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Confirmatory method for detection 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in urine samples using gas chromatography-mass spectrometry (GC/MS)

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ABSTRACT

Gas chromatography mass spectrometry (GC/MS) analysis of 11-Nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), the major metabolite of tetrahydrocannabinol in biological samples is reported, the THC metabolite obtained by alkaline hydrolysis from urine samples were extracted using mixture of solvents followed by trimethylsilylation. The dervatized extract was submitted to GC/MS analysis of EI-SIM mode. Different factors were studied as pH, temperature, time to establish the best conditions for the determination. The calibration curves of THC-COOH derivatized (THC-COOH-TMS) in urine samples were linear in the concentration range from 10 to 150μg/ml. the proposed method is able to detect the major metabolite of cannabis derivatives at very low level (10μg/ml) with high specificity so these analytical procedure can be used as confirmatory method in drug testing of cannabis use.

KEYWORDS

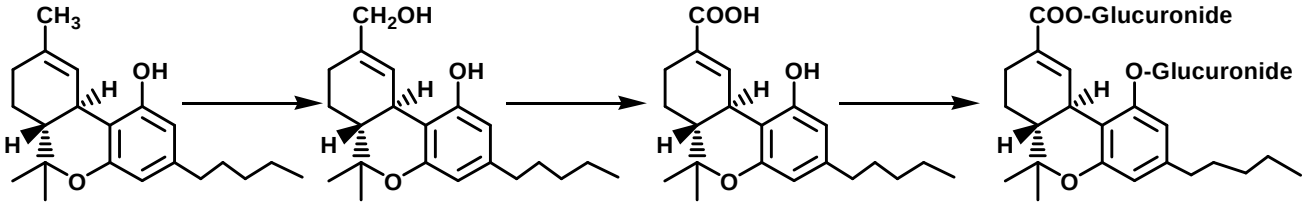
Cannabinoides;
GC/MS analysis;
THC-COOH;
Tetrahydrocann-abinol;
Biological samples.

INTRODUCTION

Considering the increasing abuse of marihuana or hashish in the last years, the detection of a long-term exposure by urine analysis.

As rule, the cannabis constituents are Tetrahydrocannabinol (THC), Cannabinol, Cannabidiol, that can be isolated from cannabis plants[1-3] and are also present in cannabis smoke, only the first demonstrate narcotic action.

This action is dose dependents, driving performance is seriously compremised because the perception of



Δ9-THC (THC) | 11-Hydroxy-Δ9-THC | 11-Nor-D9-THC-9-carboxylic acid (9-carboxy-THC) | 9-Carboxy-THC mono-/Diglucuronide

Figure 1 : Metabolic pathway of THC

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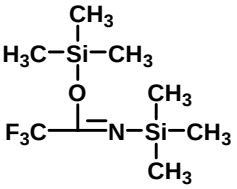

Figure 2 : Structure of N,O-bistrimethylsilytrifluroacetamide (BSTFA)

time and space is disturbed if inhaled, THC is absorbed faster and in larger amount than when taken orally and owing to its strong lipophilic nature, it is rapidly spread throughout the system and less than 1 % of unchanged THC is recovered in the urin. When smoked, intial metabolism occurs in the lung, whereas this takes place in the liver when hashish is taken orally. Seventy-two hours after smoking, approximately 50 % of the inhaled THC is excreted as metabolites and the remaining 50% distributed throughout the body. Where it is mostly absorbed by the fatty tissue and excreted slowly over the next few days. Execration is mainly via urine 25 % and faces 65 % Although over 20 metabolite is 11-Nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) which is converted to di-glucuronide conjugates and these the major forms of the metabolites excreted in urine[4]. (The metabolic pathway of THC is summarized in figure 1.

Various method for analysis of cannabinoids have been developed using radioimmunoassay (RIA)[5-7], fluorescencepolarisationimmunoassay(FPIA)[7], using According to the recent EU recommendation on drugs of abuse testing and the Mandatory Guidelines for Federal Verkplace Drug Testing Programs (USA) samples should be screened by validated Immunoassays and specific substance should be confirmed by chromatographic method using mass spectrometric detection.

