Automated Extraction of 11-nor-\(\Delta^9\)-Tetrahydrocannabinol Carboxylic Acid from Urine Samples Using the ASPEC XL™ Solid-Phase Extraction System*

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Abstract

The analysis of 11-nor-\(\Delta^9\)-tetrahydrocannabinol-carboxylic acid (THCCOOH, the major metabolite of cannabis) in urine with gas chromatography and mass spectrometry (GC–MS) and solid-phase extraction (SPE) sample preparation is well documented. Automated SPE sample preparation of THCCOOH in urine, although potentially advantageous, is to our knowledge poorly investigated. The objective of the present study was to develop and validate an automated SPE sample-preparation step using ASPEC XL suited for GC–MS confirmation analysis of THCCOOH in urine drug control. The recoveries showed that it was not possible to transfer the protocol for the manual SPE procedure with the vacuum manifold to the ASPEC XL without loss of recovery. Making the sample more lipophilic by adding 1 mL 2-propanol after hydrolysis to the urine sample in order to overcome the problem of surface adsorption of THCCOOH led to an extraction efficiency (77%) comparable to that reached with the vacuum manifold (84%). The reproducibility of the automated SPE procedure was better (coefficient of variation 5%) than that of the manual procedure (coefficient of variation 12%). The limit of detection was 1 ng/mL, and the limit of quantitation was 4 ng/mL. Precision at the 12.5-ng/mL level was as follows: mean, 12.4 and coefficient of variation, 3.0%. Potential carryover was evaluated, but a carryover effect could not be detected. It was concluded that the proposed method is suited for GC–MS confirmation urinalysis of THCCOOH for prisons and detoxification centers.

Introduction

The Dutch Laboratory for Drugs and Doping performs urinary analyses for drugs of abuse for prisons, detoxification centers, and hospital emergency testing. Screening for amphetamines, barbiturates, benzodiazepines, cocaine, cannabis, ethanol, methadone, opiates, and phenycyclidine is routinely performed using the EMIT immunoassay technique. In 1999, the total number of screenings was approximately 100,000 of which approximately 19,000 concerned cannabis. Approximately 20% of all urines analyzed for cannabis had a positive test result. Confirmation analysis is performed in accordance with the guidelines of the Substance Abuse and Mental Health Services Administration (SAMSHA) (1-4) and the European Union (5,6). In case of confirmation of cannabis the major metabolite 11-nor-\(\Delta^9\)-carboxy-\(\Delta^9\)-tetrahydrocannabinol-carboxylic acid (THCCOOH) is determined using gas chromatography–mass spectrometry (GC–MS).

The analysis of THCCOOH in urine with GC–MS and solid-phase extraction (SPE) sample preparation are well documented (7-14). However, to our knowledge, only one recent study evaluated the automation of SPE sample preparation of THCCOOH in urine (15). The increased volume of samples in our laboratory for THCCOOH confirmation prompted the investigation of the possibility of using automated sample preparation, thus eliminating what is generally considered the most labor-intensive and error-prone step in an analytical method. Potential advantages of automating sample preparation are increased throughput, improved reproducibility, and minimal personal exposure to hazardous materials. The ASPEC XL met many of our needs. Although the time required to process an individual sample by manual or automated SPE (approximately 30 min) remains the same, an automated system can work 24 h per day.

The objective of the present study was to develop and validate an automated SPE sample-preparation step suited for GC–MS confirmation analysis of THCCOOH in urine drug control.

Materials and Methods

Reagents

All reagents were analytical grade or better. THCCOOH urine calibrators were purchased from Dade Behring (Leusden, The
was adjusted to 2.5 by adding 2 mL 50mM phosphate buffer (pH
Amsterdam, The Netherlands). The column used was a
acetonitrile/0.1M HCl (2:3 v/v) solution. The cartridge was dried
samples were slowly eluted with 3 mL of a mixture of
and conditioned with 3 mL methanol and 3 mL 50mM phosphate
2.5). The SPE cartridges were mounted on the vacuum manifold
in order to evaluate the extraction efficiency. The initial eluent was
100 ng/mL were used
to get an impression of the robustness.

