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Automated Extraction, Derivatization and GC/MS Determination of Tetrahydrocannabinol and Metabolites in Whole Blood Using Disposable Pipette Extraction

William E. Brewer, Sparkle T. Ellison, Stephen L. Morgan
Department of Chemistry and Biochemistry, University of South Carolina, 631 Sumter Street, Columbia, SC 29208, USA

Fred Foster, Jacqueline A. Whitecavage
Gerstel, Inc., 701 Digital Drive, Suite J, Linthicum, MD 21090, USA

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Abstract
Disposable Pipette Extraction (DPX) has been shown to be a rapid, efficient and reproducible technique for performing solid-phase extraction (SPE) of drugs from biological specimens.

DPX is based on pipette tips that incorporate loosely contained sorbent material. The sorbent is suspended in the sample solution using turbulent air bubble mixing for efficient contact between the phases, resulting in higher recovery and faster extraction. Elution can be performed with small volumes of solvent to minimize extract dilution. DPX is readily automated using the GERSTEL MultiPurpose Sampler (MPS).

The analyzed 0.5 mL blood specimens were protein precipitated with acetonitrile and centrifuged. The rest of the analysis was completely automated using a dual rail

GERSTEL MPS 2 combined with a Cooled Injection System (CIS) inlet for large volume injection (LVI). Automated extractions were performed using 1 mL DPX-RP (reversed phase) tips, and the total time required per sample, was approximately 6 minutes.

The CIS inlet was used for sample introduction, solvent evaporation (analyte concentration), and chemical derivatization. A 50 µL aliquot of sample extract and 20 µL of derivatizing reagent were introduced directly into the CIS. The combined system provided excellent throughput for analysis of THC and metabolites.

Derivatization of THC, OH-THC and COOH-THC was performed by using a mixture of BSTFA and MTBSTFA. Limits of detection were determined to be 0.5 ng/mL for THC and OH-THC and 2 ng/mL for COOH-THC. Coefficients of variation were below 5 % for all 3 analytes. Extraction efficiencies were found to depend mostly on the initial protein precipitation step. Recoveries for the DPX extraction (post precipitation) were close to 80 % for THC.

INTRODUCTION

The analysis of tetrahydrocannabinol (THC) and its metabolites, hydroxy-THC (OH-THC) and carboxy-THC (COOH-THC), in whole blood is widely performed using one of the most tedious and time consuming methods in forensic toxicology. The analytical challenge is to reach the required limit of detection of 1 ng/mL for THC in this complex sample matrix. Most solid-phase extraction (SPE) methods produce extracts that require separate chromatographic analysis of THC and COOH-THC due to possible interfering sample matrix components. This means that extraction and GC/MS determination of THC and its metabolites is more time consuming than most other forensic analysis methods.

Recently, Disposable Pipette Extraction (DPX) has been found to be a rapid and efficient SPE method for the analysis of THC and metabolites in whole blood [1]. The sorbent is loosely contained inside the pipette tip; therefore, sample solutions are mixed with the sorbent to provide efficient extraction without the risk of channelling or of variations in solution flow rates. Also, less solvent is required for elution which means that less time is required for the concentration step. Using reversed phase DPX extraction mechanisms (DPX-RP), the GC/MS analysis has been shown to be efficient for the simultaneous extraction and analysis of THC and its metabolites.

This research was performed with the aim of developing a fast automated method for GC/MS determination of THC and its metabolites in whole blood. The only manually performed steps in this analysis involved spiking the samples with internal standard, protein precipitation with acetonitrile, and centrifugation. The rest of the method was completely automated, including extraction, concentration and chemical derivatization.

EXPERIMENTAL

Materials. BSTFA and MTBSTFA were purchased from Sigma Aldrich.

Instrumentation. Analyses were performed using a 6890N GC equipped with a 5975 (inert XL) mass selective detector (Agilent Technologies), PTV inlet (CIS 4, GERSTEL) and MPS 2 Prepstation with DPX option (GERSTEL).

Figure 1. A picture of the dual rail GERSTEL MPS 2 with DPX automation platform. The left MPS is equipped with a 100 uL syringe for injecting eluent and derivatizing reagents, and the right MPS is equipped with a 2.5 mL syringe for performing DPX extractions.