Immunoassay screening can lead to false positive results because of antibody cross-reactivity with molecules of similar structure hence the need for a confirmation method[8]. Immunoassays for THC metabolites are usually calibrated to give positive results for samples concentration (50µg/ml(cut-off concentration, so the conformation method must be more sensitive.

GC-MS is an excellent method for determination of metabolite of hashish in urine. The paper describes a method to confirm and determine urinary THC-COOH, this method include a hydrolysis of conjugates by

TABLE 1: Recovery and precision for THC-COOH-TMS at three target concentrations

	Intra day (n =5)		Inter-day (n =15)	
THC-COOH-TMS (µg/ml)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
10.00	94	0.12	94	0.17
20.00	98	0.07	93	0.22
30.00	97	0.14	96	0.10

NaOH, extraction and derivatization of THC-COOH.

The hydrolysis is necessary to free the metabolite and this can be performed either by alkaline NaOH[9-18] or enzymatic hydrolysis, alkaline hydrolysis is considered more efficient and reproducible than acid or enzymatic hydrolysis[19].

The extraction procedure should be efficient and selective. A good recovery is important, since the total amount of cannabinoids present is very low. Due to the low sensitivity of the mass detector to THC-COOH in the injection port due to temperature, derivatization are used to reduce the thermal decomposition to the mass spectrum and also used to stabilize ions formed in the mass spectrometer to favor structurally informative fragmentation mode[20], so the Derivatization of THC-COOH was needed in this method N,O-bistrimethylsilytriflutoacetamide (BSTFA). Figure 2 BSTFA is used as derivatizing agent[4].

EXPERIMENTAL

Chemicals

All chemicals used through the work were of analytical reagents grade. The reagent included 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) (1gm/mL) was obtained from Sigma-aldrich gmbh steinheim, germany. Stock solutions of THC-COOH were prepared in methanol (100 ug/ml) and stored at 5°C, thesilylation reagent BSTFA obtained from Sigma-aldrich gmbh steinheim, germany, Ethyl acetate (BDH), n-Hexane, potassium hydroxide (10N), (BDH), HCl (2N), (BDH).

Urine samples

Some precautions have been considered in urine samples, the sample was collected in duplicate in two 30 mL plastic bottles. Each plastic bottle was filled at least 2/3 full. Immediately after collection, the tempera-