Equipment
SPE. Manual SPE was performed on a Waters Vacuum mani-
fold (Etten-Leur, The Netherlands). Automated SPE was per-
formed on a Gilson® ASPEC XL (Meyvis, Bergen op Zoom, The
Netherlands).
HPLC. HPLC analysis was carried out with a Merck Hitachi
L-6200A pump and an AS-2000A autosampler coupled on an
L-4500 diode-array detector (DAD) (all components Merck,
Amsterdam, The Netherlands). The column used was a
LiChroCART®125-4 HPLC-cartridge, LiChrospher® 100 RP-18
dapped (5 μm) (Merck, Amsterdam, Netherlands).
GC-MS. GC–MS analysis was carried out with an HP 5890 GC
(Hewlett-Packard, Amstelveen, The Netherlands) equipped with an
HP 7673A automatic injector and interfaced to an HP 5971A
MSD. The column was a 12-m HP-1 (cross-linked methyl
siloxane) capillary with a 0.22-mm internal diameter and a 0.33-
μm film thickness (Hewlett-Packard).

Methods
Hydrolysis. Prior to analysis all samples were hydrolyzed for
15 min at 60°C after the addition of 300 μL 10M KOH solution,
2 mL water, and 25 μL internal standard (4 μg/mL THCCOOH-
d) to a 3-mL urine sample. After hydrolysis, the tubes were
cooled, and 350 μL acetic acid (96%) was added.
Manual SPE on vacuum manifold. After hydrolysis, the pH
was adjusted to 2.5 by adding 2 mL 50mM phosphate buffer (pH
2.5). The SPE cartridges were mounted on the vacuum manifold
and conditioned with 3 mL methanol and 3 mL 50mM phosphate
buffer (pH 2.5). After conditioning, the sample was poured slowly
down the cartridge. The cartridge was then washed with 2 mL
acetonitrile/0.1M HCl (2:3 v/v) solution. The cartridge was dried
under full vacuum for 1 min. The cartridge was washed with 0.5
mL hexane and then dried under full vacuum for 5 min. The
samples were slowly eluted with 3 mL of a mixture of
hexane/ethylacetate (1:1, v/v). The extracts were dried under
nitrogen at 40°C (11).
SPE ASPEC XL. The initial protocol on the ASPEC XL was a
direct translation of the manual protocol for the vacuum mani-
fold. As is shown in the Results section, it was necessary to add
1 mL of 2-propanol to the samples after hydrolysis. The details of
the protocol can be obtained from the authors upon request.
HPLC procedure. A low-pressure HPLC gradient program was
used to evaluate the extraction efficiency. The initial eluent was
10mM phosphate buffer/acetonitrile (53:47, v/v, pH 3.4) at a flow
rate of 1 mL/min. After 20 min, 70% of the initial eluent was
replaced with acetonitrile. After extraction the dry residues were
reconstituted in 100 μL of the initial eluent. Injections (30 μL)
took place at ambient temperature. Detection took place at a
50mM phosphate buffer (pH 2.5) was added. After extraction the dry residues were
5. THCCOOH quality-control material, Lyphochek® Urine Toxicology Control, was purchased from Bio-Rad
Veenendaal, The Netherlands). SPE cartridges (Bakerbond SPE
NARC-1™ 3 mL) were obtained from Baker (Deventer, The
Netherlands). Derivatizing agent (BSTFA + 1% TMCS) was pur-
chased from Alltech (Breda, The Netherlands).

Validation
The final automated SPE method was validated in combina-
tion with GC–MS analysis. A calibration line was generated using
THCCOOH concentrations of 5, 12.5, 25, 50, 100, and 150
ng/mL. Each concentration was analyzed twice. The intra-assay
accuracy and repeatability of the assay were assessed by
extracting six aliquots of four concentration levels of THCCOOH
in urine, specifically 5, 12.5, 25, and 100 ng/mL. The interme-
diate precision of the assay was assessed by extracting six ali-
quots of a commercially purchased quality-control material
(Lyphochek® Urine Toxicology Control) on six different days at
four concentration levels, specifically 6, 30, 61, and 121 ng/mL.

