ELSEVIER

Journal of Chromatography B, 726 (1999) 121–131

JOURNAL OF CHROMATOGRAPHY B

Supercritical fluid extraction of methyltestosterone, nortestosterone and testosterone at low ppb levels from fortified bovine urine

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Received 26 June 1998; received in revised form 8 January 1999; accepted 11 January 1999

Abstract

A multi-residue supercritical fluid extraction (SFE) method is proposed for the isolation of nortestosterone, testosterone and methyltestosterone from bovine urine. Prior to SFE, bovine urine was hydrolyzed and then fortified with the three steroids at 100 ng/ml and 50 ng/ml each for HPLC analysis and 25 ng/ml and 12.5 ng/ml each for GC–MS analysis. The samples then were mixed with an adsorbent material, placed in an SFE extraction vessel prepacked with a 3-ml SPE column containing neutral alumina and the testosterones were extracted from the urine matrix using unmodified supercritical CO_2 at 27.2 MPa and 40°C. The steroids were retained in-line on the neutral alumina sorbent in the SPE column while co-extracted artifactial material was trapped off-line after CO_2 decompression. After SFE, the SPE column was removed from the extraction vessel, and the trapped steroids were eluted from the neutral alumina sorbent with 3 ml of a methanol–water mixture. Eluates were used directly without post-SFE clean-up either for HPLC analysis (detection limit 50 ng/ml) or for GC–MS analysis (detection limit 5 ng/ml after steroid derivatization). The multi-residue SFE recoveries ($n=6$) for nortestosterone, testosterone and methyltestosterone from hydrolyzed bovine urine by GC–MS analysis were 90.8±6%, 93.9±3% and 92.5±5%, respectively for each steroid at the 12.5 ng fortification level.

Keywords: Steroids; Methyltestosterone; Nortestosterone; Testosterone

1. Introduction

Throughout the countries of the European Union (EU) the use of xenobiotic anabolic steroids is forbidden as growth promoting agents in stockbreeding. However, for over 30 years a variety of steroids have been illegally used in Europe for this purpose. Urine is one of the matrices used in EU regulatory laboratories to detect and confirm the illegal use of these compounds in food-producing animals. The determination of anabolic steroid residues in biological matrices such as urine is difficult because many endogenous steroids and dietary components contained in this fluid interfere with the detection of the target analytes. Consequently, most existing

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00039-0

analytical methods now in use employ a combination of various analytical procedures; pre-treatment by liquid–liquid or solid-phase extraction (SPE), followed by extract clean-up using immunoaffinity chromatography (IAC) and/or high-performance liquid chromatography (HPLC) and the detection of the derivatized steroids by gas chromatography–mass spectrometry (GC–MS) [1–3]. Within the last several years much analytical research on anabolic steroids in urine has been directed toward the development of automated systems using IAC in on-line systems combined with HPLC [3–5]. From the standpoint of regulatory needs, less attention has been focussed on the development of alternative non-IAC techniques for steroid analysis.

Supercritical fluid extraction (SFE) is a potentially attractive alternative to conventional liquid–liquid, SPE and IAC techniques for the recovery of anabolic steroids. Because of their lipophilic nature, steroids such as nortestosterone and testosterone exhibit modest solubility in supercritical CO_2 under relatively mild extraction conditions [6]. Investigators recently have made use of this property to report the SFE of several natural and synthetic steroids from various biological matrices [7–10]. The majority of these papers report the isolation of steroids from animal products such as muscle, liver and adipose tissue [7–10]; while only one study applied SFE to their recovery from biological fluids [11]. In that investigation, Simmons and Stewart [11] recovered several steroids in methanol modified supercritical CO_2 from serum fortified at the 1 μg/ml level. None of the SFE work on biological fluids reported to date has been at steroid levels of interest to regulatory agencies (1–100 ng/ml), a limitation that restricts the use of this technology for such applications.

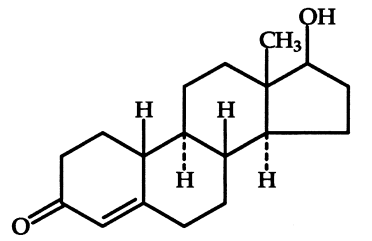
Conventional trapping techniques used to collect analytes after SFE may limit the low ng/ml level recovery of analytes from biological fluids. Typically, analytes are trapped after CO_2 decompression on either sorbent beds or in solvent-filled vials. This process may be complicated by the co-extraction of unwanted non-polar matrix components along with the target analytes, often necessitating additional post-SFE clean-up operations. We have developed alternative, in-line solute retention techniques using unmodified supercritical CO_2 in order to overcome the known problems associated with off-line trapping of trace level analytes. In earlier reports, we described the in-line SFE recovery of veterinary pharmaceuticals such as sulfonamides and melengesterol acetate [12–14]. In those investigations, target analytes solubilized in the supercritical fluid were retained on in-line sorbent beds while non-retained co-extracted solutes were collected off-line after CO_2 decompression. After SFE, the sorbent beds were removed from the extraction vessels, poured into empty SPE columns and the analytes were recovered in one step simply by eluting the SPE column with the HPLC mobile phase solvent.

