mL and 13 ng/mL in cases not involving alcohol. Two drivers confirmed positive for psychoactive cannabinoids also tested positive to other drugs; one being positive to amphetamines and the other to benzodiazepines. Six drivers were involved in single vehicle accidents while the other seven drivers were involved in multi-vehicle accidents, (Table 2).

Discussion

A number of extraction techniques were attempted throughout the course of this study to optimize the extraction and detection of low concentrations of THC and 11-OH-THC in postmortem blood. Initially solid phase techniques such as those employed by Parry [*16*] and Johansson [*17*] were used to adsorb the target compounds onto a silica gel bonded column and to remove endogenous components from blood as much as possible with a minimum loss of THC and 11-OH-THC. This procedure was found to be inadequate with poor reproducibility yielding low extraction recoveries. However, high recovery rates for THC-COOH in urine have been possible using this procedure [*15, 16, 18*].

The recovery of THC and 11-OH-THC using the method described was consistently around 40%, which is similar to that described by Foltz et al. [*11*], on which the extraction conditions described in this paper are based. The separation of THC and 11-OH-THC was achieved using a Spherisorb C8 column, which was found to have superior retentive properties to C18 and phenyl bonded columns. However the use of one mobile phase was not sufficient to separate both cannabinoids due to long run times (~ 40 min), which resulted in the THC peak being broad and eluting too late for any reliable measurements. The percentage volume of acetonitrile and methanol were therefore increased to counter this effect. This resulted in the 11-OH-THC peak eluting far too early and being masked by other endogenous peaks in blood. Consequently two different mobile phases were used to optimize separation and quantification.

The first detector was set at a value which is a few millivolts less than the potential required to begin oxidation of the analyte (+0.40 V). The use of this potential resulted in electrolysis of all substances contained in the extract which have formal potentials less than the analyte. The second detector (+0.68 V) was then used to detect the analyte at its appropriate potential removing some of the interferences in the extract.

The mean concentrations of THC and 11-OH-THC in blood of 13 drivers was found to be 5.4 ng/mL and 18 ng/mL, respectively. The high concentration of THC found in some cases is probably a reflection of recent use of marijuana as studies have shown that levels of this nature are often observed within a brief period after smoking [*19*] or, alternatively, following an ingestion (or inhalation) of a large amount of cannabis. Plasma levels of THC have been shown to fall rapidly within an hour of smoking to about 10% of peak concentration and after 3 to 4 h are typically less than 1 ng/mL [*20*]. The metabolite THC-COOH reportedly appears in blood in higher concentrations for a number of hours after smoking marijuana but well after the psychoactive effects of marijuana have worn off [*21*].

The principal limitation with the EMIT® screen is primarily that the assay detects the major urinary metabolite THC-COOH. Therefore the presence of the acid metabolite may not necessarily indicate recent use of marijuana. This was reflected by 13 of the 21 subjects being confirmed for THC and/or 11-OH-THC using HPLC. The other eight subjects had either used cannabis quite some time before the accident or had given a false immunoreactivity.

The overall incidence of cannabis determined in this study was 7%, which is similar to other studies in Australia that have quoted incidences of 6% [*3*], and 20% [*5*]. Five drivers studied who showed psychoactive marijuana had blood alcohol concentrations

(BAC) well in excess of the legal limit in Victoria (0.05%). While alcohol and THC have shown to be synergistic [*3, 22*], the high concentration of alcohol consequently could have contributed to these accidents.

A number of drivers (n = 4) confirmed positive for psychoactive cannabinoids were also involved in accidents in which other contributing factors were operating. Four drivers (driver nos 3, 5, 7, and 11) were involved in accidents where road conditions were poor (such as, wet roads, bad visibility) and two drivers also tested positive to other psychoactive drugs (amphetamines and benzodiazepines). The remaining four drivers confirmed positive for cannabis (driver nos 2, 9, 10, 13) had accidents for which there were no plausible explanations or mitigating circumstances. The concentrations of THC and 11-OH-THC in these four cases were within the range at which pharmacological effects may be expected to occur [*7, 23*].

Surprisingly in cases 9 and 10 and to a lesser extent in case 13, THC was not detected after the subject had been confirmed for 11-OH-THC (20 ng/mL and 3.5 ng/mL, respectively). The explanation for this is that the THC in the blood may have not been detected due to the detection limit of 1 ng/mL and a longer time interval from smoking to the accident allowing the 11-OH-THC to be the dominant psychoactive form in blood. It is also recognized that higher concentrations of 11-OH-THC than THC occur after oral administration but also that the ratio of THC to 11-OH-THC varies considerably following oral use [*24*].

In conclusion, a sensitive and specific HPLC assay coupled with electrochemical detection is described, suitable for the measurement of the two psychoactive components, THC and 11-OH-THC in postmortem blood. Our results confirm the presence of marijuana in up to 7% of fatal motor vehicle accidents in Victoria.

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Address requests for reprints or additional information to
Olaf H. Drummer, Ph.D.
Victorian Institute of Forensic Pathology and
Department of Forensic Medicine
Monash University
57-83 Kavanagh St.
South Melbourne, 3205
Victoria, Australia