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Inclusion complex-based solid-phase extraction of steroidal compounds with entrapped β-cyclodextrin polymer

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ABSTRACT

Although the hydrophobic interaction-based solid-phase extraction (SPE) has been widely used, the extraction yields of steroids including androgens, estrogens, and corticoids were slightly different along with the physical and chemical properties of each molecule. A new SPE technique based on the formation of an inclusion complex with β-cyclodextrin (βCD) has been achieved for comprehensive sample purification in mass spectrometric analysis of 45 endogenous or synthetic androgens, 11 endogenous estrogens, and 21 corticoids. A copolymer of βCD with epichlorohydrin was prepared by a cross-linking reaction followed by entrapment with 0.3 M $CaCl_2$ to yield an improved SPE sorbent and the hydrolyzed urine samples were applied for purification. Steroidal compounds tested on the entrapped βCD polymer were extracted with tetrahydrofuran and the overall recoveries ranged from 82% to 112% for 77 steroids in urine. Especially, the hydroxylated estrogens showed an excellent binding capacity (96–116% recovery) to βCD through hydrogen bonding between their phenolic hydroxyl and exterior hydroxyl groups. A comparison between SPE methods with βCD and Oasis HLB as a conventional cartridge showed that the extraction efficiency of polar steroids was significantly increased in the βCD experiment, which has no connection with different polarity of steroid molecules. Due to its multi-functional mechanism derived from molecular inclusion and chemical interactions, this new SPE sorbent resulted in better selectivity and extraction efficiency than that obtained using the conventionally used hydrophobicity-based SPE method.

1. Introduction

There are many naturally occurring steroids that are similar in chemical structure and they could reflect biological actions [1]. In addition, a number of steroids and their analogues have been chemically synthesized and used for clinical trials as well as performance enhancing agents in sports. To achieve the exact identification of steroids at low concentrations, the sample preparation technique must provide a good yield and selectivity. Hence, the hydrophobic interaction-based solid-phase extraction (SPE) methods have been preferentially used employing XAD™ (styrene and divinylbenzene), Sep-Pak™ C18, and Oasis HLB™ (divinylbenzene and *N*-vinylpyrrolidine) as the commercial SPE sorbents [2–5].

Although the conventional SPE has been widely used in many applications, irreproducible results by undesirable silanol activity, sorbent drying, and breakthrough polar compounds limit the utility of the technique [6]. A reliable method

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of sample purification is usually necessary to selectively isolate target analytes from complex biological specimens. Several comprehensive methods have been developed to achieve adequate extraction of diverse steroid molecules [7,8]. In these studies, urinary steroids were isolated by hydrophobic and immunoaffinity interactions from complex matrices followed by the hydrophobicity-based chromatography combined with mass spectrometry.

The β-cyclodextrin (βCD), α-1,4-linked cyclic oligosaccharide with seven glucose units containing hydrophilic exterior and hydrophobic interior cavity, is responsible for formation of inclusion complexes with guest molecules through non-covalent interactions [9–14]. The immobilization of βCD on solid supports has been attempted to obtain an SPE sorbent [15,16], and other methods based on polymerization have been also studied to improve binding affinity and selectivity [17–19]. In recent, the affinity of the cross-linked βCD polymer with cholesterol showed higher chemical affinity than the immobilized βCD on glass beads [20–22]. The cross-linked βCD polymers as a water-insoluble soft-gel allows an effective affinity, but its amount is limited because of swelling in aqueous solutions [10,23]. Although cyclodextrin SPE has been studied to improve binding affinity with steroidal compounds [12,24–27], its application was mainly focused on separation sciences combined with liquid chromatography (LC) or capillary electrophoresis (CE) [11,28–30].

To improve the binding capacity with increasing density of polymerized βCD in analysis of steroidal compounds, including both endogenous and synthetic androgens, estrogens, and corticoids, it was entrapped with $CaCl_2$ solution, which is one of the simplest methods for entrapping biomolecules [31]. This method allows also improved sample recovery as well as detectability with effective elimination of non-steroidal compounds as urinary interference prior to gas chromatography or liquid chromatography coupled to mass spectrometry (GC–MS or LC–MS)-based analysis. This improved SPE sorbent was not swelled in aqueous solution and its extraction results are compared to that observed with Oasis HLB as a representative hydrophobic interaction SPE method.

2. Experimental

2.1. Chemical

The 77 steroids examined in this study (Supplemental Table 1) were obtained from Sigma (St. Louis, CA), Steraloids (Newport, RI), NARL (Pumble, Australia), and Cologne Laboratory (German Sports University, Köln, Germany). The internal standards, 16,16,17-d_3-testosterone, 2,4,16,16-d_4-estradiol and 9,11,12,12-d_4-cortisol, were purchased from NARL and C/D/N isotopes (Pointe-Claire, Quebec, Canada). The β-cyclodextrin and a cross-linking agent epichlorohydrin were obtained from TCI (Tokyo, Japan) and Sigma, respectively. The trimethylsilylating agents, *N*-methyl-*N*-trifluorotrimethylsilyl acetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE) were purchased from Sigma. The 50% glycerol solution of β-glucuronidase extracted from *E-coli* (140 U/mL) was purchased from Boehringer Mannheim (Mannheim, Germany).