Analysis conditions.

PTV:	1 min solvent vent (150 mL/min) splitless 80°C (1 min); 12°C/min; 280°C (3 min)
Column:	30 m HP 5 (Agilent) d_i = 0.25 mm d_f = 0.25 µm
Pneumatics:	He, constant flow = 1.5 mL/min
Oven:	100°C (1.5 min); 40°C/min; 200°C; 8°C/min; 240°C (1 min); 10°C/min; 300°C (3 min)
MSD:	SIM

Analyte	Ret. Time [min]	Target Ion [m/z]	Qual. Ions [m/z]
THC-TMS	9.68	386	303, 371
THC-d_3-TMS	9.66	389	306, 374
OH-THC-2TMS	12.11	371	473, 488
OH-THC-d_3-2TMS	12.09	374	476, 491
COOH-THC-TMS/ TBDMS	14.83	371	473, 515
COOH-THC-d_3-TMS/TBDMS	14.81	374	476, 518

Initial Sample Preparation

- Deuterated internal standard containing d_3-THC, d_3-OH-THC and d_3-COOH-THC was added to 0.5 mL samples of blood resulting in concentrations of 5 ng/mL of each compound.
- The samples were protein precipitated using 0.75 mL acetonitrile, centrifuged, and the resulting supernatants were transferred to clean, labeled sample tubes.
- 1mL of deionized water and 50 µL of 0.1 M HCl were added to each tube.
- All tubes were then placed onto the GERSTEL MPS 2 sample tray.

Automated DPX

Automated DPX was performed using 1 mL DPX-RP (reversed phase) tips from DPX Labs, LLC.

- Multiple extractions (3 in total) of approximately 0.7 mL each were performed with the GERSTEL MPS 2.
- After extracting the analytes from the sample solution, a wash step was performed by adding 0.5 mL of 10 % acetonitrile in water to the top of the DPX tip and dispensing it to a waste container.
- Elution of the analytes was performed by introducing 0.7 mL of CH_2Cl_2 to the DPX tip from the top and collecting the eluent in a clean GC vial.
- The total time required for performing the extraction and all associated steps in the MPS 2 was approximately 6 minutes per sample.

Automated injection of the extract combined with analyte concentration and derivatization.

The derivatizing reagent contained 33 % BSTFA and 33 % MTBSTFA in acetonitrile. A "sandwich" injection of derivatizing reagent and eluent was performed by the left rail of the MPS 2 by first aspirating 20 µL of derivatizing reagent, then aspirating 20 µL of air, then aspirating 50 µL of eluent. The injection speed was 1.32 µL/s, allowing the solvent to evaporate and concentrate the analyte inside the injection port.

Results and Discussion

The DPX method was chosen for automated extractions because it can be performed in just a few minutes (6 min). For high throughput analysis, the MPS 2 enables parallel processing of a sample during the chromatographic analysis of the preceding sample.

The GERSTEL MPS with automated DPX option prepares samples on a just-in-time basis. Whenever the GC becomes ready after a run, the next sample is ready to be injected. Only one sample is prepared at a time, but since the process takes less than 6 minutes, and thereby less than the GC run, the GC never has to wait idly for the next injection. This "just-in-time" sample preparation provides the highest possible throughput while ensuring that all samples are treated in the exact same way. Prepared samples never have to wait prior to injection.

For determination of THC and THC metabolites in whole blood, high sensitivity is required (LOD of 1 ng/mL for THC and OH-THC and 2 ng/mL for COOH-THC). Whole blood extracts contain possible interferences, and the analytes must be derivatized. In order to achieve the best possible efficiency of the complete process, the GERSTEL Cooled Injection System (CIS) was used simultaneously as a sample inlet, a concentration device and derivatization reactor for the analytes during the injection process.

The DPX extraction was performed using 1 mL DPX-RP tips which were fitted with "transport adaptors" for automation with the GERSTEL MPS 2. The transport adaptors enable the MPS to introduce wash and elution solvents through the "top" of the DPX

tip, an approach that has proven to provide cleaner extracts and higher recoveries. Especially when the LVI technique is used, it is important that the extracts are clean in order to prevent buildup of residue in the inlet liner which could have deleterious effects on subsequent chromatographic analyses.

