Journal of Chromatography A, 1204 (2008) 87–92

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Validated gas chromatographic–mass spectrometric analysis of urinary cannabinoids purified with a calcium-hardened β-cyclodextrin polymer

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ARTICLE INFO

Article history:
Received 5 March 2008
Received in revised form 16 July 2008
Accepted 17 July 2008
Available online 5 August 2008

Keywords:
Cannabinoids
β-Cyclodextrin
Solid-phase extraction
Inclusion complex
GC–MS

ABSTRACT

A comprehensive solid-phase extraction (SPE) technique based on the formation of an inclusion complex between β-cyclodextrin (βCD) and cannabinoids including Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) was developed in gas chromatographic–mass spectrometric (GC–MS) analysis. A βCD/epichlorohydrin copolymer was prepared and then 'hardened' in aqueous solution with 0.3 M $CaCl_2$ to yield a stable particulate copolymer, which was used as a novel SPE sorbent. An internal standard THC-COOH-d_9 was added to urine samples containing 3 cannabinoids and then purified with the hardened βCD polymer. The cannabinoids were extracted from the hardened βCD using tetrahydrofuran. Resulting extracts were evaporated and derivatized with MSTFA/NH_4I/dithioerythritol (500:4:2, v/w/w) and analyzed by GC–MS in selected-ion monitoring (SIM) mode. Overall recoveries ranged from 85% to 102%, with a detection limit of 0.2 μg L^{-1} for the three cannabinoids tested. The precision (% CV) and accuracy (% bias) of the assay were 1.2–5.1% and 93–111% in 0.2–50 μg L^{-1} calibration range, respectively ($r^2 > 0.9997$). Twenty actual samples positive by fluorescence polarization immunoassay were also quantitatively analyzed. The devised technique based on the calcium-hardened βCD sorption of cannabinoids and subsequent GC–SIM/MS resulted in better selectivity and extraction efficiency than is possible using the conventional hydrophobicity-based SPE methods.

1. Introduction

THC (Δ^9-tetrahydrocannabinol) is one of the most widely used drugs of abuse. It occurs naturally in the marijuana plant *Canabis sativa* and it is rapidly metabolized to 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) in liver and other tissues (Fig. 1) [1–5]. Cannabinoids accumulate in body fat due to their lipophilic characters, and are slowly excreted from the body. In addition, urinary THC-COOH is an indicator of THC, and can be detected in infrequent users for a few days and in frequent users for weeks or even months after administration [6,7].

Analysis of cannabinoids in biological samples, such as urine, plasma, hair, and oral fluid, is commonly performed using GC–MS (gas chromatography–mass spectrometry)-based methods [7–12]. These comprehensive techniques have an acceptable sensitivity, but they described sample preparation procedures using the solid-phase extraction (SPE) followed by a multi-step liquid–liquid extraction combined with GC–MS analysis. SPE is mainly used to isolate cannabinoids from biological specimens, and the simplicity of work-up procedures is an important forensic issue when cannabinoids are likely to be present at low concentrations in urine [9,10,13]. General SPE procedures based on silica sorbents are pH dependent, and thus, sample losses are inevitable as individual cannabinoids have different optimal extraction pH values [14,15]. Therefore, pH durable sorbents have been extensively used, but sample preparations are time consuming and require activation of sorbents. Thus, a reliable selective sample preparation technique is necessary to isolate cannabinoids from complex biological specimens.