For SPE experiments, a hydrophilic sorbent, Oasis HLB (60 mg, 3 mL; Waters, Milford, MA) was preconditioned with methanol followed by deionized water, and tested as a conventional method. All organic solvents used as the analytical and HPLC grades were purchased from Burdick & Jackson (Muskegan, MI). Deionized water was prepared by Milli-Q purification system (Millipore, Billerica, MA).

2.2. Standard solution and spiked urine samples

Each stock solution of all reference standards was prepared at a concentration of 1000 mg/L in methanol and working solutions were made up with methanol at varied concentrations in the range of 0.1–10 mg/L. All standard solutions were stored at −20 °C until used. To evaluate the binding capacity of an SPE sorbent, a quality-control sample at 40 μg/L urinary concentration of all steroids studied which was prepared in house from steroid-free urine [5]. After optimizing the SPE procedure, the first-morning urine obtained from a healthy male volunteer was also spiked with synthetic steroids to measure sample recoveries and the level of each steroid was relatively increased depending on the urinary concentration endogenously presented. Fluoxymesterone and oxandrolone positive urine samples were provided by the World Anti-Doping Agency (WADA) for re-accreditation on the anti-doping analytical test.

2.3. Preparation of entrapped β-cyclodextrin polymer

The entrapped βCD polymer was prepared based on previous reports [10,20]. To obtain different types of polymerized βCD with epichlorohydrin, different molar ratios of epichlorohydrin and βCD (1:1, 2:1, 5:1, 10:1, 25:1, and 50:1) were tested.

2.5 g of βCD was added into NaOH solution (2.5 g in 7.5 mL water) and 4.4 mL of epichlorohydrin was added dropwise for 20 min. The molar ratio of epichlorohydrin to βCD was kept at 25:1. After 4 h stirring at room temperature, the solution was mixed with water to quench the reaction, and the solid gel was collected by filtration. The gel was immersed in 0.3 M $CaCl_2$ solution for 30 min and washed with water and ethanol. The immersed gel was then vacuum-filtered and dried at 70 °C over-night. The resulting product was a brittle white solid which was ground in a mortar to yield small particles powder (<1.0 mm), which were used in subsequent experiments.

2.4. Scanning electron microscopy

The morphology of entrapped βCD polymer was examined at a magnification of 3000 using a JEM-1011 instrument (Jeol, Tokyo, Japan) operated at 80 kV, equipped with a MegaView III camera. For the measurements, samples were prepared by deposition of a drop of the solution onto carbon-coated copper grids.

2.5. Optimization of SPE experiments

To maximize binding capacity, the effects of both mixing temperature (25 and 60 °C) and time (5, 10, 20, 30, 60 and 120 min) of entrapped βCD polymer with the hydrolyzed urine samples were tested on each condition. Then, the extraction solvent

for isolation inclusive steroid molecules from βCD was evaluated with five different organic solvents, ethanol, methanol, diethyl ether, ethyl acetate, and THF. Different volumes of THF, an optimized extraction solvent were also examined with 0.5, 1 and 3 mL. And diethyl ether, ethyl acetate, and *n*-hexane as additional organic solvent were tested to partition water-miscible THF.

2.6. *Urinary sample pretreatment*

The urine sample (2 mL) was diluted with phosphate buffer (0.2 M, pH 7.2; 1 mL) and incubated with β-glucuronidase (50 μL) for 1 h at 55 °C. After cooling at room temperature, entrapped βCD polymer (0.5 g) was added, and then the mixture was shaken for 10 min and centrifuged for 5 min at 3000 rpm. After discarding the aliquot, phosphate buffer (1 mL) and tetrahydrofuran (THF; 3 mL) were added to residual βCD polymer, and sonicated for 10 min. For extraction of inclusive steroid molecules under pH 9.6, 5% K_2CO_3 (0.7 mL) and *n*-hexane (2 mL) were added. The solution was shaken (5 min), centrifuged (5 min, 2500 rpm), and the organic layer was taken. The organic solvent was evaporated by an N_2 evaporator at 40 °C and further dried in a vacuum desiccator for at least 30 min. In analysis of 21 corticoids and 2 hydrophilic steroids (gestrinone and tetrahydrogestrinone), the dried residue was reconstituted with 100 μL of 10% acetonitrile and injected 10 μL into a LC–MS system. For other steroids, the dried residue was derivatized with 50 μL of MSTFA/NH_4I/DTE (500:4:2, v/w/w) for 20 min at 60 °C and then injected 2 μL for GC–MS analysis. The general procedure with a conventional SPE sorbent [5,32,33] were also processed and compared.

2.7. *Instrumental conditions*

The GC–MS analysis of androgens and estrogens was performed with an Agilent 6890 plus GC interfaced to an Agilent 5973 MSD (Agilent; Palo Alto, CA). For glucocorticoids and two hydrophilic steroids, a Surveyor LC system coupled to a Finnigan TSQ Quantum Discovery mass spectrometer (Thermo; Waltham, MA) was used. All instrumental parameters are listed in Supplemental Table 2.

2.8. *Measurement of the binding capacity*

To evaluate binding capacities of different types of βCD-based SPE techniques, the removal rate of all steroids presented in quality-control samples was measured by GC–MS and LC–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 or LC–MS without extraction steps.