In a separate investigation, we reported an improved technique for in-line analyte collection [15]. In that study a PTFE sleeve assembly was developed that enabled us to fit a standard 3-ml SPE column directly in the extraction vessel in place of the loose sorbent bed, thus eliminating the need for post-SFE removal and transfer of the sorbent bed to an empty SPE column. This assembly was used to develop a preliminary method for isolating three anabolic steroids from animal tissues and greatly facilitated extraction vessel packing, minimized post-SFE sample clean-up operations and standardized the extraction to conform with modern processing instrumentation. In the present study, the use of in-line solute trapping on SPE columns has been extended to include the recovery of three anabolic steroids; nortestosterone, testosterone and methyltestosterone (Fig. 1) from bovine urine. Studies were carried out at concentration levels of interest to EU regulatory agencies. The advantage of the selectivity achieved by in-line SPE column trapping was illustrated by a direct comparison of the in-line vs. off-line recovery of the three steroids. HPLC–UV was used as a preliminary means to evaluate the efficacy of the SFE method while the final, low ppb level steroid recovery data were verified by GC–MS.

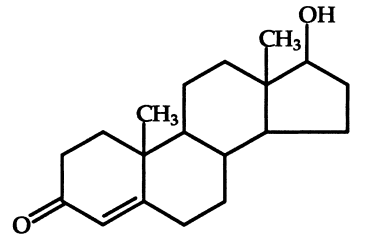
2. Experimental

2.1. Chemicals and reagents

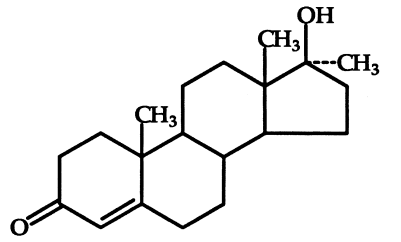
Hydromatrix (Celite 566), was obtained form Varian-Sample Preparation Products (Harbor City, CA, USA). Polypropylene wool and neutral alumina, activity grade I Brockmann, 150 mesh (19 997-4)



Nortestosterone



Testosterone



17-Methyltestosterone

Fig. 1. Chemical structures of three anabolic steroids.

were from Aldrich (Milwaukee, WI, USA). Polypropylene frits (14 mm diameter) were a product of Applied Separations (Allentown, PA, USA) or were punched from 35 mesh medium polypropylene sheets (H 13638, Bel-Art Products, Pequannock, NJ, USA) in the laboratory using an appropriately sized stainless steel cork borer. Alumina basic, Brockmann activity grade I, 80–200 mesh and Florisil F101, 100–200 mesh were obtained from Fischer Scientific (Fairlawn, NY, USA). Silica gel, 70–230 mesh, was obtained from E. Merck (Cherry Hill, NJ, USA). Octadecylsilica (14% load), the standard 1-, 3- and 6-ml disposable polypropylene SPE columns used for in-line and off-line analyte trapping and the PTFE sleeves used to retain 1 and 3 ml SPE columns in the extraction vessels were all products of Applied Separations (Allentown, PA, USA). Helium and SFC grade carbon dioxide pressurized with a helium headspace were from Scott Specialty Gases (Plumsteadville, PA, USA). Methanol, high-purity solvent, was a product of Baxter Health Care (Muskegon, MI, USA). Isooctane was purchased from J.T. Baker (Phillipsburg, NJ, USA). β-Nortestosterone (code H150126), 17β-testosterone (code H146342), 17α-methyltestosterone (code 90M0223) the internal standard 17β-hydroxy-17α-deutero-18-dideuteroestr-4-en-3-one (17β-nortestosterone-d3) and drug-free bovine urine (coded 94M0042/94M0032) were gifts from the RIVM/ARO Community Reference Lab. (Bilthoven, Netherlands). β-Glucuronidase type H-3, crude solution from *Helix pomatia* (β-glucuronidase activity approximately 100 000 Fishman units per ml at pH 5.0, sulfatase activity <1000 Roy units per ml) was from Sigma (St. Louis, MO, USA). Heptafluorobutyric acid anhydride (HFBA) was obtained from Pierce (Rockford, IL, USA).

