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Determination of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in urine using high-performance liquid chromatography and electrospray ionization mass spectrometry

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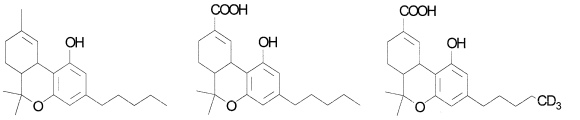
Abstract

High-performance liquid chromatography with electrospray ionization mass spectrometry was used to determine 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in urine. After basic hydrolysis of conjugates, the compound was extracted using SPEC-PLUS-3ML-C_{18} solid-phase extraction columns. A deuterium labelled internal standard (d_3-THC-COOH) was added prior to hydrolysis. Separation was performed on a reversed-phase Zorbax Eclipse XDB-C_8 analytical column (150×3.0 mm I.D.) using a gradient program from 60 to 80% acetonitrile (4 m*M* formic acid) at a flow-rate of 0.5 ml/min. The compounds were detected by single ion monitoring of *m/z* 345 and *m/z* 348 for the protonated molecules $[THC\text{-}COOH+H]^+$ and $[d_3\text{-}THC\text{-}COOH+H]^+$, respectively. The precision and accuracy were tested on spiked urine samples in the range 2.5–125 ng/ml. The mean recovery was 95% ($n=58$), coefficients of variations were 2.2–4.3% and the limit of detection 2 ng/ml. Diagnostic qualifying ions of THC-COOH (*m/z* 327 and *m/z* 299) and d_3-THC-COOH (*m/z* 330) were generated using up-front collision-induced dissociation. The relative ion intensities in clinical samples ($n=21$) were within ±20% deviation compared with standards. Using this tolerance and the presence of the ions *m/z* 327 and *m/z* 299 at the correct retention times as the acceptance criteria for identification of THC-COOH positive samples, the limit of detection was 15 ng/ml. The LC–MS method complies with the current recommendations on drugs of abuse testing, in which mass spectrometric detection is emphasized.

Keywords: 11-Nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid

1. Introduction

Verification of cannabis abuse is usually done by measuring trace levels of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) and its glucuronide ester in urine (Fig. 1). THC-COOH is



THC THC-COOH d_3-THC-COOH

Fig. 1. Structures and abbreviations of the compounds described in the text. Δ^9-tetrahydrocannabinol (THC), 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) and deuterated internal standard (d_3-THC-COOH). Stereochemistry is not shown.

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one of the major metabolites of Δ^9-tetrahydrocannabinol (THC), the psychoactive component in Cannabis products (marijuana, hashish). Cannabis is one of the most frequently used illicit drugs, and therefore often encountered by the clinical laboratories involved in routine analysis of drugs of abuse.

Clinical toxicological investigations serve a number of purposes primarily in the fields of Hospital Emergency, Toxicology and Drug Rehabilitation Programmes, but also as services for psychiatric clinics, general practitioners, prison- and military personnel, employers (workplace drug testing) and various health professionals.

According to the recent EU recommendations on drugs of abuse testing and the Mandatory Guidelines for Federal Workplace Drug Testing Programs (USA) samples should be screened by validated immunoassays and specific substances should be identified by chromatographic methods using mass spectrometric detection [1,2]. Immunoassay screening can lead to 'false-positive' results because of antibody cross-reactivity with molecules of similar structure, hence the need for a confirmative method. Although a quantitative analytical approach is not always strictly necessary for interpretation of the results, laboratories are still advised to determine drug concentrations to improve overall performance [1]. Immunoassays for THC metabolites are usually calibrated to give a positive result for sample concentrations $\geqslant$50 ng/ml ('cut-off' concentration). The confirmation method must be more sensitive, and the specific detection limit recommended for analysis of THC-COOH in urine is 15 ng/ml [1,2]. The urinary excretion of THC metabolites is prolonged as the cannabinoids are retained in human adipose tissue. With the methodology and sensitivity described above, THC metabolites can be measured for up to several weeks, depending on the frequency of abuse [3].

Various aspects of analytical methods for determination of THC-COOH in urine e.g. hydrolysis of conjugates, extraction, derivatization, choice of internal standards and identification by mass spectrometry have been reviewed [4]. The preferred method used for verification of drugs in urine is isotope dilution gas chromatography and mass spectrometry (GC–MS) [5]. In a recent survey of drugs of abuse testing and analytical approaches in the EU, about 50% of the 269 participating laboratories used GC–MS for identification of specific substances [6,7]. Other chromatographic methods used 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 detectors (ECD, FID, NPD). Generally these methods lack either specificity or sensitivity and the use of a coeluting deuterated internal standard is not possible. If TLC or HPLC methods were to be applied as single analytical methods independently of an initial screening, they could give rise to high rates of 'false-negative' results [6].