3. Results

For the host–guest applications of βCD in urinary steroid analysis, two immobilization-based βCD techniques [21,32] were initially tested. One immobilized βCD method resulted in 35–55% removal rates of urinary steroids while the other cross-linking immobilized βCD with epichlorohydrin showed little improved results as 46–68%. Then, the non-immobilized βCD-epichlorohydrin copolymerization [18,33] was successfully introduced to give a good removal rates ranged from 62% to 85% for all urinary steroids tested. Different types of βCD gel were obtained by changing the molar ratios of epichlorohydrin and βCD. These water-insoluble soft gels were obtained by reaction with epichlorohydrin and βCD (25:1 and 50:1 molar ratios), while the ratios of 10:1 produced a viscous liquid gel. When the molar ratio was less than 10:1, the resulting polymer tended to remain soluble in water. Since no significant difference was found in binding capacity of urinary steroids between 25:1 and 50:1 ratios controlled polymers, the 25:1 molar ratio was chosen.

Although the water-insoluble gel was successfully applied, its binding was not acceptable to be a comprehensive SPE method because of capacity swelling in an aqueous sample such as urine. Immersion of polymerized βCD gel in 0.3 M $CaCl_2$ solution therefore produced the modified rigid polymer (Fig. 1). After the immersing condition was optimized in different concentrations of $CaCl_2$ solution, the white solid product was finally obtained. Then $CaCl_2$ entrapped βCD polymer was ground to a powder with about 0.5–1.0 mm particle size for an SPE experiment. There was no swelling during sample preparation when this entrapped βCD polymer was adopted. As the effects of both mixing temperature and time of entrapped βCD polymer with steroids were also tested, they were not as much different in binding capacity. The minimum amount, 0.5 g of the βCD polymer was used because amounts larger than 0.5 g were enough to maximize the removal rates estimated to be >95%, based on replicate experiments. 0.5 g of entrapped βCD polymer was enough to adsorb all urinary steroids from 10 mL of urine.

The quantitative extraction of inclusively bound steroidal compounds with βCD is necessary since the levels of steroids in urine are found in a wide range of concentrations. In extraction protocol carefully evaluated with five different organic solvents, the entrapped βCD polymer showed the highest recovery yields (82–112%) of all steroids when using the THF extraction method, while diethyl ether and ethylacetate extractions ranged from 30% to 82%. In general, alcoholic solvents have been used to elute steroid molecules from conventional SPE sorbents [2–6]. However, ethanol and methanol extractions resulted in poor recoveries (41–82%) and swelling of entrapped βCD polymer in alcoholic solvents, may responsible for decreasing the recovery in sample preparation. Different volumes of THF were also examined and it was found that 3 mL of THF was enough to isolate inclusive steroid molecules from βCD.

Since the extraction solvent THF is water miscible, the additional organic solvent was needed in sample preparation and *n*-hexane was used to mix with THF. When the relatively polar organic solvents, such as diethyl ether and ethylacetate, were used, analytical backgrounds derived from sample matrix was increased and it was responsible for decreasing both selectivity and sensitivity. The overall extraction efficiency was not significantly increased along with increasing the mixing time and temperature tested. In addition, de-conjugated steroids were more effectively recovered than steroid glucuronides.

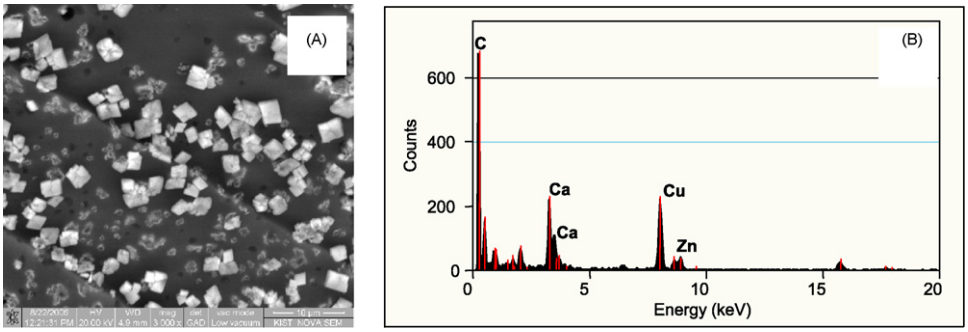

Fig. 1 – Scanning electron microscope analysis of entrapped β-cyclodextrin (βCD) polymer. (A) The morphology at a magnification of 3000 of copolymer of βCD with epichlorohydrin treated with 0.3 M $CaCl_2$ solution is presented. The entrapped βCD polymer by $CaCl_2$ has relatively homogenous structure. (B) Compositional analysis shows presence of Ca^{2+} ions on the surface of polymerized βCD powder.

