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Solid-phase extraction of 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid from human urine with gas chromatographic–mass spectrometric confirmation

VANDANA DIXIT and VYAS M. DIXIT*

Varian Sample Preparation Products, 24201 Frampton Avenue, Harbor City, CA 90710 (U.S.A.)

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ABSTRACT

A selective and sensitive technique has been developed for detecting and identifying 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in human urine. Using a new, "mixed-mode", bonded silica gel, solid-phase extraction column cartridge, THC-COOH was selectively isolated from urine components. Following extraction, the presence of THC-COOH was confirmed and quantitated using gas chromatography–mass spectrometry (GC–MS) or gas chromatography–flame ionization detection. A linear quantitative response curve for THC-COOH was generated over a concentration range of 10 to 300 ng/ml. Overall extraction efficiency averaged >85% and the quantitative response curve exhibited a correlation coefficient of 0.999. The limit of detection and identification using GC–MS for the drug metabolite was found to be six times below the present NIDA guidelines cut-off concentration of 15 ng/ml.

INTRODUCTION

Marijuana and related cannabis products are used by a significant portion of our society. The major psychoactive component in marijuana is tetrahydrocannabinol which is highly lipophilic. In human tetrahydrocannabinol is accumulated in tissues rich in lipids and then slowly released from these tissues in such a way that it is barely detectable in urine. The major metabolite of tetrahydrocannabinol is 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid, which is excreted in urine mainly as a glucuronic acid conjugate. [1,2].

Radioimmunoassay [3–5], gas chromatography [6], high-performance liquid chromatography [7,8], thin-layer chromatography [9–11], and gas chromatography–mass spectrometry (GC–MS) [12–14] procedures have been developed for monitoring urine for the drug metabolite. Due to the inherent unspecificity of most of these techniques, caused by cross-reactions with endogenous or exogenous components, positive results should be confirmed by GC–MS. Current methods of sample preparation, including liquid–liquid extraction [15,16] and other solid–phase extraction (SPE) techniques [7–9,12], yield low recovery, provide incomplete removal of interfering urine components, or require long preparation times.

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This article reports a sensitive, specific, qualitative, and quantitative extraction procedure for 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) from urine using Bond Elut Certify II™, a new bonded silica gel SPE column. Bond Elut Certify II is a chemically modified silica gel material exhibiting three different types of interactions: hydrophobic, polar, and ion exchange. Due to the mixed-mode properties and selective nature of Bond Elut Certify II, the THC-COOH extracts are extremely clean. This cleanliness is due to the fact that many of the interferences are removed during the column rinse process, while some of the pigments present in the urine are irreversibly retained on the Bond Elut Certify II extraction column and THC-COOH is selectively eluted.

EXPERIMENTAL

Materials

Bond Elut Certify II extraction columns and a Vac Elut® vacuum manifold (AI 6000) were provided by Varian Sample Preparation Products (Harbor City, CA, U.S.A.). A vortex mixer was obtained from Scientific Industries (Bohemia, NY, U.S.A.). A Reacti Therm™ heating module and a Reacti Vap™ evaporator were purchased from Pierce (Rockford, IL, U.S.A.).

Equipment

Gas chromatography–flame ionization detection (GC–FID) was performed on a Varian Model 3500 instrument (Walnut Creek, CA, U.S.A.). The GC system was equipped with a split–splitless injector and a 30 m × 0.25 mm I.D., 0.25 μm film thickness, DB-5 capillary column. The oven temperature was programmed at 180°C, increased at a rate of 20°C/min and held at 280°C. The injector and detector temperatures were set at 300°C. The Finnigan MAT ITS 40 GC–MS–data system (San Jose, CA, U.S.A.) used for data analysis was equipped with a Varian Model 3400 gas chromatograph, an ion trap quadrupole analyzer, a COMPAQ DESK PRO386/20e Model 40 personal computer (PC), and a Finnigan A200S autosampler. All temperature programming was performed through the personal computer which was equipped with complete data processing and library search programs.

Reagents

THC-COOH was purchased from Alltech Applied Science (Deerfield, IL, U.S.A.) and 11-nor-9-carboxy-Δ-8-THC-D_6 (internal standard, I.S.) was obtained from ElSohly Labs. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce. HPLC-grade methanol, hexane, and ethyl acetate were purchased from E.M. Science (Cherry Hill, NJ, U.S.A.). Certified negative human urine was obtained from Fisher Scientific (Tustin, CA, U.S.A.) and certain volumes of urine were spiked with known amounts of THC-COOH. All other chemicals were purchased from Fisher Scientific.