Although GC–MS is an excellent method, the major disadvantage is the elaborate sample preparation and the need to use various derivatization techniques for non-volatile and thermolabile compounds. THC-COOH can undergo decarboxylation in a hot GC-injector, and the compound is therefore measured as a derivative [4]. Drugs of abuse and their metabolites are by nature polar compounds with carboxylic, hydroxylic and amino groups, and therefore better suited for liquid chromatography. Today the combination of liquid chromatography and mass spectrometry (LC–MS) has become a routine analytical tool in pharmaceutical and biotechnological applications.

LC–MS is also gaining importance in analytical toxicology [8], although the technique was only available (in 1994) at less than 0.1% of the European Clinical and Forensic laboratories involved in drugs of abuse testing [6]. The new designs of liquid phase atmospheric pressure ionization interfaces, such as electrospray (ES) and atmospheric pressure chemical ionization (APCI) are very suitable for a broad spectrum of acidic and basic drugs.

To our best knowledge no applications of LC–MS have been published for routine analysis of THC-COOH in urine. LC–MS has been used for the profiling of Cannabis products by Rustichelli et al. [9]. Backstrom et al. used supercritical fluid chromatography (SFC) coupled to APCI-MS for the same purpose [10].

This paper describes a new LC–MS method using a single quadrupole instrument for determination of THC-COOH in urine. It is intended for routine verification in clinical and forensic laboratories. The

method includes basic hydrolysis, solid-phase extraction, reversed-phase chromatographic separation and detection by electrospray ionization mass spectrometry (ES-MS). Sample concentrations are calculated relative to a deuterated internal standard. Up front collision-induced fragmentation is used to generate confirmative fragment ions of THC-COOH, and a criterium for identification of positive samples is discussed.

2. Experimental

2.1. Chemicals

11-nor-Δ^9-Tetrahydrocannabinol-9-carboxylic acid (THC-COOH) was obtained from Sigma (St. Louis, MO, USA). Trideuterium labelled internal standard: 6a,10a, 7,8-tetrahydro-6,6-dimethyl-9-carboxy-3-(5,5,5-trideuteropentyl)-6H-dibenzo[*b,d*]pyran-1-ol= d_3-THC-COOH, was obtained in methanol (100 μg/ml) from Radian (Austin, Canada). The isotopic purity of d_3-THC-COOH was 99.89%. Stock solutions of THC-COOH and d_3-THC-COOH were prepared in acetonitrile (5 μg/ml) and stored at 4°C. All other chemicals were of analytical reagent grade and were used without further purification. The utilized glassware was made of non-silylated borosilicate.

2.2. Instrumentation

The liquid chromatograph was a Hewlett-Packard (Palo Alto, CA, USA) 1100 Series system with vacuum degasser, binary pump, thermostatted column department and autosampler. The data system used for acquisition, storage and calculation was HP LC/MSD CHEMSTATION. The analytical column was a reversed-phase Zorbax Eclipse XDB-C_8, 150×3.0 mm I.D., 5 μm, from Hewlett-Packard. Mobile phases were: (A) 4 m*M* formic acid in water (LiChrosolv, Merck); and (B) 4 m*M* formic acid in acetonitrile. The mobile phase conditions were as follows: 60% B for 0.5 min followed by a linear gradient to 80% B in 2.5 min. Then 80% B for 4 min and a linear gradient to 60% B in 3 min. Between each run was 5 min conditioning at 60% B. Flow-rate was 0.5 ml/min. Column department temperature was 60°C. Injection volume was 5 μl.