Stock solutions of the steroids (1 mg/ml each) were prepared in methanol and stored at −20°C. Working solutions were prepared by diluting each stock solution ten-fold. A standard solution of the three steroids (5 ng/μl each) in methanol was prepared from the working solutions and was used to fortify the urine. The internal standard was a solution of 17-β-nortestosterone-d3 (5 ng/μl) in methanol. To adjust the urine pH, a 2 *M* acetate buffer solution (pH=5.2±0.1) was prepared by dissolving 25.2 g acetic acid and 129.5 g sodium acetate in 800 ml of water, which then was diluted to a final volume of 1 l.

2.2. Instrumentation

Supercritical fluid extractions were carried out on a prototype instrument developed jointly by this laboratory and Applied Separations. The design of this instrument has since been patented and is now in commercial production [16]. The SFE apparatus was configured for the parallel processing of two extraction vessels. The extraction vessels (66015, 24 ml capacity, 14 mm I.D., Keystone Scientific, Bellefonte, PA, USA) were connected to the system using

Keystone Scientific hand-tightened, slip-free connectors. The restrictors were micrometering valves (10RMM2812, Autoclave Engineers, Erie, PA, USA) which were encased in an aluminum block fitted with a cartridge heater and a thermocouple. The micrometering values were fitted with a special adapter that enabled standard 6-ml SPE columns to be attached directly to the micrometering valve without the aid of fittings and connecting tubing. A detailed description of the metering valve/SPE interface has been reported elsewhere [17]. A Floline SEF-51 flow meter/gas totalizer obtained from Scott Specialty Gases was used to measure flow-rates and volumes of decompressed CO_2.

The isocratic HPLC system used for off-line and in-line sorbent trapping analysis consisted of an ESA solvent delivery module Model 5700 (Bedford, MA, USA) and an Applied Biosystems 785A programmable absorbance detector (Foster City, CA, USA). The steroids were detected at a UV wavelength of 254 nm. A Hewlett-Packard Model 3396A integrator-recorder (Valley Forge, PA, USA) was used for data acquisition. The analytical HPLC column (150×4.6 mm I.D.), which was operated at ambient temperatures, was packed with Supelcosil LC-18, 5 μm particles (Supelco, Bellefonte, PA, USA) and was protected by a reversed-phase guard column packed with Perisorb RP-18, 30–40 μm mesh (Upchurch Scientific, Oak Harbor, WA, USA). The mobile phase was methanol–water (65:35, v/v) with a flow-rate of 1.0 ml/min.

A second HPLC system was used to validate the results of the extractions performed using in-line alumina SPE column trapping. That system consisted of an LKB 20150-010 pump (LKB, Woerden, The Netherlands), an LKB 2157-020 autosampler, an LKB 2155 column oven set at 25°C and a Spectroflow 757 absorbance detector set at 254 nm (Kratos, Hendrik Ido Ambacht, The Netherlands). Data acquisition was obtained on a Maxima system (Interscience, Breda, The Netherlands). Analyses were performed using a Spherisorb ODS-2 (C_{18}, 5 μm) 100×3 mm glass column (Chrompack, Middelburg, The Netherlands) with a flow-rate of 0.4 ml/min. The isocratic mobile phase was methanol–water (60:40, v/v).

The GC–MS system consisted of a Model 5890 Series II Plus gas chromatograph, Model 7673 autoinjector and a Model 5972 mass-selective detector that was controlled by Chemstation software (Hewlett-Packard). Steroids were analyzed on a HP-5, crosslinked, 5% phenyl methylsilicone column (30 m×0.25 mm I.D.) with a film thickness of 0.25 μm having a helium carrier gas flow of 1 ml/min. The GC–MS operating conditions were: splitless injector held at 225°C; oven temperature program: 100–240°C at 20°C/min then 240–280°C at 3°C/min with a final hold time of 2 min; a transfer line temperature of 280°C and an inlet column pressure maintained at a constant 0.073 MPa.

2.3. Pre-SFE sample preparation and extraction vessel packing

2.3.1. Sample preparation

The pH of a 5-ml bovine urine sample was adjusted to 5.2 with dilute acetic acid or NaOH, which was followed by the addition of 1 ml of the 2 *M* acetate buffer (pH 5.2) and 0.1 ml of the β-glucuronidase solution from *H. pomatia*. This mixture then was incubated for 2 h at 37°C to deconjugate the bound steroids [1]. For the experiments using HPLC as the detection means, the deconjugated urine was fortified with the 5 ng/μl standard mixture at either 100 ng/ml or 50 ng/ml of each steroid and processed as outlined below. For the experiments using GC–MS as the detection means, 5 ml of the resultant hydrolyzed urine was fortified with the 5 ng/μl standard steroid mixture at two concentration levels: 12.5 μl (=12.5 ng steroid/ml hydrolyzed urine) or 25 μl (=25 ng/ml). Two ml of this hydrolyzed, fortified urine solution was pipetted into a beaker containing 2.0 g of Hydromatrix. The contents of the beaker were gently blended together with a spatula until a free flowing granular material was obtained.