Initially, derivatization was performed using BSTFA. However, it was found that BSTFA provided varying degrees of derivatization efficiency for COOH-THC in particular, depending on the manufacturer and lot of the BSTFA reagents. Poor reproducibility was observed both for conventional "off-line" derivatization and for derivatization in the inlet as used in the work described here. Use of MTBSTFA seemed to correct the problem, but the sensitivity obtained for THC and OH-THC was significantly reduced. Through a series of targeted studies, we found that combining the two reagents resulted in high derivatization efficiencies for all three analytes enabling us to simultaneously determine all 3 analytes in a single chromatographic analysis.

The results of the study indicate good reproducibility and recovery (Table 1). It should be noted that the protein precipitation caused some loss of analytes so the actual DPX extraction recovery was much better. There are three factors that limit the DPX extraction efficiency. One is that the solution pH could be lowered further by addition of strong acid to improve the recovery of COOH-THC, but this would create interference problems for the determination of THC. A second factor is that CH_2Cl_2 was used for elution, and this is not the most suitable solvent for COOH-THC. However, this solvent is ideal for use with LVI and it provided better results when performing concentration and derivatization in the CIS inlet. The third factor is that reducing the sample volume that is extracted would provide higher recovery. For example, multiple extractions of a total of 0.5 mL provided higher recovery than a single extraction of a 0.7 mL sample. Multiple extractions would of course require more time in total, leading to reduced productivity and throughput.

Table 1. Recovery data for THC and its metabolites.

Compound	Concentration [ng/mL]	Recovery [%]
THC	5	51
OH-THC	5	59
COOH-THC	20	41

The most significant findings in this study are the reproducibility and limits of detection (LOD), which are listed in Table 2. The results show very good reproducibility based on the calibration plots (Figures 2-4), with coefficients of variation (CVs) of less than 5 % for all 3 analytes. The LOD was found to be 0.5 ng/mL for both THC and OH-THC, based on a signal-to-noise (S/N) of 5 for the lowest intensity qualifier ion. In Fig. 5, the qualifier ion of 303 has a S/N of approximately 10:1 at 0.5 ng/mL, clearly showing that this analyte is readily detected at this low concentration.

Table 2. Reproducibility and LOD data.

Compound	C. V [%]	LOD [ng/mL]	LOQ [ng/mL]
THC	2.0	0.5	1.0
OH-THC	3.2	0.5	1.0
COOH-THC	3.1	2.0	2.0

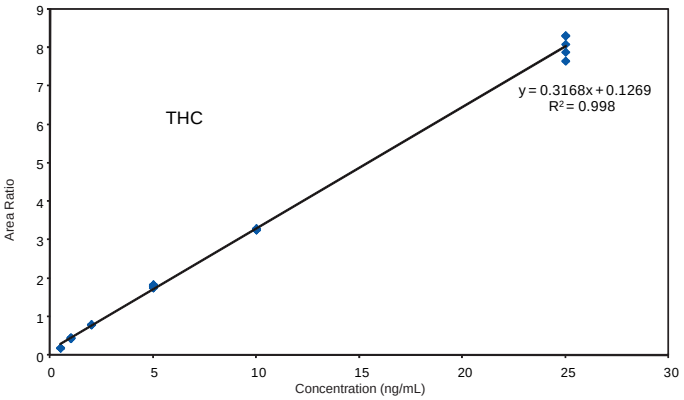

Figure 2. Calibration plot of THC extracted from whole blood.

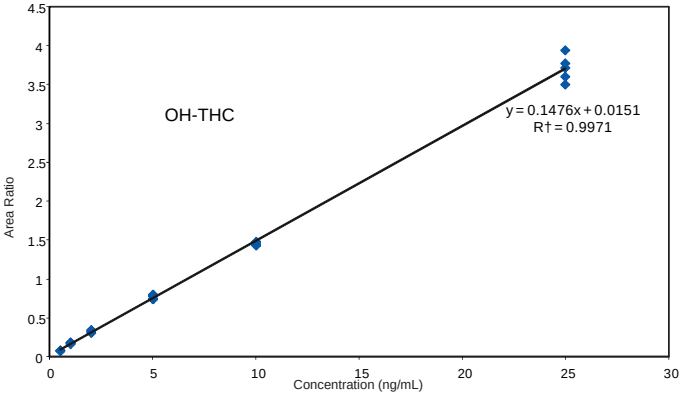

Figure 3. Calibration plot of OH-THC extracted from whole blood.