The robustness of the ASPEC XL was tested by analyzing 200
patient urine samples on seven days. On each day 41 samples
were processed. The first four samples were used for calibration
at 12.5, 25, 50, and 150 ng/mL. After these calibrators, 15 patient
urines were processed, followed by a standard (100 ng/mL) and a
blank urine sample. After these quality-control samples, 15 more
patient urines were processed. These were followed by four com-
mmercially available control samples (6, 30, 61, 121 ng/mL), 100-
ng/mL standard, and a blank urine sample. The concentrations
measured on these seven days for standard 100 ng/mL were used
to get an impression of the robustness.
In addition, the potential for THCCOOH carryover was evalu-
ated. There are two places during the analysis protocol where
urine samples or their extracts, calibrator, or control come into
contact with the same surfaces. The first is when the sample is transferred from the test tube to the SPE cartridge mounted on the deck of the ASPEC XL, when the sample needle aspirates the sample. The needle and tubing connected to it come into contact with each specimen, calibrator or control. After every step the ASPEC XL was programmed to perform a washing step. The other place where carryover may occur is when the derivatized extract is actually injected by the autosampler into the GC-MS system. The same syringe was used to inject every sample. After each injection the autosampler of the GC-MS system performed a washing step. The carryover of the total analytical procedure was evaluated using the results from the blank urine samples that were analyzed in the sequence described here after 100-ng/mL standard. The blank urine samples were monitored for the target ions 371 for THCCOOH and 374 for THCCOOH-d3.

Results

Extraction efficiency

Table I summarizes the efficiency of the extraction procedures of THCCOOH from urine samples using Bakerbond SPE NARC-1 3-mL cartridges on a vacuum manifold (A), ASPEC XL with manual protocol batch-wise (B), ASPEC XL with manual protocol sequential mode (C), and ASPEC XL sequential mode with 2-propanol addition after hydrolysis (D). The recoveries show that it was not possible to transfer the protocol for the manual SPE procedure with the vacuum manifold (A) to the ASPEC XL without loss of recovery (B). Because THCCOOH was known to be adsorbed to surfaces we tried to shorten the time the urine sample was in contact with the tubing of the ASPEC XL and the SPE cartridge. This was done by performing the extraction in a sequential mode (C). Extraction efficiency increased but still did not reach the level of the vacuum manifold procedure. Making the sample more lipophilic by adding 1 mL 2-propanol after hydrolysis to the urine sample (D) led to an extraction efficiency (77%) comparable to the one reached with the vacuum manifold (84%). The repeatability of the automated SPE procedure was better (coefficient of variation 5%) than that of the manual procedure (coefficient of variation 12%).

Validation

Figure 1 illustrates the extracted ion chromatograms for m/z 371 after automated SPE with ASPEC from blank urine, 12.5-ng/mL calibrator, and a patient urine (THCCOOH 21.0 ng/mL). The calibration line was linear for 0–150 ng/mL. The limit of detection, according to ICH Guidelines defined as 3.3 times the standard deviation of the slope of the calibration line, was 1 ng/mL; the limit of quantitation, defined as 10 times the standard deviation of the slope of the calibration line, was 4 ng/mL. The validation parameters are provided in Table II. At the con-

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<th>Table I. Extraction Efficiency and Reproducibility of the Different Extraction Procedures</th>
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<td>Procedure</td>
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<tr>
<td>A. Manual SPE on vacuum manifold</td>
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<td>B. ASPEC XL with manual protocol batch-wise</td>
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<td>C. ASPEC XL with manual protocol sequential</td>
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<td>D. ASPEC XL sequential with 2-propanol addition</td>
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Figure 1. Selected ion chromatogram (m/z 371) for blank urine (A), calibrator 12.5 ng/mL (B), and patient urine 15.4 ng/mL (C). Retention time for THCCOOH, 12.86 min.

<table>
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<th>Table II. Validation Parameters using ASPEC XL*</th>
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<tr>
<td>Parameter</td>
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<tr>
<td>Intra-assay accuracy*</td>
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<tr>
<td>Repeatability*</td>
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<td>Intermediate precision*</td>
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* All experiments were performed six times.