2.3.2. Extraction vessel packing

One end of the SFE extraction vessel was sealed with an end cap and then pre-chilled at 4°C until needed (the sealed end became the outlet when the vessel was installed in the oven and for identification purposes was labeled ''Top''). Next, the finger grip flange of a standard, commercial 3-ml SPE column was trimmed so that it fit the I.D. of extraction vessel. After this operation the column was packed

with 2.0 g of neutral alumina and inserted into the PTFE sleeve adapter. The SPE column/PTFE sleeve assembly was inserted into the cooled extraction vessel and seated in the sealed end using a tamping rod as shown in Fig. 2B. This was followed by a plug of polypropylene wool or a polypropylene frit, which was inserted into the vessel and positioned above the SPE column/sleeve assembly with the aid of the tamping rod. Next, the pre-prepared sample/Hydromatrix mixture was poured into the vessel and tightly compressed, followed by another frit or plug of polypropylene wool, which in turn was followed by 1.5 g of Hydromatrix and a final frit or plug of polypropylene wool. The entire contents of the vessel were tightly compressed and the vessel was sealed with the second end cap.

2.4. Supercritical fluid extraction method for in-line SPE column trapping

Prior to SFE operations, two empty 6.0 ml SPE columns were packed with 1.0 g neutral alumina each and then were attached to the interface adapters of the dual micrometering valves [17]. The Luer fittings of the SPE columns were connected in parallel through Tygon tubing to two Floline SEF-51 flow meter/gas totalizers (Horiba, Sunnydale, CA, USA). The micrometering valves were heated to 110°C. At this time, two extraction vessel were installed vertically in the oven with the ends labeled ''Top'' connected to the upper fittings as shown in Fig. 2B and pre-pressurized with CO_2 to 25.5 MPa. The oven temperature was set to 40°C and held for 10 min to equilibrate the vessels. At the end of this period the outlet valves were opened, the pressure readjusted to 27.2 MPa and the flow of expanded gas set at 2.8±0.06 l/min. This flow-rate was maintained until a volume of 50 l was registered on the flow totalizer. The extraction vessels containing the desired steroids were removed from the SFE oven, the ''Top'' endcap unsealed and the SPE column/sleeve assemblies removed. The 3-ml SPE columns were separated from the PTFE sleeves and the trapped steroids were recovered as described below. (The 6-ml SPE columns were retained on the micrometering valves after system depressurization, except in the early stages on this investigation since they contained only co-extracted artifactual material).

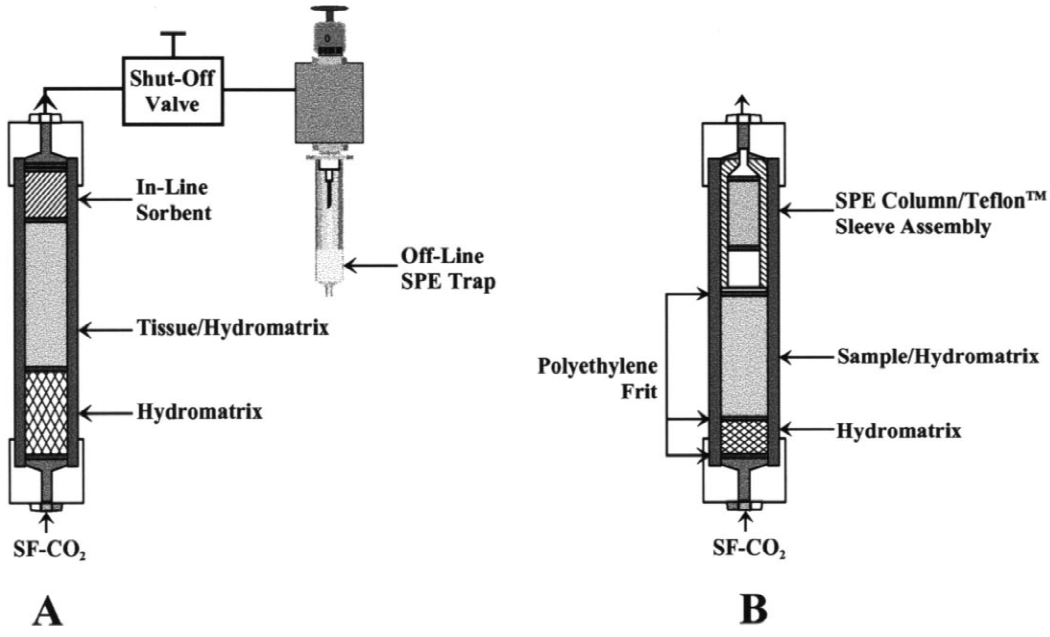


Fig. 2. Schematic of SFE flow pathway configured: (A) with extraction vessel containing an in-line alumina sorbent bed connected to the micrometering valve/SPE column interface assembly and (B) extraction vessel containing the SPE column/PTFE sleeve assembly.