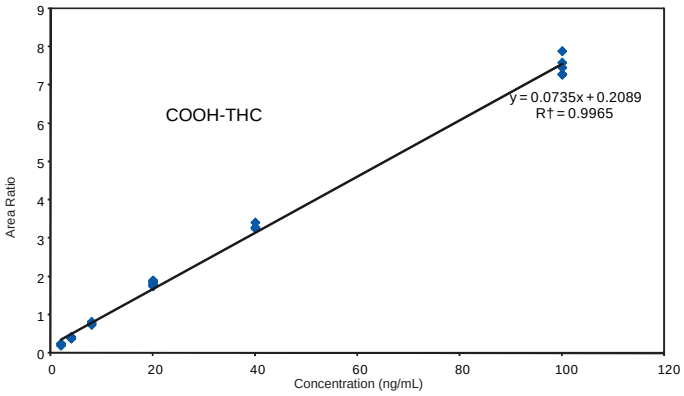

Figure 4. Calibration plot of COOH-THC extracted from whole blood.

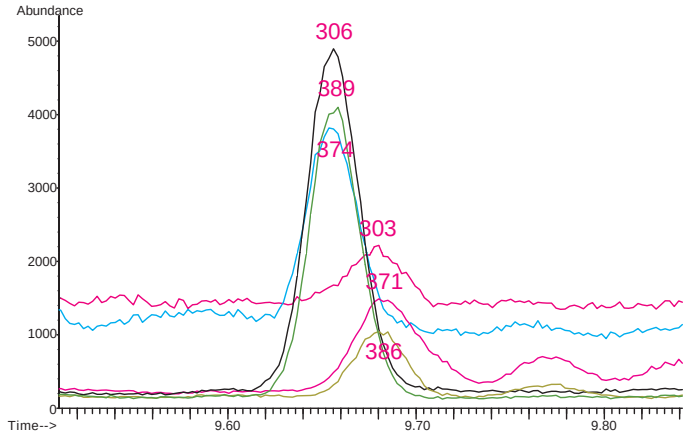

Figure 5. Extracted ion chromatogram of THC-TMS at 0.5 ng/mL, showing target and qualifier ions.

For OH-THC (Fig. 6), the qualifier ion of 474 has a S/N of about 8 at 0.5 ng/mL. The target ion, however, has a much greater S/N ratio enabling detection at concentrations below 0.1 ng/mL. Please note that part of the 372 ion signal intensity is being contributed to d_3-OH-THC, and it is readily apparent at the low concentration of 0.5 ng/mL. If it is deemed necessary to have a 3rd qualifier ion for OH-THC, then a lower concentration of 2 ng/mL for d_3-OH-THC (instead of 5 ng/mL) should enable detection of this ion without interference at the LOD of 0.5 ng/mL.

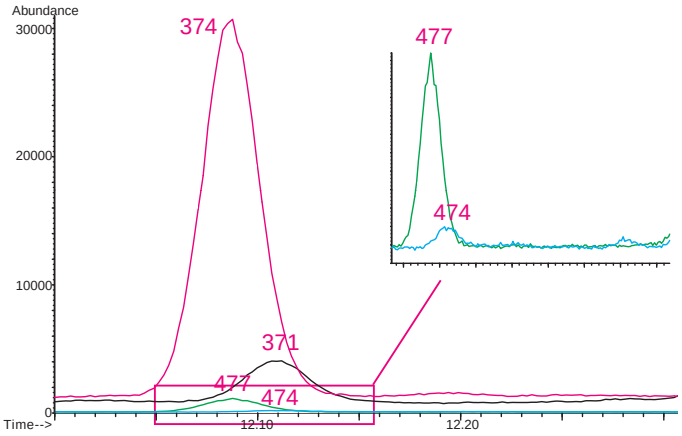

Figure 6. Extracted ion chromatogram of OH-THC-2TMS, showing target and qualifier ions.