β-Cyclodextrin (βCD) is an α-1,4-linked cyclic oligosaccharide that possesses seven glucose units, which in aqueous media

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doi:10.1016/j.chroma.2008.07.083

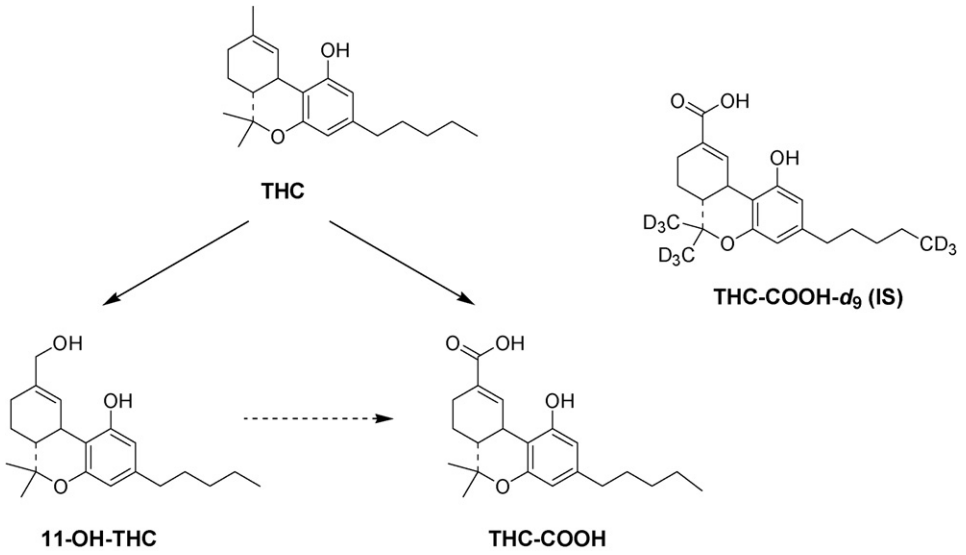

Fig. 1. Major metabolic pathways of Δ^9-tetrahydrocannabinol and the internal standard used.

presents a hydrophilic surface that conceals a hydrophobic interior. These hydrophobic regions are responsible for its absorbing hydrophobic guest molecules to form inclusion complexes [16–18]. Although cyclodextrin SPE has been shown to effectively binding steroids [19–21], its practical applications center on the isolation of hydrophobic species usually in combination with liquid chromatography (LC) or capillary electrophoresis (CE) [22–25]. Moreover, although βCD-based soft gels, like SPE sorbents, bind steroids effectively, their practical applications are limited because of their gel-like characteristics, i.e., their intrinsic swelling in aqueous solutions [16,26].

The inclusion-complex-based SPE with βCD was successfully applied with urinary steroids [27] and it could be expanded to include other steroidal compounds and possibly other biological compounds as universal extraction sorbents. Due to the structural similarities of cannabinoids and steroids, inclusion complexes of cannabinoids in βCD have been administered sublingually to improve bioavailability [28–31]. To investigate the binding affinity of βCD polymer as an alternative SPE sorbent for urinary cannabinoid analysis, the present study was designed and tested with the calcium-hardened βCD polymer. However, no study has been conducted on the use of these complexes for urinary cannabinoids analysis. Here, we describe a method for the quantitative analysis of THC, 11-OH-THC, and THC-COOH in human urine using calcium-hardened βCD polymer.

2. Materials and methods

2.1. Chemicals

THC, 11-OH-THC, THC-COOH and THC-COOH-d_9 (internal standard, IS) were obtained from Cerilliant (Round Rock, TX; Fig. 1). *N*-Methyl-*N*-trifluorotrimethylsilyl acetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE) were purchased from Sigma (St. Louis, MO). β-Cyclodextrin (βCD) and epichlorohydrin were obtained from Sigma–Aldrich (St. Louis, MO). A 50% glycerol solution of β-glucuronidase from *E coli* (140 U mL^{-1}) was purchased from Roche (Mannheim, Germany). All organic solvents used were of analytical or HPLC grade and were purchased from Burdick & Jackson (Muskegan, MI).

Stock solutions of cannabinoid reference standards were prepared at 10 mg L^{-1} in methanol, and working solutions were made up in methanol at concentrations from 10 to 1000 μg L^{-1}. All standard solutions were stored below −20 °C until required and there was no degradation during 3 months of method validation. The urine samples used for calibration and quality control (QC) were prepared by spiking drug-free urine with these working solutions.