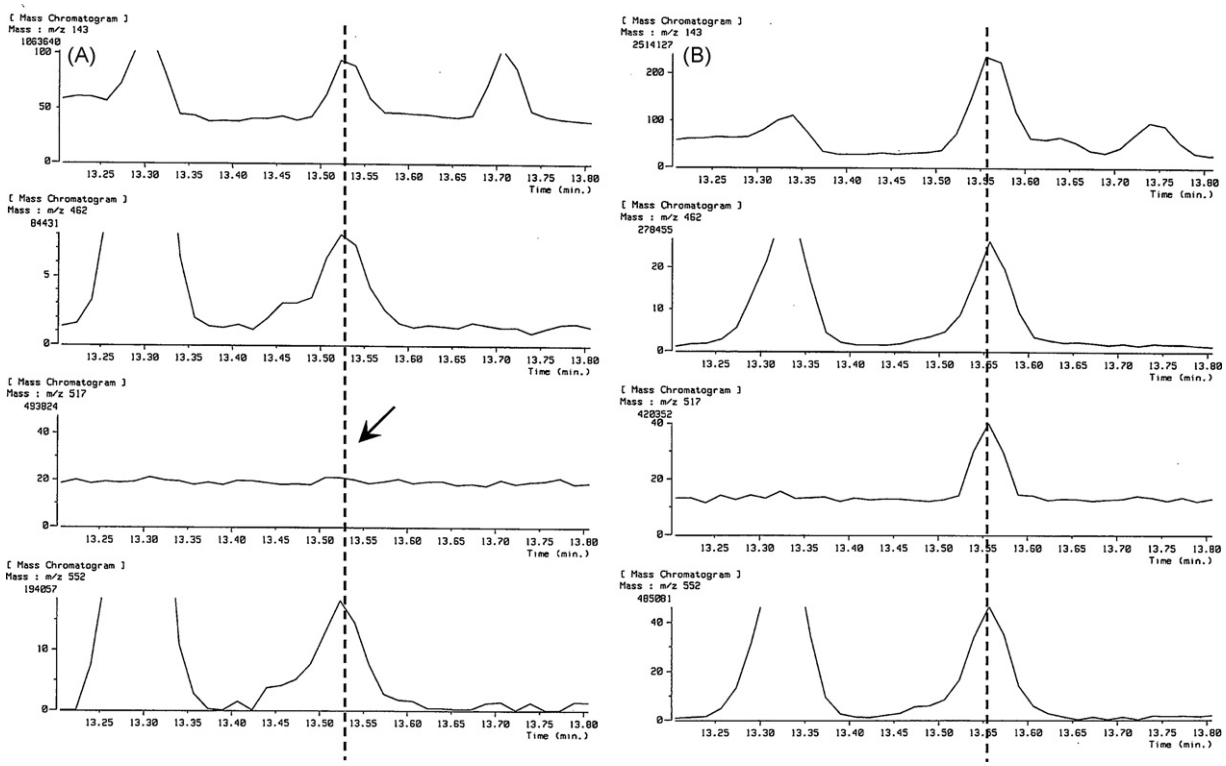

Fig. 2 – Extracted ion chromatograms of fluoxymesterone-M1 purified with (A) Oasis HLB and (B) entrapped βCD polymer. The spectrum of fluoxymesterone-M1 as its tetra-TMS derivative has characteristic ions at *m/z* 143 (D-ring cleavage), 462 (M^+–180), 517 (M^+–90–35), and 552 (M^+–90). No peak at *m/z* 517 was detected in sample purified with Oasis HLB, while chromatograms at four ions monitored were clearly detected in βCD experiment.

The SPE with entrapped βCD polymer was therefore processed after enzymatic hydrolysis with β-glucuronidase.

The adjusted SPE protocol for various steroidal compounds was used and the sample recoveries were slightly different along with the physical and chemical properties of each molecule (Table 1). The hydrophobic interaction SPE procedure used in steroid analysis [2–5] was also carried out with Oasis HLB and compared. The overall recoveries were increased in the βCD experiment as compared to the HLB protocol. A comparison between SPE methods with βCD and Oasis HLB shows clearly that the extraction efficiency of polar steroids was significantly increased in the βCD experiment (Supplemental Table 3). To verify this method with real urine samples, the extraction efficiencies for 14 androgens, 11 estrogens, and 2 corticoids as endogenous steroids were evaluated in triplicate and showed better results in βCD experiments, which are similar to those of control urine.

When the SPE experiments were applied to positive samples provided by WADA, the present method gave the reliable results for detection of urinary metabolites of polar steroids. The improved detectability of fluoxymesterone-M1, which shows very poor extraction yield in Oasis HLB experiment, was achieved by purification with βCD polymer (Fig. 2). In comparing the use of the βCD polymer, chromatogram did

Table 1 – Recoveries of steroids treated by entrapped βCD polymer ($n = 4$)