The detector was a Hewlett-Packard 1100 LC-MSD equipped with an atmospheric pressure ionization electrospray (API-ES) interface. Selected ion monitoring (SIM) was performed in positive mode for the ions m/z 345 $[\text{THC-COOH}+\text{H}]^+$ and m/z 348 $[\text{d}_3\text{-THC-COOH}+\text{H}]^+$ (dwell time: 169 ms). Capillary voltage was 4500 V and the cone voltage was 95 V. Drying gas was 99% pure nitrogen from a nitrogen gas generator (Whatman, UK) in line with a Junair 25 *M* air compressor (Junair, Nørresundby, Denmark). Drying gas temperature was 275°C and gas flow-rate 6.8 l/min. Nebulizer pressure was 36 p.s.i. (1 p.s.i.=6894.76 Pa). Spray chamber parameters were optimized in flow injection mode, using standards dissolved in 4 m*M* formic acid in acetonitrile–water (80:20, v/v). Experiments with up-front CID was optimized in flow injection mode and performed during analysis in SIM-mode using the following combinations [m/z/cone voltage (V)]: 348 (95), 345 (95), 330 (130), 327(140) and 299 (150), dwell time: 90 ms. Mass calibration (100–1000 a.m.u.) was checked daily and performed using autotune macros and calibrators from the manufacturer; electron multiplier voltage: 1711 V.

2.3. Sample preparation

2.3.1. Hydrolysis

Urine (2 ml) was mixed with 100 μl 6 *M* NaOH and 10 μl internal standard d_3-THC-COOH (5 μg/ml). The sample was hydrolysed at 60°C in a heating unit for 20 min. After cooling to room temperature, 500 μl glacial acetic acid were added, and the sample was centrifuged at 1000 *g* for 1 min.

2.3.2. Solid-phase extraction

A vacuum manifold was used for solid-phase extraction. SPEC-PLUS-3-ML-C_{18} (15 mg) columns (Ansys, Irvine, CA, USA) were conditioned with 150 μl methanol for 1 min (without vacuum applied). The sample supernate was transferred to and percolated through the column (flow-rate: 2–3 ml/min). The column was washed with 750 μl methanol–water (50:50, v/v) and air dried in the manifold for a minimum of 5 min at 20 in. Hg. Finally the analytes were eluted with 800 μl hexane–ethyl acetate (3:1,

v/v). The eluate was evaporated to dryness in a TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at a water bath temperature of 45°C using laboratory air at 20 p.s.i. for 5 min. Analytes were redissolved in 50 μl acetonitrile, vortex shaken (5 s) and transferred to autosampler microvials.

3. Validation

The method was validated for precision and accuracy by analysis of human urine samples spiked at six levels (2.5, 5, 10, 25, 50 and 125 ng/ml) in replicates of nine or ten samples. The urine samples were spiked with THC-COOH from a stock solution (5 μg/ml) and stored in glass bottles at 4°C for a maximum of 3 days.

The limit of detection (LOD) for m/z 345 [THC-COOH+H]$^+$ was determined at a signal-to-noise ratio (S/N) of $\geqslant$3 in the final extracts of spiked samples (2.5 ng/ml) diluted hereafter with extracts from blank runs. The LOD using qualifying ions were determined at $S/N \geqslant 3$ for m/z 327.

To test for specificity, possible interference from other matrix compounds and deviation of relative ion intensities, 21 authentic clinical samples from frequent Cannabis users were analysed. The samples were initially tested positive with the enzyme multiplied immunosassay technique (EMIT d.a.u. cannabinoid assay) (Behring Diagnostics, San Jose, CA, USA). Some of the samples contained metabolites of methadone and benzodiazepines (n=11), morphine (n=1), amphetamine (n=2) and phenobarbital (n=1). They were stored at 4°C until analysed.

Internal quality control samples (urine, conc. 15 ng/ml) were prepared by the following procedure: drug free urine was adjusted to [OH^-]=0.1 M with 10 M NaOH. After centrifugation at 1000 g for 10 min, the supernate was separated and spiked with a stock solution of THC-COOH. The quality control samples were analysed in double in three series containing seven clinical samples and one drug-free urine sample (blank).

External quality control urine, Liquichek urine toxicology control level C3 (Lot No. 68210) and level S2 (Lot No. 66410) (Bio-Rad Laboratories, Hercules, CA, USA) were analysed in triplicate by the final method.

4. Quantification

The calibration curve consisted of seven calibration points at the concentrations 125, 250, 500, 1000, 2000, 4000 and 10 000 ng/ml. The internal standard amount was 1000 ng/ml. Calculations were based on peak area ratios of [THC-COOH+H]$^+$ to corresponding internal standard. Least-squares linear regression was used to fit the curves. For determination of LOD for m/z 345 [THC-COOH+H]$^+$ an extra calibration point of 50 ng/ml was used.