Specimen preparation

Spiked urine (3 ml), an I.S., and 300 μl of 10 *M* potassium hydroxide solution were added to a large test tube. The mixture was vortexed and hydrolyzed for 15 min at 60°C. The sample was cooled, and 165 μl of glacial acetic acid and 2 ml of a mixture of 100 m*M* sodium acetate buffer (pH 7.0)–methanol (95:5) were added. The specimen was adjusted to pH 4.5–6.5 with glacial acetic acid and vortex-mixed.

Extraction

Bond Elut Certify II tubes were connected to a Vac Elut and conditioned with 2 ml of methanol. Excess methanol was removed by washing with 2 ml of 100 m*M* sodium acetate buffer (pH 7.0)–methanol (95:5). The hydrolyzed urine specimen containing THC-COOH was added to each column and passed through the bed at a low flow-rate by applying vacuum at approximately 51–76 mmHg. The column was washed with 10 ml of 50% aqueous methanol and the sorbent was dried for 10 min under full vacuum (380 mmHg). The column was further washed with 2 ml of ethyl acetate and dried under full vacuum for 30 s. All washes were discarded.

The tips of the Vac Elut delivery needles were wiped and a rack with labeled collection tubes was placed in the Vac Elut. The drug metabolite was eluted with 2 ml of hexane–ethyl acetate (75:25) containing 1% acetic acid. The Vac Elut was disassembled and the labeled test tubes were removed and placed in the Reacti Therm evaporator. The solvent was evaporated to dryness under a slow stream of nitrogen at room temperature. Toluene (500 μl) was added and evaporated under a slow stream of nitrogen at room temperature. A second evaporation with an additional 500 μl of toluene was performed again to remove residual acetic acid and water. The samples were derivatized prior to analysis.

Derivatization

Method 1. The trimethylsilyl (TMS) derivative of THC-COOH was prepared in order to increase the volatility for GC–FID analysis. The evaporated sample was reconstituted in 50 μl of BSTFA [with 1% trimethylchlorosilane (TMCS)]. The vial was capped and heated for 15 min at 90°C. The vial was cooled, and 1 μl of the sample was injected into the GC–FID system.

Method 2. The methyl derivative of THC-COOH was prepared for GC–MS analysis. A 70-μl volume of the alkylating reagent (prepared from 50 μl of 25% methanolic tetramethylammonium hydroxide in 1000 μl of dimethylsulfoxide) was added to a dried extracted sample and vortex-mixed for 30 s. The mixture was left at room temperature for 2 min. Methyl iodide (5 μl) was added and vortex-mixed. The mixture was again left at room temperature for 5 min. A 200-μl volume of 0.1 *M* hydrochloric acid and 1 ml of isooctane were added to this mixture and vortex-mixed for 3 min. The upper organic layer was placed in a test tube, dried under a stream of nitrogen at 50°C, and reconstituted in 20 μl of isooctane for further analysis.

Analytical recovery and precision

An aliqout of 15, 150, 300, or 450 μl of a methanolic solution of the THC-COOH (2 μg/ml) was spiked into a 3-ml blank urine sample to provide a concentration of 10, 100, 200, or 300 ng/ml. Multiple samples (n = 4) at these concentrations were used to evaluate the recovery of the analyte. The drug was extracted through Certify II columns and the peak area of the extracted drug was divided by the peak area for the same concentration of unextracted standard to provide absolute recovery. Average recovery, standard deviation and coefficient of variation (C.V.) of the drug were calculated at each concentration to show the precision of the method.

Standard curve preparation

The linear curve was generated by plotting the peak area of the analyte against analyte concentration and the best-fit line was generated ($y = mx + b$) to show the linearity of the graph.

Study with real life samples

Validation of Certify II columns and extraction procedure was performed by an independent laboratory[a] using marijuana-positive urine specimens. The I.S. used for this study was 11-nor-9-carboxy-Δ-9-THC-D_3. The dried extracts were derivatized by following the TMS procedure (method 1) and the samples were analyzed by a Hewlett Packard GC–MS instrument.