2.2. Synthesis of polymerized β-cyclodextrin powder

The βCD polymer was synthesized based on previously reported methods [27,32,33]. The βCD monomer (2.5 g) was added to NaOH solution (2.5 g in 7.5 mL water) and 4.4 mL of epichlorohydrin was added dropwise over 20 min (a molar ratio of epichlorohydrin to βCD of 25:1). After stirring for 4 h at room temperature, the solution was mixed with water to quench the reaction. The gel formed was collected by filtration, immersed in 0.3 M $CaCl_2$ solution for 30 min, and washed with water and ethanol. This calcium treated gel was then vacuum-filtered and dried at 70 °C overnight. The calcium-hardened βCD polymer (a brittle white solid) was ground to a particle size of <1.0 mm, and used in subsequent experiments. The inclusion-complex-based SPE with βCD can be applied without having to incur irreproducible results and low recoveries caused by sorbent drying, pH limitations, and breakthrough when polar steroidal compounds were tested [27].

To evaluate binding capacities of different types of βCD based SPE techniques, the removal rate of all cannabinoids presented in QC samples was measured by GC–MS in triplicate. The removal rate was expressed as a percentage of the response of an extracted sample to which all reference standards at the same amounts had been added and a solution containing these standards directly injected into GC–MS without extraction steps.

2.3. Gas chromatography–mass spectrometry

The GC–MS analysis was performed using selected-ion monitoring (SIM) mode using an Agilent 6890 Plus gas chromatograph equipped with an Ultra-2 capillary column (25 m × 0.2 mm i.d., 0.33 μm film thickness; Agilent Technologies; Palo Alto, CA) and interfaced with an Agilent 5973N MSD. An electron energy of 70 eV and ion source temperature of 230 °C were used. Each sample