5. Results and discussion

The conjugation of THC-COOH with glucuronic acid is variable, and even though it is possible to measure intact drug glucuronides after electrospray ionization, we consider the use of a basic hydrolysis step as a safe approach. The applied solid-phase extraction column is widely used for THC metabolites as described in detail by the manufacturer [11]. It was chosen partly because of low organic solvent consumption (<1.5 ml/sample) and because it was relatively simple to use. The procedure was used as a reference method in a study by Singh and Johnson [12]. A high concentration factor (40) was necessary to ensure a low limit of detection.

The optimal parameters of classical reversed-phase chromatography are not directly compatible with electrospray ionization. The use of volatile organic buffers in LC–MS are essential, and to ensure high ionization efficiency in electrospray, ions have to be preformed in solution. This is done by adjusting the pH in the mobile phase to favour detection of either protonated or deprotonated molecules. The use of acidic or basic mobile phases, however, has a major influence on the retention of molecules on reversed-phase columns (e.g. C_{18} columns), which are the most commonly used type today. As a consequence, new chromatographic methods often have to be developed for LC–MS application. Tyrefos et al. reported that ammonium acetate and trifluoroacetic acid in low concentrations considerably reduced the electrospray signal for opiate drugs and their glucuronides [13]. In this study a constant mobile phase concentration of 4 mM formic acid was used and this

gave reproducible retention times (±0.5%) and adequate sensitivity in electrospray.

Liquid chromatography was used in the gradient elution mode. The flushing out of matrix compounds is important because high concentrations of drugs and metabolites (e.g. methadone) are usually present in samples from drug abusers. From the ion chromatograms (Fig. 2) it can be seen that the signals from $[THC\text{-}COOH+H]^+$ at m/z 345 and $[d_3\text{-}THC\text{-}COOH+H]^+$ at m/z 348 (retention time: 5.42 min) are well separated from interfering peaks.

Preliminary tests carried out with standards indicated that both positive and negative mode electrospray were suitable techniques for detection of THC-COOH. ES in positive mode was chosen, however, because of compatibility with other methods for drugs of abuse used in this laboratory. The method showed good within-run precision with coefficients of variations (C.V.) from 2.2 to 4.3% in the concentration range tested (2.5–125 ng/ml) (Table 1). This includes the working range for tetrahydrocannabinol screening, where identification must be conclusive. Standard curves were, because they reflected relative response factors, very reproducible,