The amount of the drug present in the sample was calculated by determining the following ratio for a known standard as well as unknown sample:

$$\text{ratio} = \frac{\text{molecular abundance response for } m/z \text{ 371}}{\text{molecular abundance response for } m/z \text{ 491}}$$

Ion 371 represented the loss of a $COOSi(CH_3)_3$ group from the parent ion of the drug and m/z 491 was the molecular ion for the I.S. Next, factor F was calculated by utilizing the following formula:

$$\text{factor } F = \frac{\text{THC-COOH concentration (ng/ml) for standard}}{\text{371/491 ratio for standard}}$$

Finally the concentration of THC-COOH present in positive specimen was determined by multiplying factor F with the ratio for ions 371/491 obtained from the sample.

RESULTS AND DISCUSSION

The SPE procedure described here provides high recovery and clean extracts of THC-COOH from human urine. The bonded phase selectively retains and

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[a] E. Chaffin, Doctors Clinical Lab., 4531 Reading Road, Cincinnati, OH, U.S.A.

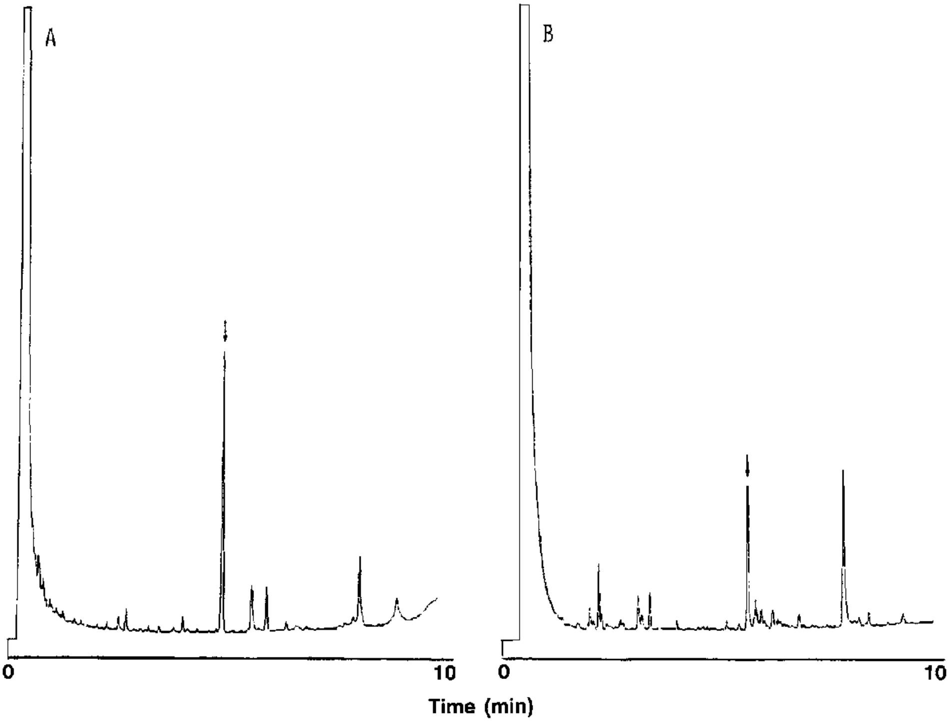

Fig. 1. (A) GC FID trace of the TMS derivative of THC-COOH at 300 ng/ml. (B) GC-FID trace of the methyl derivative of THC-COOH at 300 ng/ml.

elutes the drug metabolite by utilizing its multiple interaction properties. The column rinse with 50% aqueous methanol followed by ethyl acetate removes polar and non–polar organic interferences. Due to the relatively non-polar nature of the elution solvent, the THC metabolite is selectively eluted while leaving other ionic impurities on the column. The use of silanized glassware is not necessary throughout the extraction procedure [17]. Chromatograms obtained from blank urine samples are clean and no interfering peaks are found at the elution times of the I.S.[a] and THC-COOH.

Fig 1 shows the GC–FID traces of TMS and methyl derivatives at 300 ng/ml. The recoveries and precision data for THC-COOH are listed in Table I. These are overall absolute recoveries calculated from spiked urine samples at four concentration levels (10, 100, 200, and 300 ng/ml). The average absolute recovery was found to be greater than 85% over the four concentrations with a standard deviation of 8.0. The relative standard deviation (C.V.) was calculated to be

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[a] 11-Nor-9-carboxy-Δ-8-THC-D_6 is a commercially available I.S. developed and sold by ElSohly Laboratories (Oxford, MI, U.S.A.).