Steroids	Recovery (%)	S.D.	Steroids	Recovery (%)	S.D.
Synthetic androgens			Androgens		
Calusterone-M	92.5	10.0	11keto-A	96.4	9.6
Bolasterone-M	98.3	11.5	11keto-E	92.7	9.3
Bolasterone	95.7	4.2	11β-OH-A	92.4	8.8
Boldenone-M	95.6	7.2	11β-OH-E	93.1	9.1
Boldenone	89.9	15.6	ααβ-diol	93.1	8.3
Clostebol-M	93.8	4.7	αββ-diol	93.1	12.3
Mibolerone	98.1	5.9	Androstenediol	95.7	10.6
Fluoxymesterone-M1	105.4	6.4	Androstenedione	96.0	7.5
Fluoxymesterone-M3	97.8	6.9	Androsterone (A)	90.5	8.4
Ethisterone	93.5	4.5	DHEA	98.2	7.2
Drostanolone-M	96.8	11.4	DHT	98.1	9.8
Formebolone-M	96.4	11.1	Epitestosterone	97.6	6.1
16β-OH-furazabol	98.9	11.6	Etiocholanolone (E)	93.4	9.9
Methyltestosterone-M1	92.4	4.6	Testosterone	92.8	7.3
Methyltestosterone-M2	91.5	4.6	Estrogens		
Methandienone-M1	99.2	8.0	Estrone (E1)	98.5	11.6
Methandienone-M2	90.5	5.2	17β-Estradiol (E2)	97.9	5.8
Metenolone-M1	96.2	13.9	Estriol	105.1	10.8
19-Norandrosterone	91.8	3.1	2-OH-E1	98.6	3.9
19-Noretiocholanolone	90.3	0.7	2-OH-E2	100.6	0.6
6β-OH-turinabol	86.2	9.6	4-OH-E1	106.4	3.9
3′-OH-stanozolol	90.7	8.3	4-OH-E2	102.8	9.8
Epioxandrolone	108.4	3.6	16α-OH-E1	96.5	6.8
Oxandrolone	103.2	1.0	2-MeO-E1	99.6	9.9
Oxymesterone	97.2	8.6	2-MeO-E2	100.4	11.6
Ethylestrenol	100.9	4.3	17-Epiestriol	102.5	8.9
α-Trenbolone	104.5	16.0	Synthetic corticoids		
α-Norbolethone	92.6	4.8	Triamcinolone	112.4	12.4
β-Norbolethone	96.7	9.3	Prednisolone	107.2	9.8
Gestrinone	82.4	9.9	Prednisone	109.3	8.0
Tetrahydrogestrinone	85.2	8.0	Fludrocortisone	99.5	18.6
			6α-Methylprednisolone	96.7	15.8
			Betamethasone	95.0	17.4
			Dexamethasone	94.1	17.2
			Flumethasone	105.3	19.7
			Beclomethasone	92.7	15.3
			Triamcinolone-A	91.2	16.1
			Desonide	93.4	11.8
			Flunisolide	86.7	9.2
			Flurandrenolide	82.9	17.8
			Fluocinolone-A	91.4	15.7
			Desoximethasone	93.3	14.2
			Fluocortolone	89.6	17.9
			Budesonide	85.7	11.2
			Fluocinonide	82.6	12.0
			Amcinonide	83.3	10.6
			Cortisol (F)	99.1	9.5
			Cortisone (E)	104.2	10.9

not show interfering ion at *m*/z 517, while the Oasis HLB extract contained background noise. In another example with oxandrolone-positive urine sample, two corresponding peaks presented a different behavior. As shown in Fig. 3A, a synthetic androgen oxandrolone and its metabolite epioxandrolone were clearly detected without any significant interfering peaks at *m*/z 255, 343, and 435 through SPE with entrapped βCD polymer. These two compounds have been extracted from urine with a lower recovery than with other urinary steroids when Oasis HLB was introduced because of its polar structure (Fig. 3B). In addition, the extraction efficiencies of some polar steroids including fluoxymesterone-M1, oxandrolone and epioxandrolone were twofold improved against a hydrophobic interaction method (Supplemental Table 3).

In general, estrogens and corticoids are more polar than androgens and thus prone to be lost during the hydrophobicity-based SPE procedures. Estrogens and corticoids were strongly retained in the βCD polymer and then extracted quantitatively (>91%). The extraction recoveries of all steroids by the βCD method were similar, while the SPE with Oasis HLB showed a trend toward decreasing recoveries along with increasing polarity of steroids molecules. However,