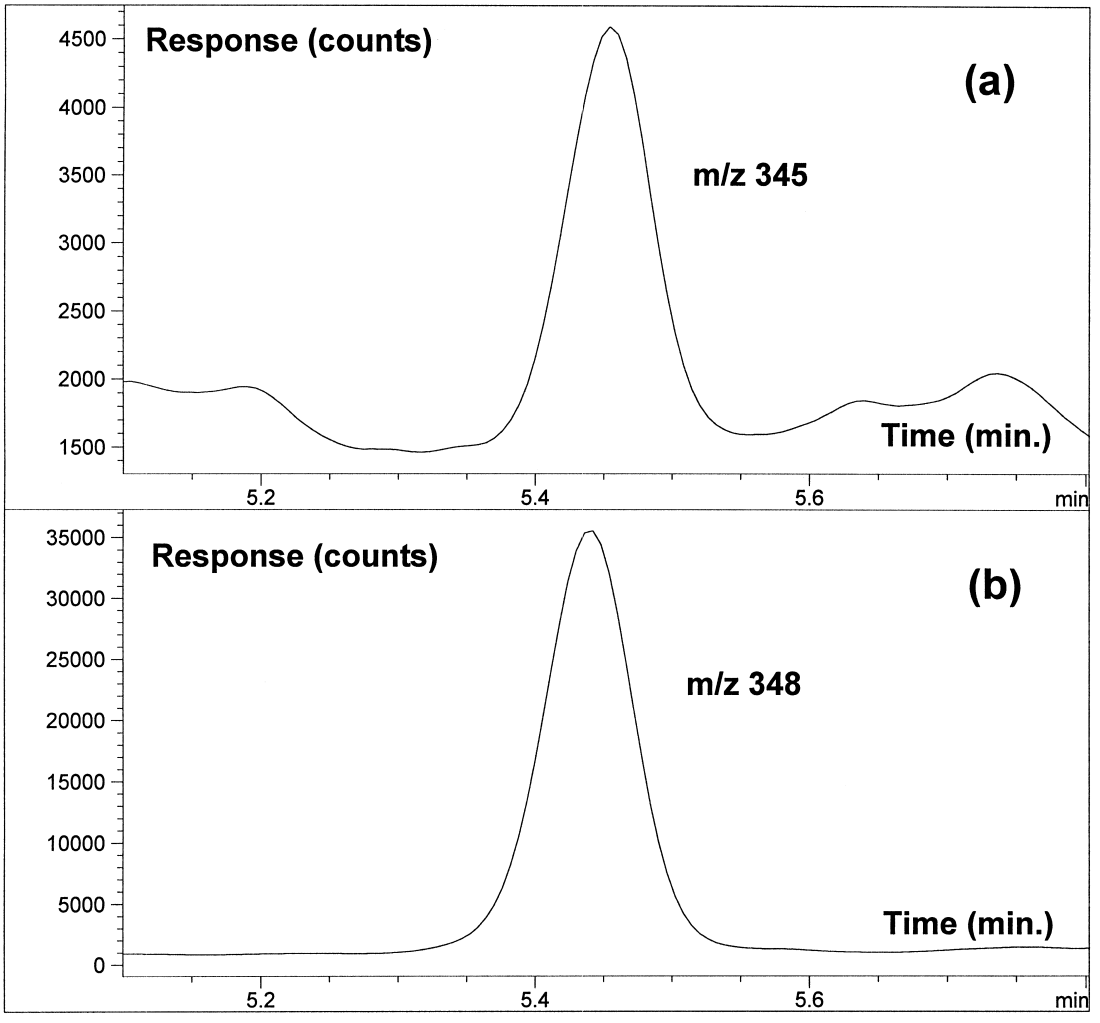

Fig. 2. Ion chromatograms obtained by analysis of a spiked urine sample (2.5 ng/ml). (a) m/z 345 from $[THC\text{-}COOH+H]^+$; (b) m/z 348 from internal standard $[d_3\text{-}THC\text{-}COOH+H]^+$. Note the slight difference in retention times of the two components.

Table 1
Within-run precision and efficiency of extraction from spiked urine samples

Concentration (ng/ml)	*n*	Mean recovery (%)	SD (ng/ml)	C.V. (%)
2.5	10	92	0.10	4.3
5	9	93	0.10	2.2
10	9	92	0.28	3.0
25	10	91	0.68	3.0
50	10	98	1.77	3.6
125	10	101	4.28	3.4

demonstrating correlation coefficients (r^2) of 0.999 or better. The equation obtained was as follows: $y = 0.9294x + 0.0083$, where y is the area ratio and x is the amount ratio, analyte to internal standard, respectively. The mean recovery of the method was 95% ($n = 58$) and the limit of detection was 2 ng/ml. This is comparable to GC–MS routine methods, although research methods for pharmacokinetic studies of THC-COOH in urine and plasma are more sensitive, particularly the negative ion chemical ionization methods (NICI), where detection in the pg/ml range is possible [14]. In this study it was the significant chemical or chromatographic noise at low concentrations of THC-COOH (<15 ng/ml) rather than electronic (detector) noise that determined the limit of detection. Changing the SIM resolution mode (instrumental options: low/high) or raising the electron multiplier voltage did not improve the limit of detection.

An important issue in LC–MS applications is the loss of spectral information in mass spectra obtained with the soft ionization techniques. This could be considered as a major drawback compared with traditional GC–MS methods, where several fragment ions are monitored and the ratios of intensities used as acceptance criteria with ±10–20% maximum difference allowed relative to standards. The presence of these qualifying or diagnostic ions at the correct retention time is a much safer identification criteria than relying on a single signal only. Four or more ions would be ideal from a theoretical point of view, but it may not always be possible in trace analysis.

If LC–MS instruments with iontrap or triple quadrupole detectors are used, the acquisition of MS–MS spectra in either full scan or selected ion monitoring (SIM) mode should lead to unambiquous results. With a single quadrupole LC–MS instrument it is nevertheless also possible to generate fragment ions by increasing the potential difference between the capillary end and the source skimmer, termed up-front collision-induced dissociation (CID) [15]. These fragmentation data are equivalent to those obtained from MS–MS spectra (although the parent ion is not selected), and can be acquired during SIM, with a different cone voltage for each m/z monitored. Up-front CID is dependent on instrument variables, and the fragmentation pattern and relative ion intensities of a given compound may not be reproducible on different instruments. We believe that more experience is needed to evaluate the practical advantage of this option in routine analysis of biological samples.