TABLE I

GC–FID DETERMINATION OF ABSOLUTE RECOVERY OF THE TMS DERIVATIVE OF THC-COOH FROM URINE

Numbers given represent the mean values for the drug quadruplicate at each concentration.

Concentration (ng/ml)	Absolute recovery (mean ± S.D., n = 4) (%)
10	89 ± 8.7
100	86 ± 7.8
200	87 ± 8.1
300	93 ± 4.8

7.2%. Lot-to-lot reproducibility data (Table II) also produced good results for four different lots of Bond Elut Certify II giving an average recovery of 93% with a standard deviation of 4.8. The C.V. was calculated to be 5.1%.

A standard curve was generated from the analysis of spiked urine samples. The plot of the quantitative reponse for THC-COOH *versus* the concentration over a range of 10 to 300 ng/ml was found to be linear ($y = 1056.25x + 14533.84$, $r^2 = 0.999$, $n = 4$).

The National Institute on Drug Abuse (NIDA) requires a quantitative GC–MS confirmation of THC-COOH at 15 ng/ml. A 5-ml urine sample volume provides the detection of THC-COOH at 2.5 ng/ml, six times below NIDA cut-off concentrations. Fig. 2 shows the total ion chromatogram of THC-COOH extracted at a concentration of 2.5 ng/ml and derivatized as methyl ester. The total spectrum (Fig. 3) shows the presence of the molecular ion peak (M^+, 372) and the base peak at 313 for M–$COOCH_3$. The single ion current profiles obtained at m/z 372 and 313 (base peak) for the metabolite are illustrated in Fig. 4. The ratio of these ions stays constant over a varying range of concentrations. A mass spectral comparison of this spectrum to the spectrum from the National Institute of Stan-

TABLE II

LOT-TO-LOT REPRODUCIBILITY DATA FOR THE GC–FID DETERMINATION OF ABSOLUTE RECOVERY OF THE TMS DERIVATIVE OF THC-COOH AT 300 ng/ml FROM URINE

Recovery (%)		C.V. (%)
Mean	S.D.	
87	7.5	8.6
92	7.6	8.3
97	9.0	9.3
97	4.6	4.7

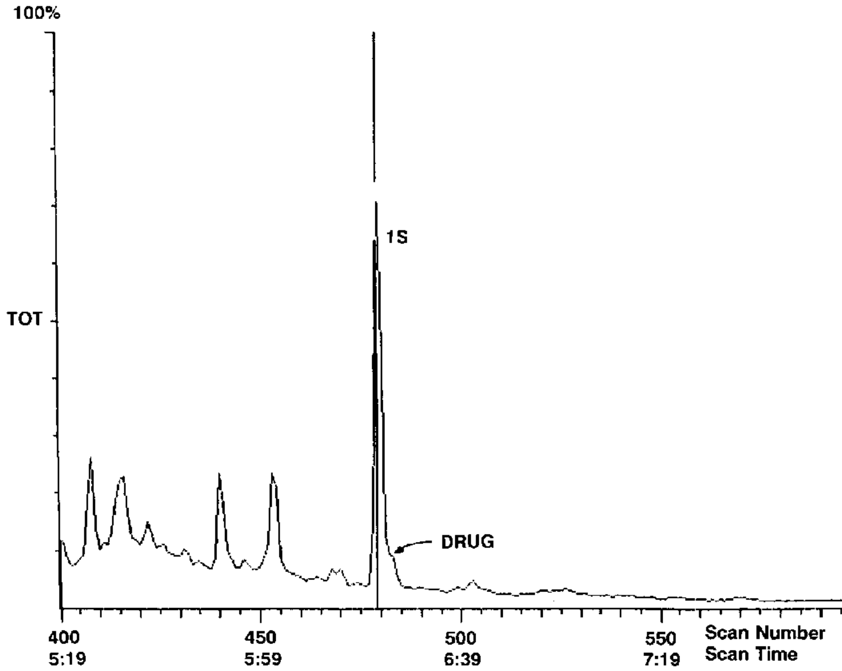

Fig. 2. Total ion chromatogram of the methyl derivatives of THC-COOH (2.5 ng/ml) and internal standard (50 ng/ml).