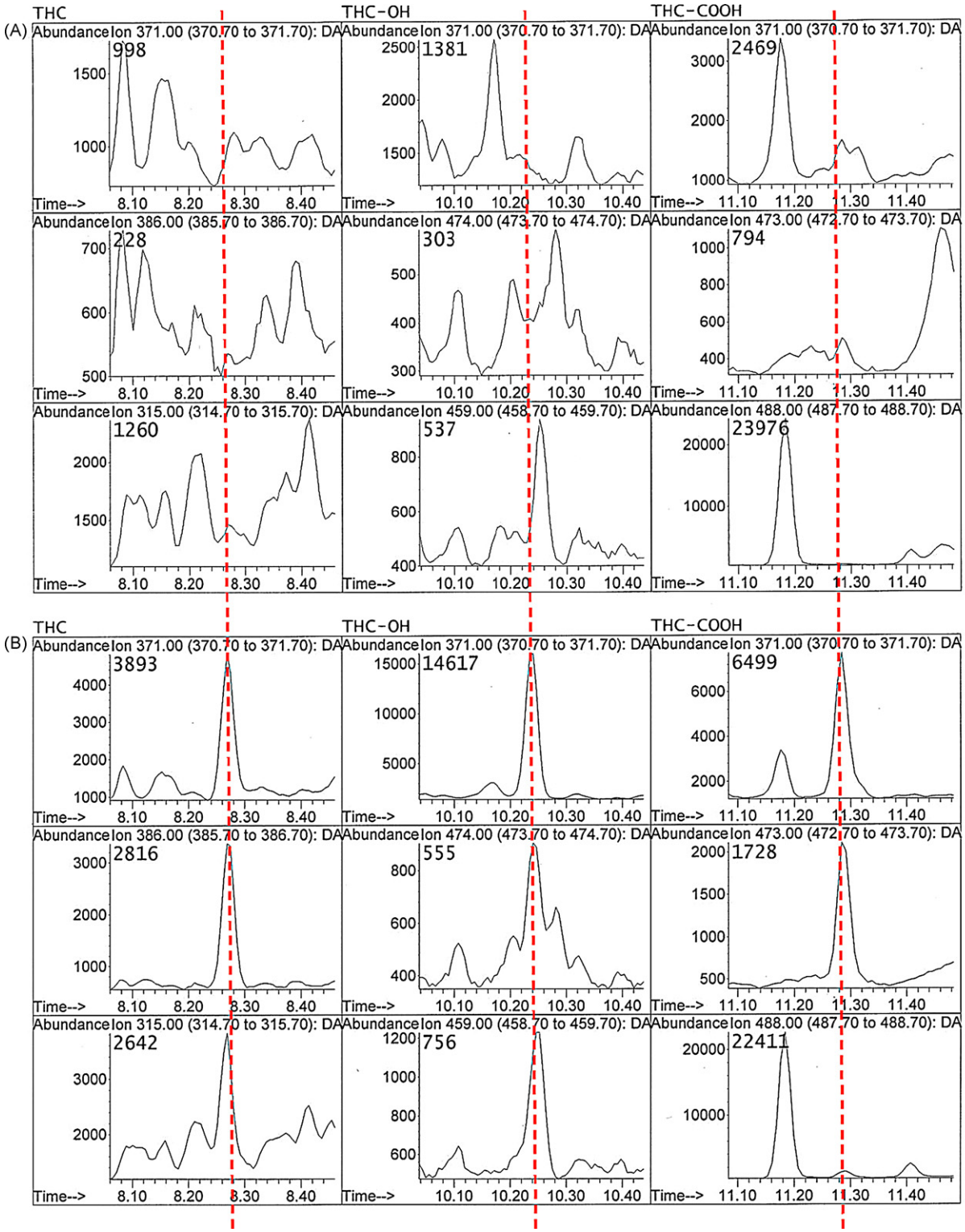

Fig. 2. Detection windows for the analysis of cannabinoids as trimethylsilyl derivatives obtained from (A) a urinary blank and (B) a spiked urine sample at urinary concentration of 2 μg L^{-1}. Ions were selected at *m/z* 386 (THC) and 371 (THC-OH and THC-COOH) for quantification. Three characteristic ions of each cannabinoid were monitored.

(2 μL) was injected in split mode (10:1) at 280 °C, using the following GC conditions: initial oven temperature 200 °C ramped to 240 °C at 8 °C min^{-1}, and then to 310 °C at 10 °C min^{-1} and held for 5 min. Helium was used as carrier gas at a column head pressure of 173 kPa (column flow: 0.8 °C min^{-1} at an oven temperature of 200 °C).

Characteristic trimethylsilyl (TMS)-derivatized cannabinoid ions were produced and identified by comparing retention times and the height ratios of three characteristic ions, i.e., *m/z* 371, 386, 315 for THC, *m/z* 371, 474, 459 for 11-THC-OH, and *m/z* 371, 473, 488 for THC-COOH. In the case of the THC-COOH-d_9 only *m/z* 380 was monitored.

Table 1
Intra- and inter-day validation results of the overall method

		Intra-day ($n=3$)			Inter-day ($n=4$)		
	Concentration ($\mu g\,L^{-1}$)	Mean ± SD ($\mu g\,L^{-1}$)	CV (%)	Accuracy (%)	Mean ± SD ($\mu g\,L^{-1}$)	CV (%)	Accuracy (%)
THC							
$r^2 = 0.9999$	1	0.93 ± 0.01	1.2	93.1	1.04 ± 0.02	2.3	103.7
	5	4.90 ± 0.25	5.1	98.0	4.85 ± 0.20	4.1	97.0
	20	20.33 ± 0.27	1.3	101.7	19.65 ± 0.49	2.5	98.3
11-OH-THC							
$r^2 = 0.9997$	1	1.02 ± 0.01	1.3	102.1	1.11 ± 0.02	2.0	111.3
	5	4.76 ± 0.24	4.9	95.2	4.93 ± 0.21	4.2	98.5
	20	20.01 ± 0.35	1.8	100.1	19.71 ± 0.60	3.0	98.6
THC-COOH							
$r^2 = 0.9999$	1	1.06 ± 0.04	3.5	105.8	1.11 ± 0.06	4.8	111.1
	5	4.71 ± 0.21	4.5	94.2	4.91 ± 0.25	5.0	98.3
	20	20.43 ± 0.61	3.0	102.1	20.19 ± 0.64	3.0	101.0