The ES mass spectrum of THC-COOH and internal standard acquired at a moderate cone voltage shows two distinct and one minor fragment ion (Fig. 3). The fragmentation pattern observed here can be explained by loss of H_2CO_2 (m/z 299) and water (m/z 327), respectively. Loss of CH_3 as seen in the electron impact (EI) spectrum of THC-COOH, does not happen under electrospray conditions. The fragments at m/z 302 and m/z 330 in the spectrum of d_3-THC-COOH contain the trideuterated pentyl chain, and these ions can be mass separated in the quadrupole from isotope peaks of THC-COOH fragments.

Data acquired from repetitive flow injections of standards at different cone voltages were used to determine the optimum parameters for the monitoring of the two fragment ions of THC-COOH. The ion intensities as a function of cone voltage showed broad peak optima and were typical for up-front CID experiments. The ion at m/z 193 in the spectra of THC-COOH was not considered for use as a qualifying ion because it had a relative low intensity and was difficult to detect at low sample concentrations. The CID data obtained for the internal standard were analogous to THC-COOH. It was an important issue in this work to find a realistic limit of detection where both qualifying ions was used, and to specify the allowed deviation of ion intensities from an expected (or reference) percentage of the main peak response. Comparison of published methods is sometimes difficult, because authors do not always de-