The determination of COOH-THC was challenging when using BSTFA derivatization. This may have been caused by the quality of the commercial derivatizing reagent. The same was not the case with MTBSTFA, but this reagent did not provide as good results with THC and OH-THC. By combining MTBSTFA and BSTFA, we could reproducibly detect COOH-THC-TMS/TBDMS without any apparent difficulties. In Fig. 7, the extracted ion chromatogram of COOH-THC-TMS/TBDMS is shown at 2 ng/mL.

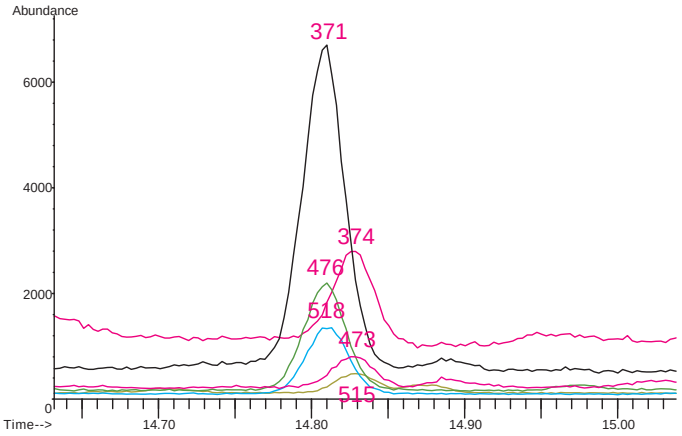

Figure 7. Extracted ion chromatogram of COOH-THC-TMS/MTBDMS, showing target and qualifier ions.

Conclusions

- DPX methods are readily automated using the GERSTEL MPS 2; Automated DPX methods are easily set up using the MAESTRO software.
- The DPX extractions are rapid, the complete procedure is performed in less than 6 minutes.
- When using the CIS inlet, automated sample introduction can be combined with concentration and derivatization.
- Limits of detection are below 1 ng/mL for THC and OH-THC, and below 2 ng/mL for COOH-THC with less than 5 % C.V. using automated DPX combined with derivatization in the CIS inlet using BSTFA and MTBSTFA.
- Automated DPX performed with the dual rail GERSTEL MPS 2 provides the highest sample throughput available.

References

[1] J. L. Schroeder, L. J. Marinetti, R. K. Smith, W. E. Brewer, B. L. Clelland and S. L. Morgan, "The analysis of Δ9-Tetrahydrocannabinol (THC) and metabolites in Whole Blood and 11-nor-Δ9-Tetrahydrocannabinol-9-carboxylic acid (THCc) in Urine using Disposable Pipette Extraction with Confirmation and Quantification by Gas Chromatography-Mass Spectrometry", Journal Anal. Tox., in press.

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GERSTEL GmbH & Co. KG
Eberhard-Gerstel-Platz 1
45473 Mülheim an der Ruhr
Germany
+49 (0) 208 - 7 65 03-0
+49 (0) 208 - 7 65 03 33
gerstel@gerstel.com
www.gerstel.com

GERSTEL Worldwide

GERSTEL, Inc.
701 Digital Drive, Suite J
Linthicum, MD 21090
USA
+1 (410) 247 5885
+1 (410) 247 5887
sales@gerstelus.com
www.gerstelus.com

GERSTEL AG
Wassergrabe 27
CH-6210 Sursee
Switzerland
+41 (41) 9 21 97 23
gerstelag@ch.gerstel.com
www.gerstel.ch

GERSTEL K.K.
1-3-1 Nakane, Meguro-ku
Tokyo 152-0031
SMBC Toritsudai Ekimae Bldg 4F
Japan
+81 3 5731 5321
+81 3 5731 5322
info@gerstel.co.jp
www.gerstel.co.jp

GERSTEL LLP
Level 25, North Tower
One Raffles Quay
Singapore 048583
+65 6622 5486
+65 6622 5999
SEA@gerstel.com
www.gerstel.com

GERSTEL Brasil
Av. Pascoal da Rocha Falcão, 367
04785-000 São Paulo - SP Brasil
+55 (11)5665-8931
+55 (11)5666-9084
gerstel-brasil@gerstel.com
www.gerstel.com.br

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