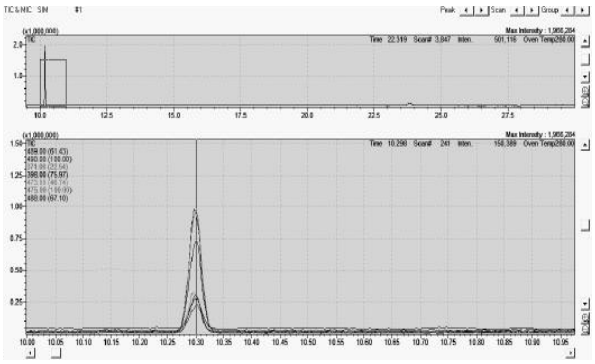

Figure 3 : The ion selected for determination of THC-COOH-TMS

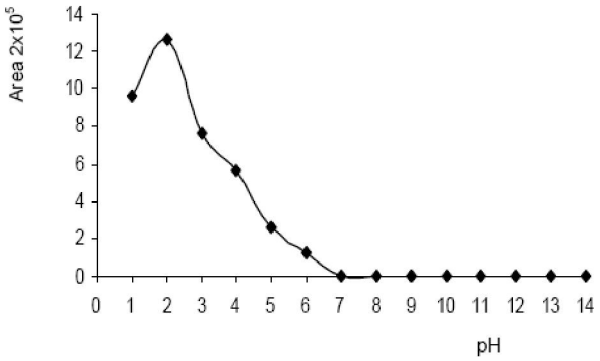

Figure 4 : Effect of pH on extraction of THC-COOH

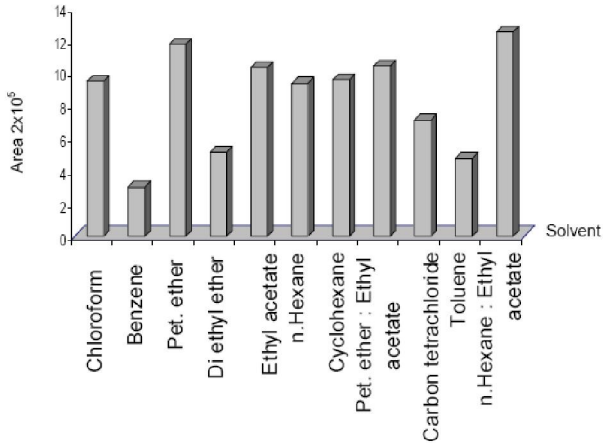

Figure 5 : Effect of solvent on extraction of THC-COOH

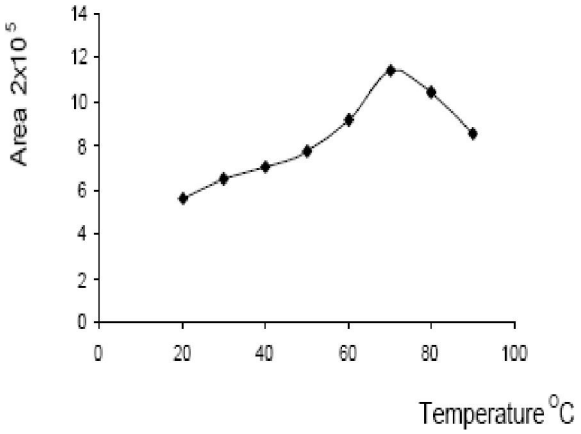

Figure 6 : Effect of Temperature on formation of THC-COOH-TMS

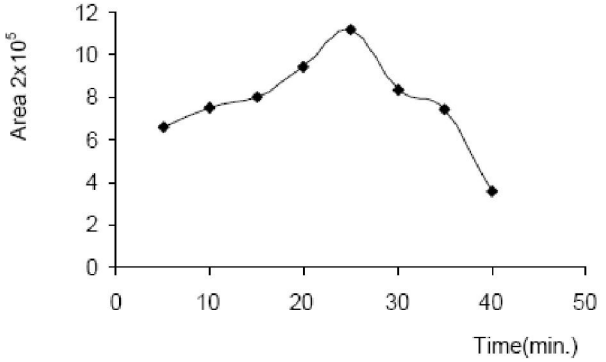

Figure 7 : Effect of Time on formation of THC-COOH-TMS

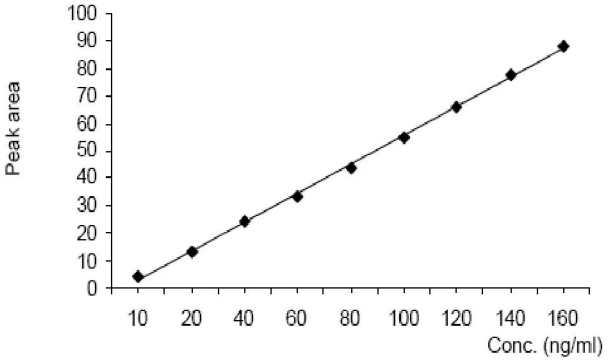

Figure 8 : Standard curve of THC-COOH-TMS

ture (32°C 38°C) within 4 min., pH, specific gravity and creatinine value of the fresh samples were recorded. Urine samples were stored at -5°C until the analysis.

Instrumentation

The analysis of extracted THC-COOH derivatized, the system performed with a Shimadzo, GC-2010 Gas Chromatograph (Shimadzo Technologies, Japan) and mass detector GCMS-QP2010 (Shimadzo Technologies, Japan), connected to a desktop computer with GCMS solution (Version 2.21.00) software. The column employed RTX-1MS (30-m×0.25-mm i.d., 0.25μm film thickness of 95% dimethyl–5% diphenyl polysiloxane copolymer column (Restek, Bellefonte,

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PA, USA). The carrier gas consisted of ultra-pure grade helium (Air Products, Parkersburg, WV, USA) at a flow rate of 1.5 ml/min. The injector temperature was held at 250°C and the detector at 300°C. For THC-TMS detection the oven started with 120°C to 2 min. and was ramped from 120 to 200°C at 50°C min and from 200 to 280°C at 15°C/min. to give a run time 29.93 min., Analysis was accomplished by selected ion monitoring of ions (high resolution mode) between 9 and 30 min. at m/z = 371, 398, 473, 475, 488, 489, 490 and EMV at 1400 as show in figure 3.