2.4. Sample preparation

Urine samples (2 mL) spiked with 20 μL of THC-COOH-d_9 (1 mg L^{-1}) were diluted with 1 mL of phosphate buffer (0.2 mol L^{-1}, pH 7.2) and incubated with 50 μL of β-glucuronidase for 1 h at 55 °C. After cooling at room temperature, 0.5 g of calcium-hardened βCD powder prepared was added and shaken for 10 min. βCD powder was then isolated by centrifugation at 3000 rpm for 8 min and added to a 1 mL acetate buffer (0.2 mol L^{-1}, pH 5.0) and tetrahydrofuran (3 mL). After sonication for 10 min, *n*-hexane (2 mL) was added, shaken for 10 min and centrifuged for 8 min at 3000 rpm. Phase separation was achieved by placing tubes in −20 °C bath to freeze the lower aqueous phase. The organic extracts were evaporated to dryness in a N_2 evaporator at 40 °C, and these residues were further dried in a vacuum desiccator over P_2O_5–KOH for 30 min. Finally, the isolated cannabinoids were derivatized with MSTFA/NH_4I/DTE (50 μL; 500:4:2, v/w/w) at 60 °C for 20 min.

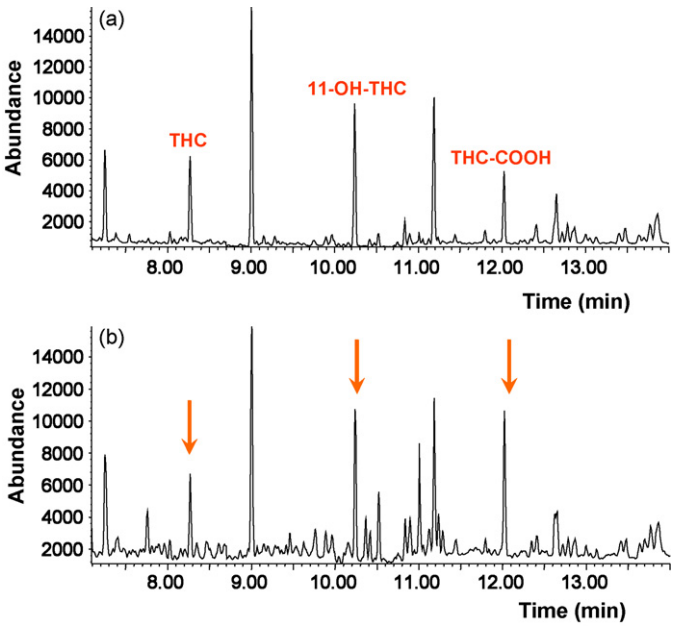

Fig. 3. Comparative total ion chromatograms of cannabinoids extracted initially with tetrahydrofuran and then further extracted using *n*-hexane and diethyl ether. (A) The chromatograms show that *n*-hexane reduces the matrix background contribution and the interference caused by matrix components as compared with (B) diethyl ether.

A routine procedure based on the use of Oasis HLB™ SPE cartridges (60 mg, 3 mL; Waters), preconditioned with methanol and water (1 mL each), was used for control purposes. Briefly, urine samples (2 mL) were spiked with 20 μL of IS (1 mg L^{-1}) in 1 mL of phosphate buffer (0.2 M, pH 7.2), and then incubated with 50 μL of β-glucuronidase for 1 h at 55 °C. SPE cartridges were placed in a device fitted with a small peristaltic pump and each was washed with 2 mL water and eluted twice with 1.5 mL of methanol. Combined methanol eluates were evaporated under a stream of nitrogen, and residues were further dried in a vacuum desiccator over P_2O_5–KOH for 30 min. Dried extracts were derivatized as described for isolated cannabinoids above.