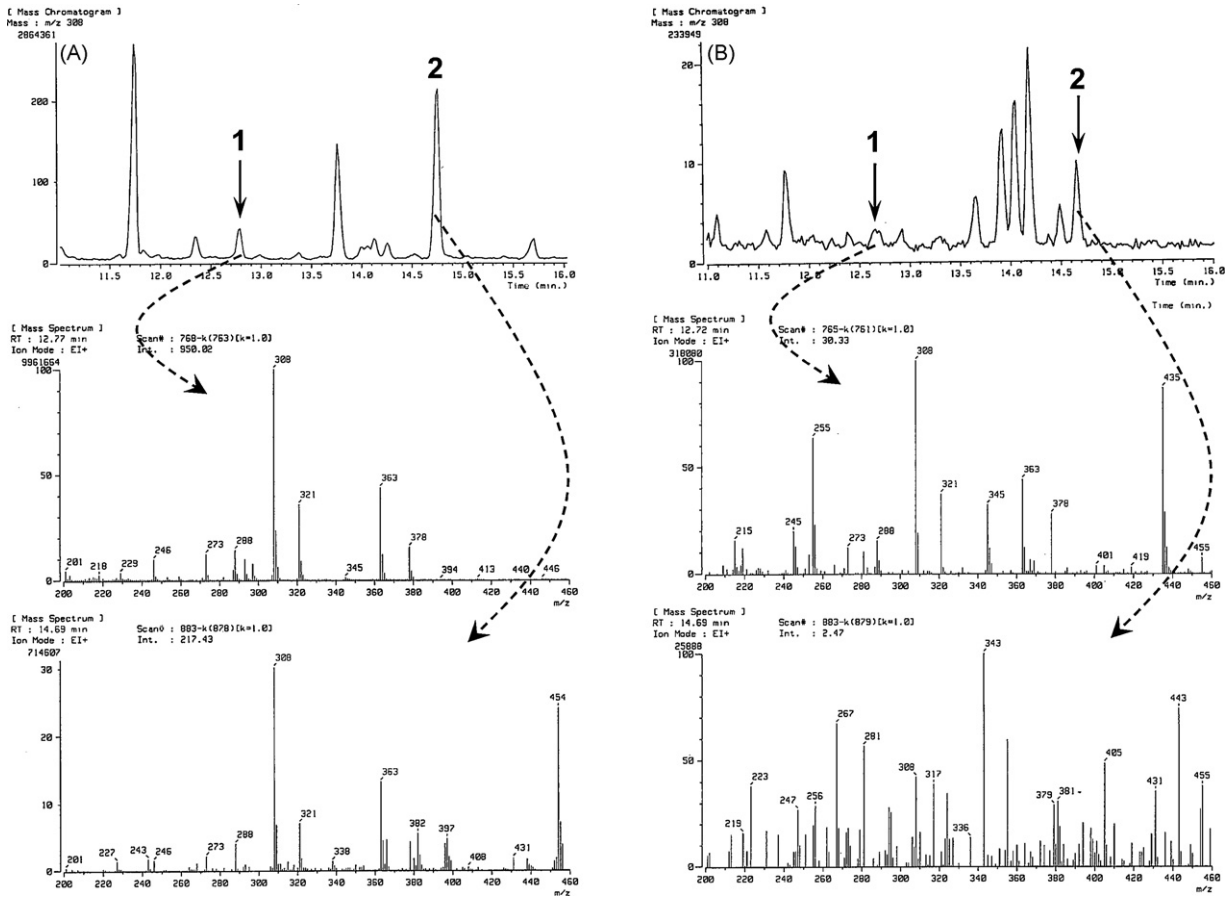

Fig. 3 – GC–MS chromatograms and mass spectra for identification of oxandrolone and its metabolite epioxandrolone using (A) entrapped βCD polymer and (B) Oasis HLB. Total ion chromatogram shows selectively detected both (1) epioxandrolone and (2) oxandrolone. The characteristic ions (*m/z* 308, 321, 363 and 378) for two compounds were clearly detected without any significant interference in the βCD experiment. The GC–MS conditions are listed in Supplemental Table 2.

standard deviations of recovery in corticoid analyses were somewhat altered compared to both androgens and estrogens, that should be more optimized (Table 1).

4. Discussion

The cyclic nature of βCD creates a hydrophobic cavity and it plays an important role in formation of inclusion complexes with guest molecules. Due to their size and hydrophobicity, steroidal compounds can form non-covalent complexes with βCD [9–14]. Therefore, the influence of different types of host–guest applications based on previous methods [18,21,32,33] was initially tested on the binding capacity using steroid-free urine samples spiked with a known amount of steroids. βCD has been also introduced for removing cholesterol from milk products [21,32,34]. However, methods used in milk products were not enough to extract urinary steroids.

βCD can be polymerized by a reaction between the hydroxyl groups of the molecules with a coupling agent epichlorohydrin to form insoluble cross-linked gels, the solubility is dependent on their molar ratios [10,23]. Although the water-insoluble gels were obtained by reaction with epichlohydrin and βCD of 25:1 molar ratio, swelling of the gel in a urine sample resulted in decreasing and irreproducible binding capacities in this study. The amount of βCD polymer as a soft-gel must be minimized because it may hinder the formation of inclusion complexes, which decreases the binding capacity. Treatment of the soft-gel with the $CaCl_2$ was therefore introduced to increase surface density (Fig. 1) and this surface modified polymer was ground to make a fine powder. Molecular entrapment with $CaCl_2$ is undertaken either for increasing stability of biomolecules or retaining the spherical shape of immobilized beads [31].

THF is one of the most polar ethers and is able to dissolve a wide range of organic compounds. The extraction strategy was to isolate as much of each urinary steroid as possible and all steroids tested were best recovered when THF was used as compared to other organic solvents. However, water-miscible THF extraction required an additional step to separate the organic layer from the aqueous layer. In subsequent experiments, low density and non-polar solvent *n*-hexane was successfully introduced to be *n*-hexane/THF phase, which is positioned as the upper layer in sample solution.

To achieve exact identification of target steroid molecules from the urine, the extraction technique must provide a good recovery and selectivity. The non-polar solvent extraction and tandem SPE procedure offered better selectivity with diminishing backgrounds, but they inevitably lead to loss of polar steroids [5]. Although extraction with polar solvents leads to increase extraction efficiency of most steroids, the selectivity is inversely decreased in MS-based analysis. In addition, many polar steroids resulted in low recoveries when hydrophobic interaction-based SPE methods were combined [5]. A dynamic equilibrium between CDs and guest molecules can be established if guest molecules are of sufficient size and have appropriate properties for the formation of inclusion complexes. Because of cavity size, αCD complexes well with aliphatic chains and molecules such as polyethylene glycol, whereas βCD is appropriate for aromatic rings [18]. In the attempt to maximize recovery of steroids, we easily established improved recoveries of all steroidal compounds tested, which have no connection with polarity (Table 1).

When polar steroids were tested with Oasis HLB, the extraction was less efficient, due to breakthrough without binding in hydrophobic interactions (Supplemental Table 3). Improved GC–MS selectivity as well as sensitivity of most steroids including polar steroids such as urinary metabolites of fluoxymesterone and oxandrolone was obtained from the βCD experiment (Figs. 2 and 3). These results could be expected because inclusion is a specific spatial interaction and it is not affected by differential polarity of guest molecules but based on the similarity of chemical structure. In addition, major interfering compound pregnanetriol was also extracted, but its characteristic ions (*m*/*z* 225, 345, and 435) were diminished by increasing peak intensity of epioxandrolone when βCD purification was processed (Fig. 3). De-conjugated steroids were more favored in inclusion complexes than their conjugates with glucuronic acid, which may indicate that the size effect appeared to be more pronounced than that of hydrophobic interaction. Although increased recovery of very polar steroids can be obtained by passage of the urine through the hydrophobic interaction cartridges at a low rate, it is still skillful, while the βCD technique could be easily adoptable.