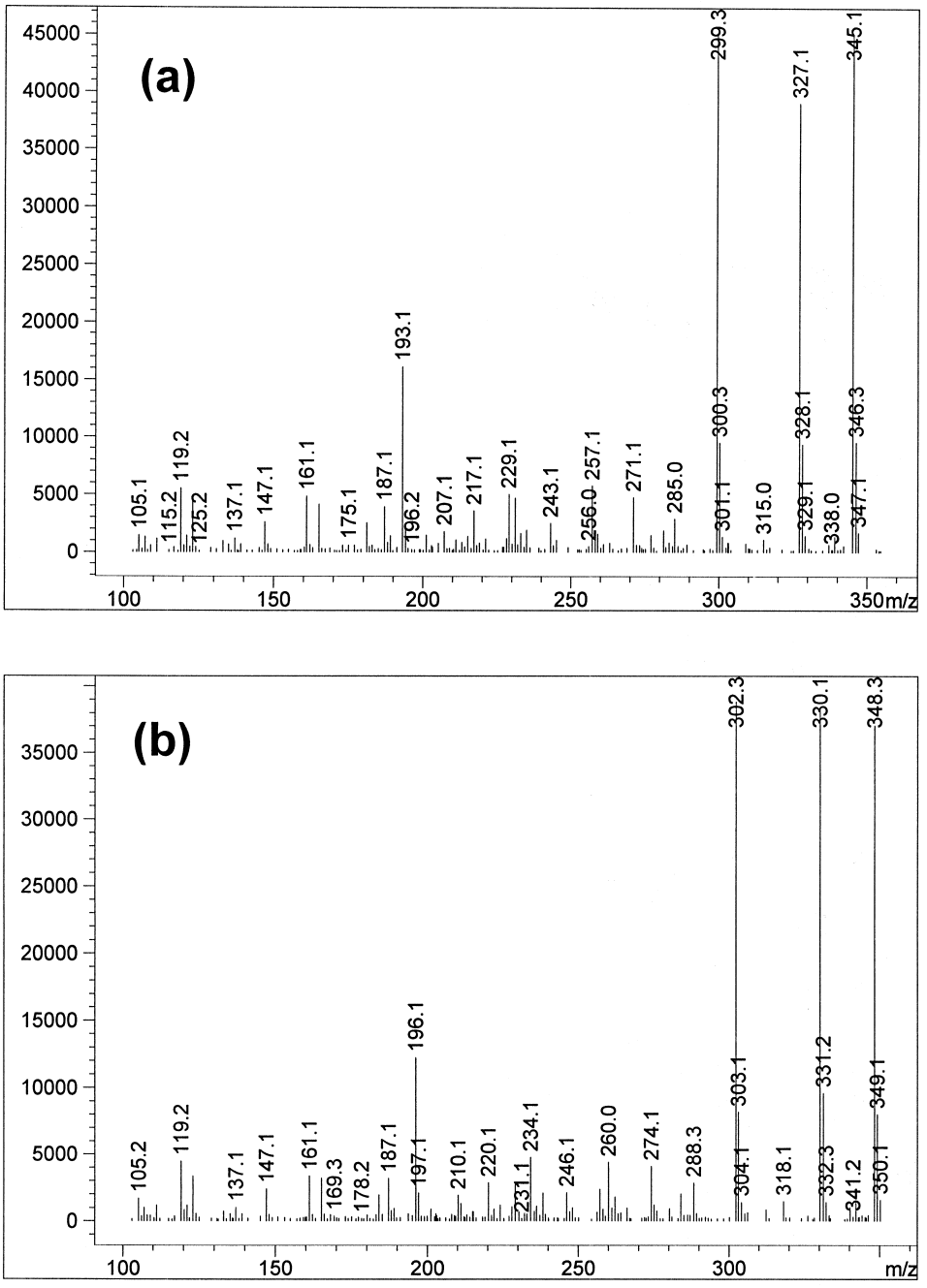

Fig. 3. Electrospray mass spectra obtained during LC–MS analysis of standards. (a) THC-COOH; (b) d_3-THC-COOH. Amount injected: 500 ng; cone voltage: 160 V.

scribe whether or not the qualifying ion criteria were met at the limit of detection.

During the analysis of 21 clinical samples, initially screened positive for cannabinoids, a SIM program was used to examine the response of two qualifying ions for THC-COOH (Q1 = m/z 327 and Q2 = m/z