2.5. Method validation

The QC samples containing all three analytes at different concentrations (1, 5 and 20 μg L^{-1}) were used over a period of 3 months. Quantification was performed using peak height ratios relative to that of the IS and calibration samples were made up at eight different concentrations (0.2, 0.5, 1, 2, 5, 10, 20, and 50 μg L^{-1}) and each sample treated with βCD powder followed by trimethylsilylation. Response linearity was measured in triplicate for the eight-point calibrations. Limits of detection (LOD) and of quantification (LOQ) were defined as lowest concentrations with a signal-to-noise (S/N) ratio of higher than 3 and 10, respectively.

Precision is expressed as coefficient of variation (% CV) and accuracy as percent relative error (% bias), and were determined from QC samples at 1, 5, and 20 μg L^{-1}. In terms of within-day repeatability, triplicates were analyzed, whereas reproducibility was measured from results obtained on four different days. Extraction recovery

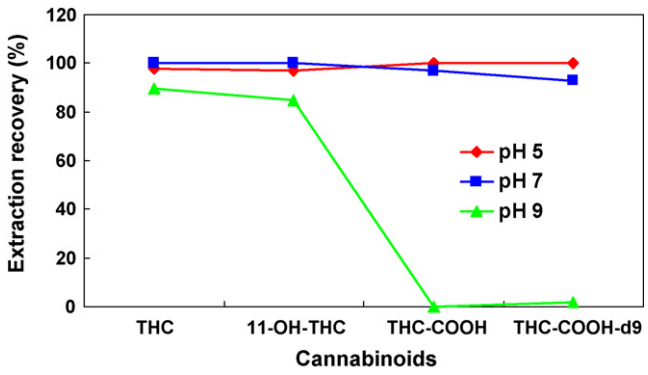

Fig. 4. Effects of extraction pH on cannabinoid analysis.

Table 2
Extraction recoveries for 5-times-recycled β-cyclodextrin powder

	Concentration ($\mu g\,L^{-1}$)	Extraction recovery, % ($n = 3$)	
		New polymerized βCD powder	Recycling polymerized βCD powder (SD)
THC	1	99.3	89.0 (7.3)
	5	89.3	84.4 (3.4)
	20	86.6	84.8 (1.2)
11-OH-THC	1	99.4	97.9 (1.1)
	5	87.1	82.2 (3.4)
	20	85.7	84.7 (0.7)
THC-COOH	1	102.1	102.9 (0.5)
	5	90.8	90.2 (0.4)
	20	88.5	80.9 (1.7)

was established using QC samples at the same three concentration levels in triplicate for each cannabinoid by adding known amounts of mixed working solutions to drug-free urine samples. Absolute recovery was calculated by comparing the peak height ratios of extracted samples versus those of their non-extracted counterparts.

2.6. Forensic applications

The present method was assessed using immunochemically THC-positive samples. Twenty urine samples were obtained from the Forensic Science Division at the Supreme Prosecutors' Office (Seoul) and stored at −20 °C until required for analysis.

3. Results and discussion

3.1. Preparation of calcium-hardened β-cyclodextrin polymer

The βCD polymer can be prepared using epichlorohydrin, which reacts with the hydroxyl groups of βCD monomer to form an insoluble cross-linked gel, the solubility of which is dependent on the molar ratios of βCD monomer and epichlorohydrin [16,26]. Although water-insoluble gels were obtained by reacting epichlorohydrin and βCD at a molar ratio of 25:1, these swelled unacceptably in urine samples, which reduced binding capacities in an irreproducible manner. To reduce this swelling in aqueous media, βCD polymer was 'hardened' using 0.3 M $CaCl_2$ solution, as described previously [34]. After optimizing this hardening process using different concentrations of $CaCl_2$, a white solid product was obtained. This hardened βCD polymer was then ground to a powder of particle size 0.5–1.0 mm and used to analyze urine samples.