2.5. Post-SFE steroid recovery and analysis

Approximately 3 ml of a MeOH–water (70:30, v/v) solution was pipetted onto the alumina sorbent bed of each SPE column. The first two ml of eluate from the column were collected and analyzed either by HPLC or GC–MS as outlined below.

2.5.1. HPLC analysis

The HPLC analytical scheme employed was based on CEC reference method M1.1 [1]. The SPE column eluates were analyzed directly without further clean-up, by injecting 50-μl aliquot volumes into the HPLC system used for the in-line sorbent bed samples and 20-μl samples into the LKB HPLC system used for the in-line SPE column samples. The concentration of each analyte was calculated from the standard curves. Standard curves were obtained over a concentration range of 2.5–200 ng/ml. Correlation coefficients were: 17β-nortestosterone (0.993), 17β-testosterone (0.992) and 17α-methyltestosterone (0.995). Retention times of the steroids were determined daily using the steroid standard mixture.

2.5.2. GC–MS analysis

The steroid confirmation method also was derived from CEC method M1.1 [1]. To each eluate from the SPE columns was added 5 μl of the internal standard containing 25 ng of nortestosterone-d3. Solvent was removed from this solution by heating the container in a 50°C water bath under a stream of nitrogen. The resultant residue was transferred to a derivatization vial with 0.5 ml of MeOH, which in turn was evaporated under nitrogen. This residue then was dissolved in 100 μl of a freshly prepared solution of HFBA–acetone (1:4, v/v), vortexed for 1 min and incubated in an oven for 1 h at 60°C. After incubation, the reaction mixture was evaporated to dryness under a stream of nitrogen at 50°C, redissolved in 100 μl isooctane and transferred to a micro autosampler vial. Two μl of this solution was injected into the GC–MS system. A blank (derivatization reagents only) and a derivatized standard mixture of the three steroids were analyzed with each set of samples in order to monitor the efficiency of the derivatization procedure and to determine the status of the GC–MS system. Molecular ions of the di-HFBA derivatives were used for quantification: 666 for nortestosterone, 669 for the internal standard nortestosterone-d3, 680 for testosterone and 694 for methyltestosterone.

3. Results and discussion

For an SFE method to be competitive with sensitive immunoaffinity techniques for steroid recovery from urine, it must be capable of reproducibly isolating these compounds from biological matrices at the low nanogram tolerance levels established by regulatory agencies [1]. We have addressed this issue in the course of developing an SFE method for the extraction of anabolic steroids from urine. Early in our investigation, we attempted to recover these steroids from urine using the conventional technique of trapping the extracted analytes after CO_2 decompression (off-line trapping). Later, we investigated techniques for retaining these compounds in the dynamic supercritical state prior to fluid decompression on sorbent beds and SPE columns contained in the extraction vessels (in-line trapping). We then used HPLC to determine the optimum SFE parameters for steroid recovery. These conditions were later employed to generate GC–MS data for detection and verification of the method.

The three anabolic steroids that are the subject of this investigation (Fig. 1) do not exist in the free state in bovine urine. Instead, they are known to exist as either sulfate or glucuronide conjugates. Therefore, prior to conducting SFE experiments, we first hydrolyzed each urine sample by a method known to deconjugate steroids [1], in order to approximate the composition of an incurred sample prepared for steroid analysis. Urine samples then were fortified with mixtures of the three anabolic steroids and prepared for SFE extraction.

In the initial SFE experiments using off-line trapping, the urine samples were extracted with supercritical CO_2 at a pump pressure of 34.0 MPa, an oven temperature of 40°C, and a flow-rate of 2.8 l/min (expanded gas). The solutes were collected after fluid decompression in 6-ml SPE columns interfaced to the micrometering valve of the SFE apparatus. The SPE columns were packed with alumina, a sorbent that was used in previous studies to efficiently retain other chemical residues [17,18].