Sample preparation

Hydrolysis

Urine (10) ml was mixed with 2 ml of 10 N KOH, the sample was hydrolysed at 50°C in a heating unit for 20 min. with occasional stirring. After cooling to room temperature, 4 ml of 5N HCL were added and added few drops of HCL until adjust pH 2[21,22].

Effect of pH

The effect of pH is studied to reach to optimum pH for the extraction metabolite THC-COOH from urine sample, so the effect of pH was study with used spiked urine with 30µg/ml as show in figure 4.

Extraction

Liquid-liquid extraction with 80 ml mixture of solvent and was shaking for two minutes and allowed to separate into two phases. The organic layer was collected and evaporated to dryness with stream of air[4]. Added small amount of the same solvent of extraction and transfer into small vial, evaporate to dryness with stream of air at room temperature.

Effect of Solvent on extraction of THC-COOH

The polarity of solvent affects on extraction efficiency. Several water immiscible organic solvents chloroform, benzene, petroleum ether, diethyl ether, ethyl acetate, n-hexane, cyclohexane, pet. Ether : ethyl acetate (7:1), carbon tetrachloride, toluene, and n-hexane : ethyl acetate (7:1). The effect of extracting solvents on extraction efficiency are represented graphically in figure 5.

Derivatization

To the vial containing urine extract after complete evaporation added 30µl of ethyl acetate and 30µl BSTFA are added the vial which is vortexed and heated at 70°C for 25 min.

Effect of temperature derivatization

The proper choice of the Derivatization agent and of its optimal amount appeared to be decisive for the sensitivity of the method. To establish the thermal stability of the THC-COOH-TMS, the effect of temperature of Derivatization on the Formation of the THC-COOH-TMS was studied by measuring Conc. of the THC-TMS formed at increased temperature intervals between 20 and 90 ^{O}C at constant time as show in figure 6.

Effect of time of derivatization

To establish the thermal stability of the THC-COOH-TMS, The effect of time of Derivatization on the Formation of the THC-COOH-TMS was studied by measuring Conc. of the THC-COOH-TMS formed at increased time intervals between 5 and 40 min. at constant temp. 70°C as shown in figure 7.

Method validation

The linearity of the method was verified using of human urine samples spiked at nine levels (10, 20, 40, 60, 80, 100, 120, 140, and 160µg/ml) spiked The urine samples were spiked with THC-COOH from a stock solution 100µg/ml), linear regression line was obtained by plotting the peak area versus the THC-COOH-TMS concentration as show in figure 8. Coefficients of variation (CV) for intra-day and inter-day precisions were calculated at three concentrations. The limit of detection was determined by estimating the minimum concentration equivalent to, or grater than, three times of the background noise. The Limit of quantification was defined as fives times the background noise.

Precision and accuracy

The intra-day precision was evaluated by replicate analysis (n=5) containing the following concentration of 10, 20 and 30µg/ml. For inter-day precision, the samples were analyzed in triplicate on 5 different days over a 3-weekperiod (n = 15) and Coefficients of variation (CV) was calculated as shown in TABLE 1. Accuracy was established by comparing the peak area ratios for amounts of THC-COOH-TMS and the peak

area ratios for the same analyte in the Standard preparation. It is expressed as a recovery percentage (Recovery %). Values were processed according SFSTP recommendations[23].

RESULT AND DISCUSSION

Several series of experiments were carried out for optimization of the different parameters of the procedure described in Section 2.4. The optimum pH of extraction of THC-COOH from urine samples is pH = 2 as shown in figure 4, (n-hexane/ethyl acetate(7:1 v/v is the best solvent to give good yield as shown in figure 5, the optimum temperature 70°C give high yield at constant time 20 min. As shown in the figure 7. 20 min. is the good time for derivatization at constant temp. 70°C.

Figure 8 shows the Standard curve was found to be liner from 10-160µg/ml, the good recovery indicates that the good conditions for extraction and derivatization as shown in table 1 and the good recovery.

TABLE 1 shows that the Good recovery from 93 to 97 % for three concentration. The method proved to be precise in terms of both intra-day and inter-day analyses, with coefficients of variation less than 10%.

The (LOD) was estimated with decreasing the concentrations as shown in figure 8 were 10µg/ml.

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