3.2. GC–MS analysis

Using the described GC–MS conditions, THC, 11-OH-THC, THC-COOH and deuterated THC-COOH were successfully separated as TMS derivatives within 14 min in SIM mode. Representative SIM chromatograms for spiked urine at the concentration of 2 $\mu g\,L^{-1}$ with each cannabinoid are presented in Fig. 2. Peak identification was straightforward and achieved using three characteristic ions. Quantitative ions at m/z 386 for THC and m/z 371 for 11-THC, and THC-COOH were used. In the case of THC, the less intense fragment at m/z 386 was chosen to improve selectivity instead of the most intense peak (m/z 371), due to urinary interference.

3.3. Optimization of sample preparation

A 0.5-g hardened βCD powder was used, because amounts greater than this did not affect binding affinity estimated to be >90% based on replicated experiments. To optimize urine/powder mixing times, we examined cannabinoid take-up for mixing times of 1 min to 2 h and decided on 10 min. The hardened βCD powder showed excellent recoveries, which ranged from 94% to 111% for the cannabinoids studied (Table 1). The devised technique was found to detect lower levels of cannabinoids in human urine. Non-glucuronic acid conjugated cannabinoids were better taken up by hardened βCD than conjugated cannabinoids, and thus, a β-glucuronidase hydrolysis step was included before the SPE procedures.

The cyclic nature of βCD creates a hydrophobic cavity that accommodates guest molecules and forms inclusion complexes. Generally, such complexes are dependent on binding between guest species and the interior surfaces of these cavities, and this binding in turn is dependent on the of factors that govern molecular interactions, i.e., van der Waals forces, hydrogen bonding, steric effects, and solvent effects. Moreover, complexation methods have been used to improve the solubilities, dissolution rates, and absorptions of many lipophilic drugs [28–31].

On a separate topic, the entrapment of steroids by hardened βCD, and found that hydroxylated estrogens are more effectively captured than other steroids, which suggest that hydrogen bonding occurred between the phenolic hydroxyls of estrogen and the exterior hydroxyls of βCD. It has previously been reported that hydrogen bonding plays a role in the binding of hydroxylated estrogens by βCD resins [20]. In addition, cannabinoids possess a phenolic ring, and have been reported to form stable "transverse" complexes with βCD, and furthermore, alkyl side chains of the cannabinoids have

Table 3
Analysis of cannabinoids in urine samples from marijuana abusers

	THC ($\mu g\,L^{-1}$)	11-OH-THC ($\mu g\,L^{-1}$)	THC-COOH ($\mu g\,L^{-1}$)
1	ND	2.52	32.56
2	ND	ND	33.19
3	ND	ND	4.91
4	ND	ND	28.93
5	ND	ND	4.76
6	ND	ND	1.54
7	1.73	ND	15.28
8	ND	ND	0.95
9	ND	ND	1.97
10	ND	ND	3.52
11	ND	1.09	20.28
12	ND	ND	5.31
13	ND	ND	5.67
14	ND	ND	5.43
15	ND	ND	12.10
16	0.36	2.34	45.25
17	ND	ND	7.98
18	ND	ND	8.21
19	ND	ND	39.55
20	ND	ND	3.95

ND, not determined.

been proposed as preferred sites for substituent groups in terms of their entrapment by βCD [29–31].