After SFE, the SPE columns were removed from the micrometering valve interface adapter (Fig. 2A) and the extracts were recovered from the sorbent with the HPLC mobile phase solvent. An HPLC chromatogram of a typical off-line collected extract is shown in Fig. 3A. Observe that no peaks for the individual steroids are discernable in this chromatogram. Instead, there is continuous UV absorption at 254 nm throughout the entire chromatographic run indicating the complexity of a deconjugated bovine urine extract. Additional clean-up of the sample extracts by SPE or varying the SFE experimental parameters failed to improve the chromatographic separation. Therefore, we discontinued the off-line approach to

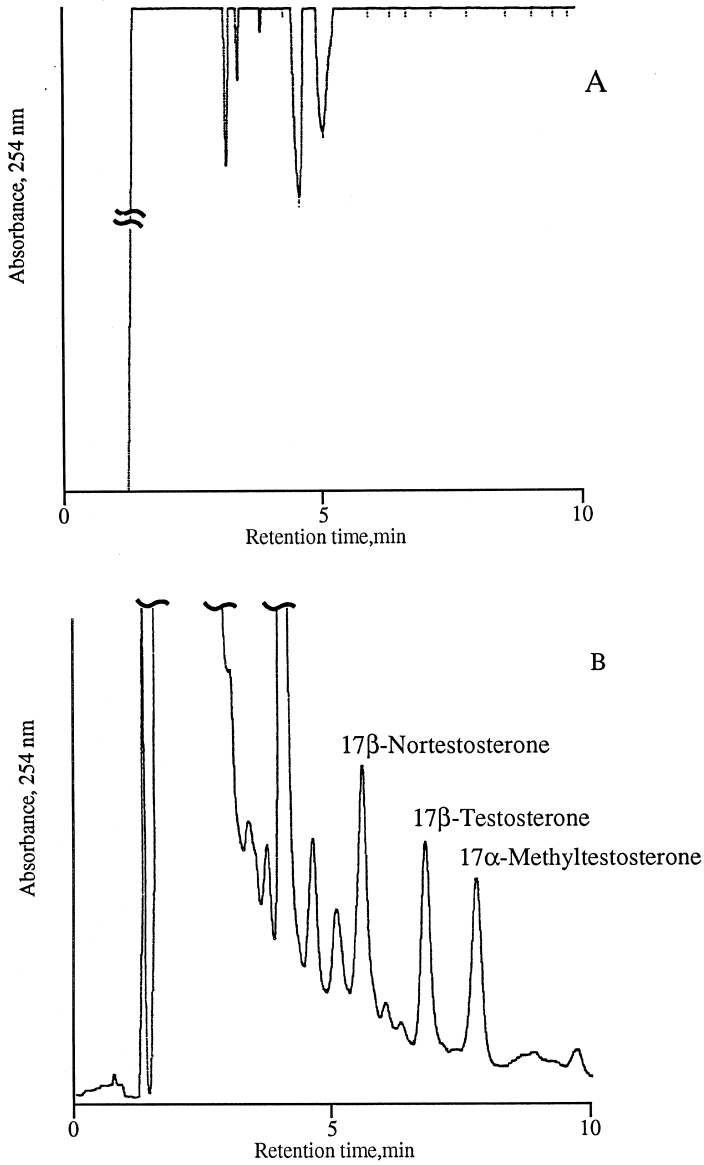


Fig. 3. HPLC chromatograms of (1) 17β-nortestosterone, (2) 17β-testosterone and (3) 17α-methyltestosterone from fortified (100 ng/ml) hydrolyzed bovine urine; (A) collected in an off-line alumina SPE column and (B) collected on an in-line alumina sorbent bed.

SFE solute trapping because the extracts obtained by this technique were too complex in composition for effective HPLC separation and detection.

We next evaluated an in-line collection technique for analyte trapping that was first proposed by Parks and Maxwell for the recovery of sulfonamides from chicken liver [12]. In that report, the investigators added a bed of neutral alumina to the extraction vessel above the sample matrix as shown in Fig. 2A. They reported that the polar sulfonamides were retained on the in-line sorbent bed while other co-extracted material such as fat and pigments were collected in the off-line SPE column. Recoveries of this drug class were high and the HPLC chromatograms were free of background interference.

In addition to the neutral alumina used in the earlier studies, we tested several different sorbent types for their ability to retain anabolic steroids on the in-line traps during a dynamic extraction. We used the same SFE experimental parameters as those described for the off-line collection experiments. These sorbents included basic alumina, florisil, silica gel and reversed-phase materials such as octadecylsilica (C_{18}), whose retentive properties were evaluated using fortified, deconjugated bovine urine. Of the sorbents tested, only basic alumina retained the steroids to the same degree as neutral alumina. However, chromatograms of the steroids recovered from this material contained interference in areas were peaks for the target analytes appeared. Breakthrough of the analytes from the other sorbent materials to the off-line SPE columns occurred frequently, resulting in poor recoveries of the steroids and a high degree of interference in the HPLC chromatograms. Therefore, neutral alumina was employed in all of the subsequent experiments using in-line trapping.