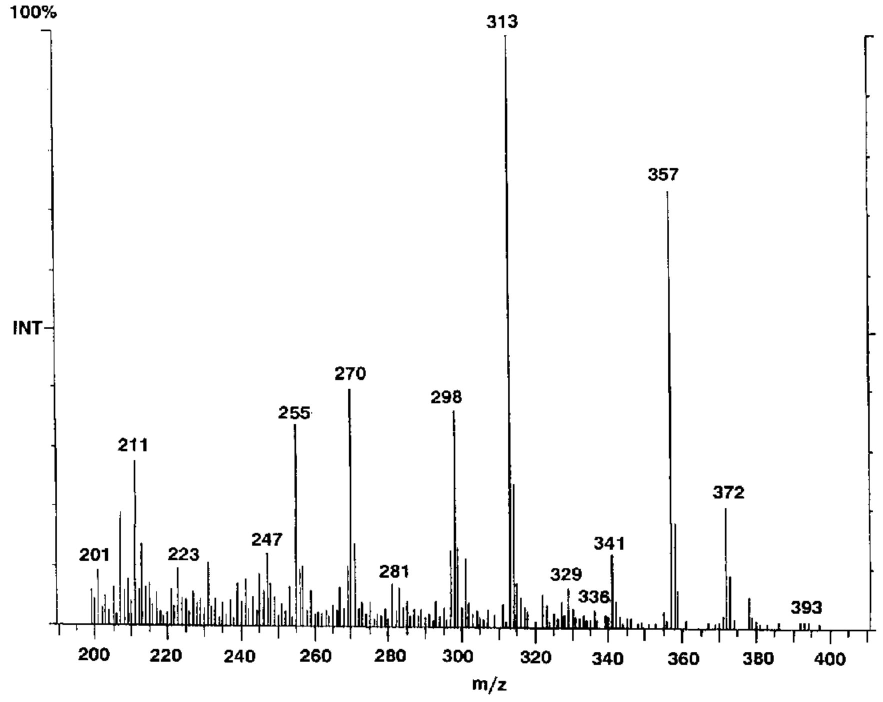

Fig. 3. Full scan mass spectrum of the methyl derivative of THC-COOH at 2.5 ng/ml.

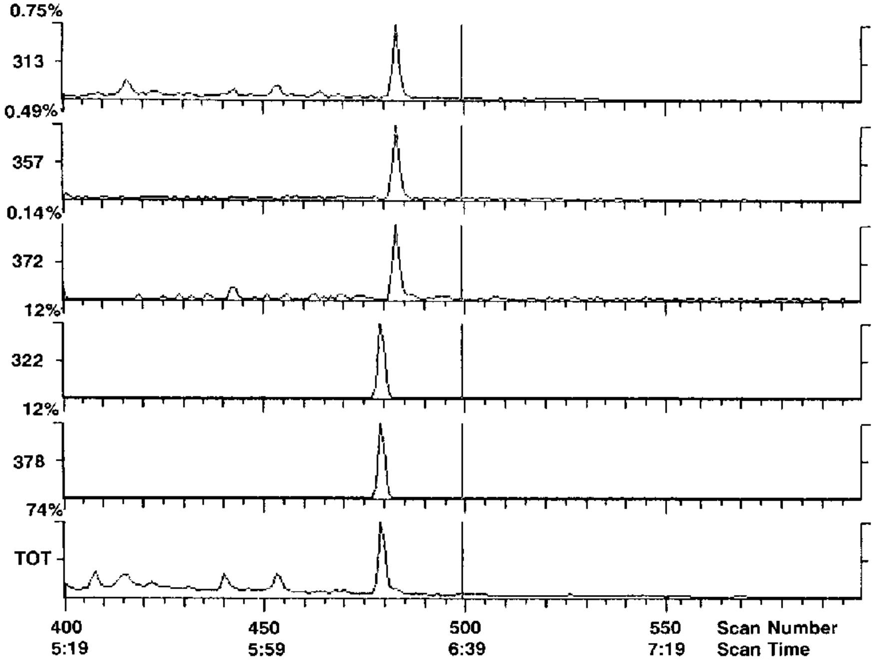

Fig. 4. The bottom trace shows the total ion chromatogram of the methyl derivative of THC-COOH. The next two traces show single ion chromatograms of the molecular ion and the base peak from the D_6 internal standard and the top three chromatograms show the single ion traces of (from bottom to top) the molecular, $[M-CH_3]^+$ and $[M-COOCH_3]^+$ ions from the analyte.