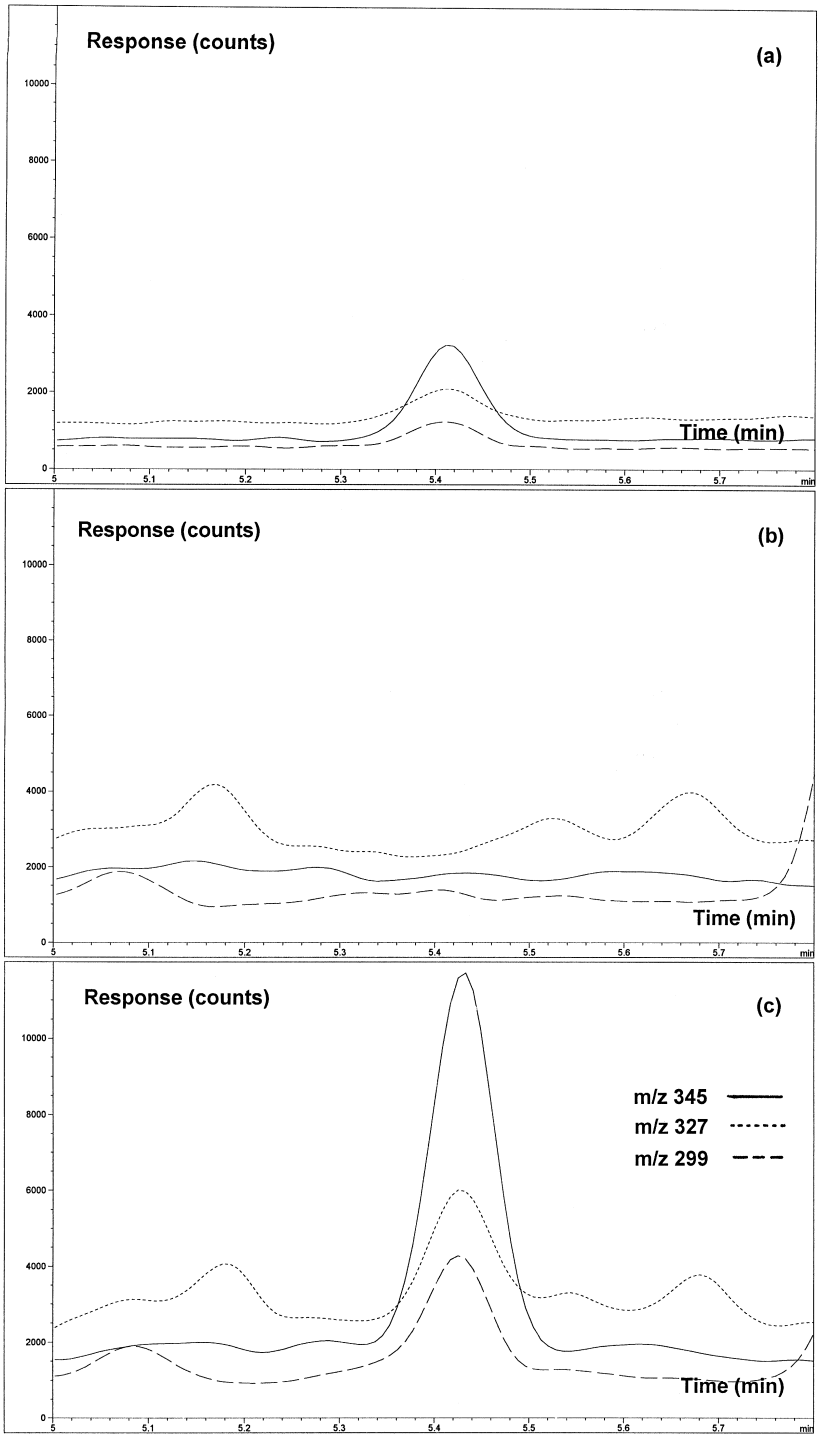

Fig. 4. Ion chromatograms of m/z 345, 327 and 299 obtained from analysis of (a) calibration standard 125 ng/ml; (b) blind sample; (c) internal quality control sample (15 ng/ml). The chromatograms are in the same scale.

299) and one qualifying ion for the internal standard (Q3 = m/z 330). The ion signals could be integrated without major interference from matrix compounds, and blank runs ($n = 3$) were free of analyte (Fig. 4). The mean relative ion intensities for Q1, Q2 and Q3 in calibration standards ($n = 7$) were 38.9, 29.0 and 39.6%, respectively. If the relative ion intensities were allowed to deviate ±20% from these reference values, all measurements in the three validation series could be approved. This tolerance is in accordance with the generally accepted guidelines for trace analysis by mass spectrometric methods using soft ionization techniques e.g. chemical ionization (CI). The majority of the samples ($n = 14$) had concentrations far exceeding the calibration curve and would have to be diluted with drugfree (blank) urine to obtain quantitative values. However, these samples were representative for the worst cases of matrix interference compounds due to the fact that the patients were multi-drug users in methadone treatment.

Internal quality control samples ($n = 6$) at a nominal concentration of 15 ng/ml were used for quality approval of the clinical samples. They were analysed in duplicate in each series with acceptable between-run precision (C.V. = 3.6%). The preparation procedure for the internal quality control samples was suggested by Joern [16], who found that loss of THC-COOH due to adsorption could be minimised if the analyte was kept in either a basic solution or an organic solvent. The claimed stability according to this preparation design is at least 18 months when stored at −80°C [16]. We only observed loss of analyte if the mobile phase was used for the final redissolution of extracts, and if laboratory water was used as the medium in recovery tests. The experience gained from the present study, indicated an acceptable stability of THC-COOH in a biological matrix at 4°C for 3–4 days using non-silylated glassware.

The limit of detection of THC-COOH using both qualifying ions was 15 ng/ml. Below this concentration it becomes difficult to distinguish the ion m/z 327 from background noise, although quantification of THC-COOH using m/z 345 is still precise at this level.

External quality control urine samples (liquid form) from Bio-Rad were later analysed by the final method. They have been used for about 6 months in our laboratory as controls for immunoassay screening. The urine contains key compounds from 12 different drug classes, and is provided at different concentration levels for both screening and confirmation purposes as recommended by the US Substance Abuse and Mental Health Services Administration (SAMHSA). The GC reference value for the S2 and C3-levels according to Bio-Rad are 17.7 and 53.0 ng/ml, respectively. Mean values ($n = 3$) from the present method were 16.6 (C.V. = 2.6%) and 58.3 ng/ml (C.V. = 0.7%), respectively. The Bio-Rad controls are manufactured by addition of drugs and metabolites to human urine; in this case by spiking with THC-COOH. However, in our opinion a quality control urine containing the THC-COOH glucuronide would be more correct to use, although it is unknown if such a reference material would be stable. To our best knowledge the THC-COOH conjugate is not yet commercially available, and would have to be custom synthesized.

6. Conclusions

The described LC–MS method complies with the current recommendations in the field of drugs of abuse testing. It can therefore be used for identification and quantification of THC-COOH in urine at low ppb levels. The performance in terms of precision and selectivity are comparable to former GC–MS methods, but the sensitivity of this electrospray technique in positive mode is lower than GC–NCI methods. In this case the advantage of LC–MS is easier sample preparation, and therefore shorter time of analysis. Our laboratory is now in the process of developing alternative LC–MS applications for other illicit drugs and pharmaceuticals.

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