In subsequent experiments, HPLC was improved further by reducing the extraction pressure from 34.0 to 27.2 MPa. The lower pressure (27.2 MPa) resulted in fewer unwanted artifacts in the retention windows where the steroids of interest appeared. Fig. 3B is an example of a chromatogram of an eluate from bovine urine fortified at 100 ppb for each steroid and obtained using this lower extraction pressure. This chromatogram may be compared with the HPLC chromatogram for the same eluate obtained after off-line trapping (Fig. 3A). In contrast to the chromatogram for the off-line extract, the peaks for the three steroids are well resolved even though they appear on the descending slope of the baseline. The percent recoveries for 17β-nortestosterone, 17β-testosterone and 17α-methyltestosterone were 131.8, 134.0 and 102.5, respectively.

The relatively high percent recoveries for these three analytes quantified by HPLC–UV indicated the presence of undetected contaminants co-eluting under the anabolic steroid peaks. In addition to this, there were some other problems associated with this in-line collection technique that adversely affected recoveries: (a) difficulty in uniformly packing the alumina in the extraction vessel, (b) occasional breakthrough of the steroids from the in-line sorbent bed, (c) possible solute contamination due to sorbent contact with the walls of the extraction vessel and (d) the potential loss of analytes in the post-SFE transfer of the alumina sorbent to the empty SPE column.

It was clear at this point in our investigation that it would be necessary to replace the in-line sorbent bed with a universally available means of analyte trapping if this technique were to gain acceptance as a routine analytical procedure. For that reason, we developed a technique for employing standard SPE columns in the SFE extraction vessels in place of the loose alumina packing used in the earlier experiments. We found that both 1-ml and 3-ml standard SPE columns would fit into 14 mm I.D. extraction vessels after the finger-grip flange on the SPE columns was trimmed to fit the vessel opening. The SPE columns then were inserted into a PTFE sleeve specifically designed in this laboratory and referred to hereafter as the SPE/PTFE sleeve assembly. This assembly, described in detail elsewhere [15], was fitted into the extraction vessel as shown in Fig. 2B, forming a pressure tight seal and forcing the supercritical CO_2 to flow directly through the sorbent bed of the SPE column. This SPE column/PTFE sleeve assembly was used in place of the in-line sorbent bed technique in all subsequent SFE experiments using fortified bovine urine.

We investigated trapping the steroids first on standard, commercial 1-ml and then on 3-ml SPE alumina columns. Standard 1 ml SPE columns hold 1 g of neutral alumina, while 3 ml SPE columns hold 2 g. We found that the amount of neutral alumina

contained in the 1 ml SPE column was not sufficient to completely retain the steroids on the sorbent bed in the supercritical state, instead; the steroids partially migrated to the off-line SPE column during the extraction process. Therefore, all subsequent tests were conducted using the 3 ml SPE columns, which were successful in completely retaining the target analytes. A series of six determinations were performed using the in-line SPE technique with bovine urine fortified at the 100 ng/ml level. The results of that study are reported in Table 1. Quantitative recovery of the three steroids was achieved at this fortification level with concomitantly low relative standard deviations (RSDs), indicating the repeatability and reproducibility possible with this technique. Total retention of the three steroids occurred even at the relatively high flow-rate of 2.8 l/min (expanded gas). This result was surprising considering the chemical structures of these anabolic steroids (Fig. 1), which might be expected to exhibit the properties of other lipid-like compounds in that they would remain solubilized in the supercritical fluid throughout the extraction process and be collected off-line after CO_2 decompression. A HPLC–UV chromatogram of an extract from a 3 ml in-line SPE column extract is shown in Fig. 4. Unlike the similar chromatogram from the in-line sorbent bed (Fig. 3B), the peaks for the steroids in Fig. 4 are baseline resolved, not part of the tailing slope of the solvent front. Each of the peaks is fully separated from any background interference allowing for more accurate quantitation of the steroids. Bovine urine samples next were fortified at the 50 ng level with the three steroids and attempts were made to analyze the recovered extracts by HPLC–UV. However, the data for recoveries at this fortification level were somewhat erratic by HPLC–UV indicating that further optimization of the method required the use of the more sensitive and selective GC–MS analysis and detection technique.

Table 1
In-line SPE column recovery[a] of three anabolic steroids from fortified, hydrolyzed bovine urine measured by HPLC–UV

	Fortification level (ng/ml)	Mean[b] (%)	RSD (%)
17β-Nortestosterone	100	97.4	3.4
17β-Testosterone	100	98.8	6.3
17α-Methyltestosterone	100	97.3	3.9

[a] SFE conditions: pressure 27.2 MPa, temperature 40°C, flow 2.8 l/min (expanded gas).
[b] Average of six determinations.