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centation of 12.5 ng/mL (concentration nearest to the 15-ng/mL cutoff), the mean concentration was 12.4 ng/mL and the repeatability was 3.0%. The results for the standard 100 ng/mL tested during routine analysis of patient samples during seven different days indicate that the method is sufficiently robust (intermediate precision 3.5%) (Table III).

The carryover of the total analytical procedure was evaluated using the results from the blank urine samples. These samples were analyzed in the previously described sequence after standard 100 ng/mL. The blank urine samples were monitored for the target ions 371 for THCCOOH and 374 for THCCOOH-d3. In all blank urine samples no THCCOOH or THCCOOH-d3 could be detected, indicating that no relevant carryover effect was present.

Discussion

The usefulness of automation of sample preparation has often discussed. Diamond et al. (16) reported that part of the answer is the cost of the automation equipment and another part is simply inertia. Analysts are trained to perform extractions in a certain way. This method is documented and validated. Changing the method to make it amenable to automation may require modification of an SPE procedure to replace the existing method. Extensive revalidation and documentation are required.

The possible advantages of automation of SPE sample preparation, as stated in the Introduction, are clear. An SPE automate can work 24 h per day. Accurate and consistent liquid handling during the SPE process eliminates the need for repeat runs. When performing manual SPE, the main points to consider are not to allow the packing material to dry during the conditioning step and to load the sample with a sufficiently low flow rate to enable a good interaction between the sorbent and the analyte of interest. Manual SPE is performed using vacuum manifolds. SPE columns are placed in the manifold cover and liquids are manually pipetted onto each column. Liquid flow rates through the columns are controlled using a vacuum pump. The difficulty of this approach is to set a vacuum of sufficient accuracy and reproducibility; small volumes are particularly difficult to control and fast flow rates can cause sorbent channeling. An alternative approach is the technique of positive air pressure, which is employed by ASPEC XL. The required liquid volume is first dispensed into the SPE column using a high-precision syringe pump. Then a defined volume of air is applied to the top of the column to push the liquid across the sorbent packing with a constant flow rate. A special polyethylene dec provides a seal between the needle and the SPE column. The air push volume controls the state of the packing bed: depending on the programmed volume, it is possible to maintain a wet packing or conversely to dry the SPE column. The ASPEC XL uses a hand held keypad computer on which protocols can be stored and copied onto 3.5-in. diskettes. For greater reliability, audit trails (log files) can be saved for each run. All common SPE steps, including cartridge conditioning, diluent addition to specimen, mixing of specimen, specimen application to the cartridge, column washing, column drying, and column elution, can be performed by the ASPEC XL. The ASPEC XL also provides automation of other sample preparation steps required prior to or following extraction (e.g., serial dilution of standard, addition of internal standards, derivatization). An evaporation module and injection module are available for injection onto a chromatographic system. The ASPEC XL uses 3-mL SPE cartridges filled with silica based packing material or disks, or 96 well standard microtiter plate format.

Manual SPE is mainly performed with disposable columns. During the conditioning, loading and washing steps, eluates are collected in waste vessels, while for the elution steps collection tubes are used. The automation of SPE with ASPEC XL is based on the mobility of a dedicated rack consisting of three parts: an SPE column holder, a drain cuvette and a collect rack. During the SPE process, the SPE column holder is moved from the back position to the front position. Conditioning, loading, and washing steps are performed in the front position over the drain cuvette. Elution is performed in the back position where the SPE columns are positioned over collection tubes. The ASPEC XL can operate in either a sequential or batch mode. In the sequential mode, a series of tasks are performed on one sample and then repeated for the next sample. In the batch mode, all samples are processed using each of the tasks in turn (17).