Liquid–liquid extraction experiments were performed using methanol, ethanol, isopropyl alcohol, ethylacetate, diethyl ether, and *n*-hexane to identify an optimal medium for extracting cannabinoids from βCD, but all were unsuitable. However, tetrahydrofuran (THF) efficiently extracted the three cannabinoids from βCD. On the other hand, the additional extracting organic solvent *n*-hexane was used because THF is water miscible. When relatively polar organic solvents (e.g., diethyl ether) were used instead of *n*-hexane, noise levels increased and selectivity and sensitivity suffered (Fig. 3). THF is a useful solvent as it is able to dissolve a wide range of organic compounds. As stated above the devised test method isolated almost all of the urinary cannabinoids present, and overall extraction efficiencies were not significantly enhanced by increasing mixing times or by changing temperatures.

The pH of media during the final extraction was also examined. Cannabinoid recoveries were 93–100% at pH from 5 to 7 for all four compounds examined. However, at values greater than pH 9, THC-COOH and d_9-THC-COOH were not detected (Fig. 4) because under such conditions phenolic groups are oxidized to quinine [7]. This was countered in practice by adjusting the pH to 5 by adding 1 mL of acetate buffer (pH 5) prior to THF extraction to enhance analyte stability.

3.4. Validation of the overall method

Method validation included evaluations of linearity, LOQ, LOD, precision, and accuracy by using calibration samples prepared from drug-free urine. The recovery tests were performed using control samples spiked with cannabinoid standard at three different concentrations (Table 1). The described method was found to be linear (correlation coefficient $r^2 > 0.9997$) over the dynamic range 0.2–50 $\mu g\,L^{-1}$ using calibration curves. The LOQ was determined as 0.2 $\mu g\,L^{-1}$, and the LOD as 0.1 $\mu g\,L^{-1}$ for all three cannabinoids.

Assay precision and accuracy were determined by analyzing three QC samples at three concentration levels (1, 5, and 20 $\mu g\,L^{-1}$) for each of the three analytes. Intra-day ($n = 3$) precisions (expressed as % CV) ranged from 1.2% to 5.1%, while accuracies (expressed as % bias) ranged from 93.1% to 105.8% for the three analytes, and inter-day ($n = 4$) precisions (% CV) and accuracies (% bias) ranged from 2.0% to 5.0% and from 97.0% to 111.3%, respectively (Table 1). Average extraction recoveries ranged from 86.6% to 99.3% for THC, 85.7% to 99.4% for 11-OH-THC, and 88.5% to 102.1% for THC-COOH for independent triplicate determinations (Table 2). These results showed that the improved extraction recoveries obtained from the SPE with Oasis HLB ranged from 81.4% to 93.6% for THC, 80.7% to 89.3% for 11-OH-THC, and 83.6% to 93.1% for THC-COOH in triplicate runs. In addition, the extraction efficiency of 5-times-recycled βCD powder was found to be as effective as unused powder at extracting cannabinoids from urine (Table 2). Our results indicate that the described hardened βCD powder can be recycled at least five times. After analyzing the QC samples treated with recycled βCD powder, the corresponding peaks of any cannabinoids were not detected.

3.5. Forensic application

The present method was applied to 20 urine samples that had been determined to be positive using an immunochemical method. The results obtained showed that THC was rapidly metabolized and sensitive detection at low concentrations of all cannabinoids tested (Table 3).

4. Conclusions

Initially, the aim of this study was simply to increase the density of βCD polymer to prevent its swelling in aqueous solution by using the calcium hardening technique. However, the resulting developed method was found to be better that the existing hydrophobicity-based SPE methods used for cannabinoid analysis. In particular, the described technique was found to efficiently entrap the urinary cannabinoids, THC, THC-COOH, and 11-OH-THC, in calcium-hardened βCD polymer. These three cannabinoids were found to form inclusion complexes with βCD, and the devised method was found to provide high cannabinoid recoveries, very low background signals, and excellent sensitivity. We suggest that the devised method is suitable for analytical toxicology purposes and for forensic use.

Acknowledgements

This work was supported by intramural grants from the Korea Institute of Science and Technology (KIST), the National R&D program of the Korea Ministry of Education, Science and Technology (MEST), and the Korea Science and Engineering Foundation (KOSEF).

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