Inclusion complexes are chemical species consisting of guest molecules in which one of the molecules, the host, can admit a guest component into its cavity, resulting in a stable encapsulation with several factors determining inclusion complex formation: hydrophilic effect, van der Waals interactions, hydrogen bonding, steric effects and solvent effects. Among the binding of steroids tested by entrapped βCD polymer, hydroxylated estrogens were more effectively captured than other steroids (Table 1), which may indicate the importance of hydrogen bonding between phenolic hydroxyl group and the exterior hydroxyl group of βCD (Fig. 4). There are reported indications that hydrogen bonding plays a role in the binding of hydroxylated estrogens by βCD resins [12]. Estrogens, having a phenolic A-ring, made βCD stable "transverse" complexes, but a corticoid prednisolone did not have a rigid complex [13]. The conformation of C- and D-ring of steroid skeleton also plays a key role in complex formation process even if the steroid species has a similar A- and B-ring structure [14].

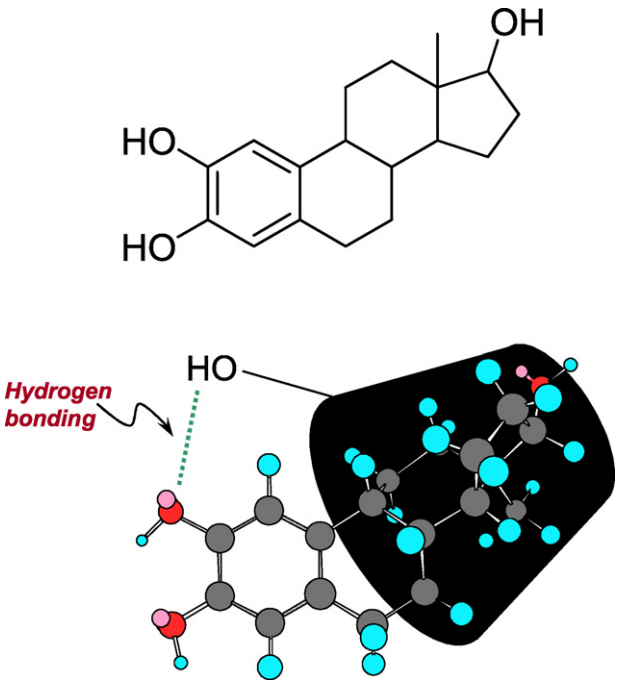

Fig. 4 – Proposed inclusion complex of βCD with 2-hydroxy estrone. Some parts of the molecule are included into the cavity of βCD, and others are on the outside. The A-ring of steroid molecule is predominantly formed complexes with the βCD and its structural feature have a great influence on the stability of the inclusion complexes through the hydrogen bonding between phenolic hydroxyl and the exterior hydroxyl groups of estrogen and βCD, respectively.

The aim of the present work was to improve the binding capacity of steroidal compounds by the surface modified polymerized βCD gel technique (described here as entrapped βCD polymer). The present 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 of polar steroids. In addition, this method can be expanded to include other steroid compounds and possibly other biological compounds as universal extraction sorbents. Steroid molecules are a diverse class of organic compounds and an optimized purification method should provide higher recoveries and selectivity as well as low background signals to improve sensitivity. Work to expand the host–guest applications of cyclodextrins to urinary steroids is underway as we strive to deliver a comprehensive method for steroid analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2008.04.008.

REFERENCES

[1] Falkenstein E, Tillmann HC, Christ M, Feuring M, Wehling M. Multiple actions of steroid hormones—a focus on rapid, nongenomic effects. Pharmacol Rev 2000;52:513–55.

[2] Schänzer W, Donike M. Metabolism of anabolic steroids in men: synthesis and use of reference substances for identification of anabolic steroid metabolites. Anal Chim Acta 1993;275:23–48.

[3] Palermo M, Gomez-Sanchez C, Roitman E, Shackleton CHL. Quantitation of cortisol and related 3-oxo-4-ene steroids in urine using gas chromatography/mass spectrometry with stable isotope-labeled internal standards. Steroids 1996;61:583–9.

[4] Choi MH, Hahm JR, Jung BH, Chung BC. Measurement of corticoids in the patients with clinical features indicative of mineralocorticoid excess. Clin Chim Acta 2002;320:95–9.

[5] Cho YD, Choi MH. Alternative sample preparation techniques in gas chromatographic–mass spectrometric analysis of urinary androgenic steroids. Bull Kor Chem Soc 2006;27:1315–22.

[6] Poole CF, Gunatilleka AD, Sethuraman R. Contributions of theory to method development in solid-phase extraction. J Chromatogr A 2000;885:17–39.

[7] Schänzer W, Delahaut P, Geyer H, Machnik M, Horning S. Long-term detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography–high resolution mass spectrometry. J Chromatogr B 1996;687:93–108.

[8] Dubois M, Taillieu X, Colemonts Y, Lansival B, De Graeve J, Delahaut P. GC–MS determination of anabolic steroids after multi-immunoaffinity purification. Analyst 1998;123: 2611–6.