Although manufacturers of SPE automates claim that manual SPE methods can easily be automated, our experience with the urinalysis of THCCOOH showed that this is not always true. In our case the physical properties of THCCOOH and mode of processing the sample were of great influence on the extraction recovery. THCCOOH is a hydrophobic molecule which is subject to adsorption to solid surfaces from aqueous solutions such as urine. The degree of adsorption is dependent upon surface-to-volume ratio, the type of surface, and handling techniques (18,19). Roth et al. (20) investigated the effects of solution composition and surface material on the loss of THCCOOH. Joern (21) described a solution to the problem of surface adsorption of THCCOOH by keeping the analyte in a basic solution or in an organic solvent. He prepared an urine sample prior to analysis for THCCOOH by adding 0.1M sodium hydroxide to prevent surface adsorption. In actual practice the first step in most published procedures is a basic hydrolysis of an urine sample; so after the urine is mixed with the base, the assay medium is the

| Table III. Robustness: Performance of the Internal Quality-Control Standard* During Routine Analysis of 200 Patient Samples on 7 Different Days |
|-----------------|-----------------|
| Day | Standard 100 ng/mL | Coefficient of variation (%) |
| 1 | 98.2 | 104.0 | 3.5% |
| 2 | 101.3 | 107.9 |
| 3 | 105.3 | 111.5 |
| 4 | 100.4 | 100.7 |
| 5 | 104.5 | 106.1 |
| 6 | 105.3 | 102.6 |
| 7 | 100.4 | 107.5 |
| Mean (ng/mL) | 104.0 | * 100 ng/mL.
same whether treated with 0.10M sodium hydroxide to prevent adsorption or untreated urine. During the development of an automated SPE procedure, it was found that the problem of surface adsorption of THCCOOH occurred during the actual sample preparation. While working with NARC 1 SPE columns it is essential that the pH of the urine sample is set at 2.5. NARC 1 is a mixed mode anionic exchange C8 phase. To bind THCCOOH to the column packing, the molecule must have a negative charge to be bound to the positively charged column packing. The acidic urine is aspirated through the needle into the plastic tubing of the ASPEC XL, resulting in a large surface-to-volume ratio. In our opinion adsorption takes place in the tubing of the ASPEC XL. While working in batch mode, extra adsorption takes place on the cartridge itself. Speeding up the extraction process by working in a sequential mode instead of a batch mode is only a partial solution. Roth et al. (20) investigated THCCOOH surface adsorption in water, urine and cannabinoids diluent (normal human urine with dimethylsulfoxide [5%], sodium chloride [0.9%], and protein [0.1%] added). They found that addition of cannabinoids diluent still gave some adsorption, but it was less than compared with the adsorption of samples without addition of cannabinoids diluent. It was decided to make the urine samples more lipophilic after basic hydrolysis by adding 1 mL 2-propanol (15%), thus giving a more lipophilic solution in comparison with cannabinoids diluent (dimethylsulfoxide polarity index 6.5 – 2-propanol polarity index 4.3) (22). The addition of 2-propanol does not affect binding of THCCOOH to the column since the adsorption is primary based on ionic binding. The excess amount of urine and 2-propanol are washed out of the column with acetonitrile/0.1M HCl (23, v/v) solution, according to the manual SPE procedure.

Another point is the claim of manufacturers that automation of sample preparation gives a higher or equal throughput of samples compared with manual vacuum manifold SPE methods. The ASPEC XL takes about 30 min to process a single THCCOOH urine sample. This is about the time necessary to process a sample with the vacuum manifold. With the manifold, however, it is possible to process 12 samples in each batch, which makes the ASPEC XL rather slow for this application.

Conclusions

Transferring vacuum manifold SPE procedures to an SPE automate is not always as easy as expected. In the case of THC-COOH physical properties of the molecule were of great influence of the extraction efficiency. Extensive revalidation and documentation are always required when transferring a manual vacuum manifold SPE procedure to an automated procedure. For our laboratory the automated extraction capability of the ASPEC XL has clear advantages over manual vacuum manifold-based SPE procedures. Human manipulations are kept to a minimum, which results in improved extraction reliability, improved repeatability, intermediate precision, and intra-assay accuracy. In addition, the procedure can operate 24 h per day.

The proposed method was suited for GC–MS confirmation urinalysis of THCCOOH for prisons and detoxification centers.

References

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