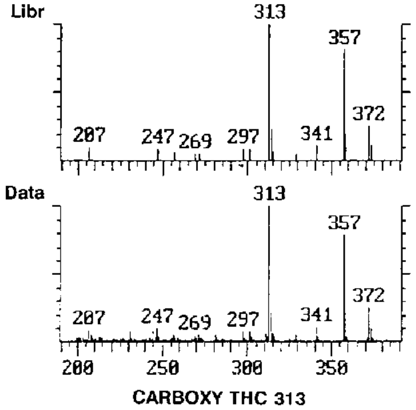

Fig. 5. Library search for the methyl derivative of THC-COOH.

TABLE III

CALCULATED CONCENTRATIONS OF THC-COOH PRESENT IN POSITIVE URINE SPECIMENS

Numbers given represent the amount of THC-COOH found in real life samples.

Sample No.	Concentration (ng/ml)	Sample No.	Concentration (ng/ml)
1	4	11	79
2	21	12	90
3	21	13	90
4	33	14	123
5	35	15	139
6	38	16	202
7	41	17	226
8	42		
9	44		
10	48		

dards and Technology's (NIST) library is shown in Fig. 5. The library search program was used to search for "purity", "best fit" and "reverse fit" of injected compounds and compared them to the stored reference data. An Incos algorithm of 1000 will be a perfect match. However, an algorithm fit of 800–1000 has become an accepted range. An excellent spectral fit of 999 was obtained from data comparison.

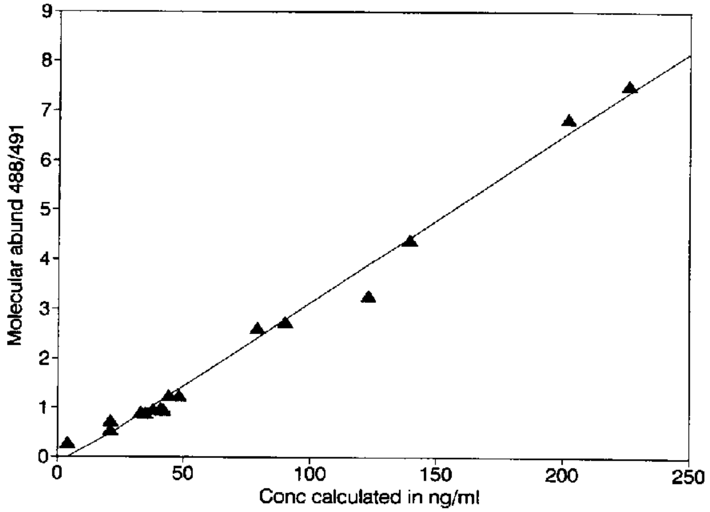

Fig. 6. Standard curve for positive urine samples.

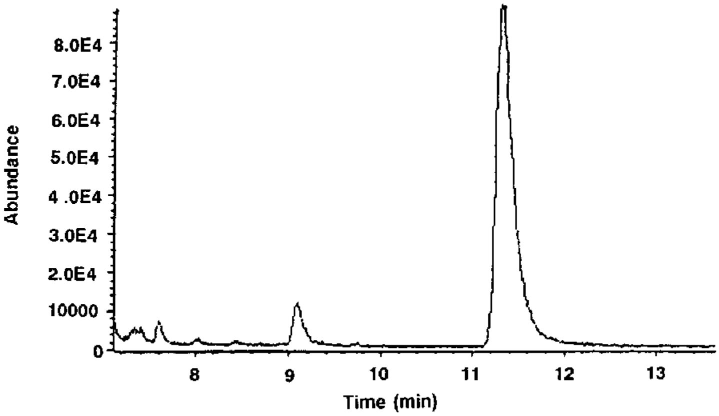

Fig. 7. Total ion chromatogram of the silyl derivative of THC-COOH from the positive urine sample at a calculated concentration of 23 ng/ml.

Verification of extraction procedure using positive urine sample

The exact concentrations of THC-COOH obtained from seventeen positive urine samples are listed in Table III. Fig. 6 shows the standard curve generated from the analysis of these urine samples over the calculated concentration range 4–226 ng/ml. The linear correlation (r^2 = 0.988) was obtained by plotting the ratio of molecular abundance for ion 488 (M^+ for the analyte) divided by molecular abundance for the I.S. (M^+, 491) against the calculated concentrations.

The total ion chromatogram (Fig. 7) obtained from the extraction of a 3-ml positive urine sample containing approximately 23 ng/ml THC-COOH clearly demonstrates the cleanliness of the procedure.

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