[9] Davis ME, Brewster ME. Cyclodextrin-based pharmaceutics: past, present and future. Nat Rev Drug Discov 2004;3:1023–35.

[10] Crini G, Morcellet M. Synthesis and applications of adsorbents containing cyclodextrins. J Separ Sci 2002;25:789–813.

[11] Deng Y, Huang HJ. Capillary electrophoretic separation and theoretical study of inclusion complexes of sulfobutyl ether β-cyclodextrin with estrogens. Int J Quant Chem 2004;100:746–52.

[12] Zhu XX, Brizard F, Wen CC, Brown GR. Binding of bile acids by polymerized cyclodextrin resins. Pure Appl Chem 1997;34:335–47.

[13] Bednarek E, Bocian W, Poznanski J, Sitkowski J, Sadlej-Sosnowska N, Kozerski L. Complexation of steroid hormones: prednisolone, ethinyloestradiol and estriol with β-cyclodextrin. J Chem Soc Perkin Trans 2002;2:999–1004.

[14] Forgo P, Vincze I, Kover KE. Inclusion complexes of ketosteroids with β-cyclodextrin. Steroids 2003;68:321–7.

[15] Akiyama T, Hishiya T, Asanuma H, Komiyama M. Molecular imprinting of cyclodextrin on silica-gel support for the stationary phase of high-performance-liquid-chromatography. J Inclusion Phenom Macrocyclic Chem 2001;41:149–53.

[16] Ponchel A, Abramson S, Quartararo J, Bormann D, Barbaux Y, Monflier E. Cyclodextrin silica-based materials: advances characterizations and study of their complexing behavior by diffuse reflectance UV–vis spectroscopy. Micropor Mesopor Mater 2004;75:261–72.

[17] Romo A, Penas FJ, Sevillano X, Isasi JR. Application of factorial experimental design to the study of the suspension polymerization of β-cyclodextrin and epichlohydrin. J Appl Polym Sci 2006;100:3393–402.

[18] Zohrehvand S, Evans CH. 2-Naphthol-containing β-cyclodextrin-epichlorohydrin copolymers: synthesis, characterization and fluorescence studies. Polym Int 2005;54:744–53.

[19] Hishiya T, Asanuma H, Komiyama M. Spectroscopic anatomy of molecular-imprinting cyclodextrin. J Am Chem Soc 2002;124:570–5.

[20] Hedges AR. Industrial applications of cyclodextrin. Chem Rev 1998;98:2035–44.

[21] Kim SH, Ahn J, Kwak HS. Crosslinking of β-cyclodextrin on cholesterol removal from milk. Arch Pharm Res 2004;27:1183–7.

[22] Breslow R, Zhang B. Cholesterol recognition and binding by cyclodextrin dimers. J Am Chem Soc 1996;118:8495–6.

[23] Xu W, Demas JN, DeGraff BA, Whaley M. Interactions of pyrene with cyclodextrins and polymeric cyclodextrins. J Phys Chem 1993;97:6546–54.

[24] Thienpont LM, De Brabandere VI, Stockl D, De Leenheer AP. Use of cyclodextrin for purification of progesterone and testosterone from human serum prior to determination with isotope dilution gas chromatography/mass spectrometry. Anal Chem 1994;66:4116–9.

[25] Zarzycki PK, Kulhanek KM, Smith R, Clifton VL. Determination of steroids in human plasma using temperature-dependent inclusion chromatography for metabolomic investigations. J Chromatogr A 2006;1104:203–8.

[26] Larsen KL, Aachmann FL, Wimmer R, Stella VJ, Kjolner UM. Phase solubility and structure of the inclusion complexes of prednisolone and 6α-methylprednisolone with various cyclodextrins. J Pharm Sci 2005;94:507–15.

[27] Sotthivirat S, Lubach JW, Haslam JL, Stella VJ. Characterization of prednisolone in controlled porosity osmotic pump pellets using solid-state NMR spectroscopy. J Pharm Sci 2007;96:1008–17.

[28] Armstrong DW, Yang X, Han SM, Menges RA. Direct liquid chromatographic separation of racemates with an α-cyclodextrin bonded phase. Anal Chem 1987;59: 2594–6.

[29] VanderNoot VA, VanRollins M. Capillary electrophoresis of cytochrome P-450 epoxygenase metabolites of arachidonic acid. 2. Resolution of stereoisomers. Anal Chem 2002;74:5866–70.

[30] Chen S. The HPLC enantioresolution of phenyl isocyanated amino acids, peptides and amino alcohols on cyclodextrin bonded phase using the acetonitrile-based mobile phase. Chromatographia 2004;59:697–703.

[31] Sahin F, Demirel G, Tümtürk H. A novel matrix for the immobilization of acetylcholinesterase. Int J Biol Macromol 2005;37:148–53.

[32] Kwak HS, Kim SH, Kim JH, Choi HJ, Kang J. Immobilized β-cyclodextrin as a simple and recyclable method for cholesterol removal in milk. Arch Pharm Res 2004;27: 873–7.

[33] Orprecio R, Evans CH. Polymer-immobilized cyclodextrin trapping of model organic pollutants in flowing water streams. J Appl Polym Sci 2003;90:2103–10.

[34] Kwak HS, Jung CS, Shim SY, Ahn J. Removal of cholesterol from cheddar cheese by β-cyclodextrin. J Agric Food Chem 2002;50:7293–8.