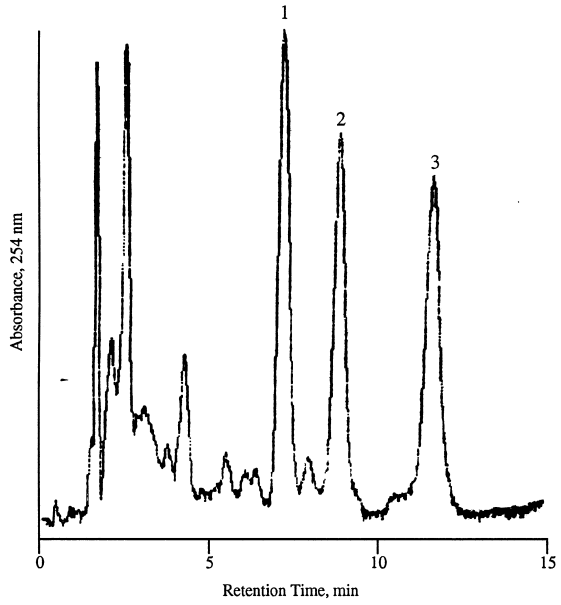


Fig. 4. HPLC chromatogram of (1) 17β-nortestosterone, (2) 17β-testosterone and (3) 17α-methyltestosterone from fortified (100 ng/ml) hydrolyzed bovine urine collected on an in-line 3 ml alumina SPE column.

For GC–MS analysis, hydrolyzed bovine urine was fortified with the steroid mixture at two concentration levels: 25 ng/ml and 12.5 ng/ml each. The SFE parameters were the same as those used at the higher fortification levels with SPE in-line recovery. The internal standard, 17β-nortestosterone-d3 was added to the sample extracts prior to derivatization to correct for sample to sample variations due to post-SFE derivatization, evaporation loss and discrimination during autosampler injection. After SFE, di-HFB derivatives were prepared directly from the extracts. Due to the absence of GC detectable artifacts in the extracts, post SFE clean-up was not required. The molecular ion chromatograms of the di-HFB derivatives of the internal standard 17β-nortestosterone-d3 and the three steroids from bovine urine fortified at the 12.5 ng level are shown in Fig. 5A–D. A GC–single in monitoring (SIM)-MS chromatogram of a control bovine urine sample is not shown, since control sample chromatograms dis-

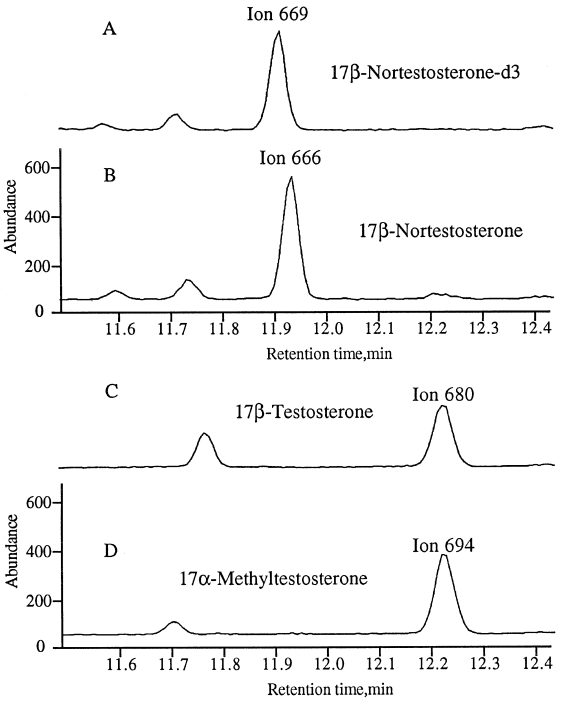


Fig. 5. GC–SIM-MS chromatograms of di-HFB derivatives of (A) the internal standard 17β-nortestosterone-d3 and (B–D) three anabolic steroids isolated from fortified (12.5 ng/ml) hydrolyzed bovine urine.

Table 2
In-line SPE column recovery[a] of three anabolic steroids from fortified, hydrolyzed bovine urine at two fortification levels measured by GC–MS

	Fortification level (ng/ml)	Mean[b] (%)	RSD (%)
17β-Nortestosterone	12.5	90.8	6.4
	25.0	95.9	4.6
17β-Testosterone	12.5	93.9	3.2
	25.0	98.7	5.6
17α-Methyltestosterone	12.5	92.5	5.0
	25.0	98.3	5.5

[a] For SFE conditions see Table 1.
[b] Average of six determinations.

played no peaks in the retention windows for the three steroids and the internal standard. Moreover, the chromatograms in Fig. 5B–D obtained from the fortified samples were free of any interference in regions where the steroids appear.

The GC–SIM-MS technique was used to quantify the recoveries of hydrolyzed bovine urine fortified both at the 25 ng/ml and 12.5 ng/ml levels. The results of those determinations are given in Table 2. The means shown in the table are the average of six determinations. At the 25 ng/ml fortification level recoveries ranged from 96–98% for the three steroids while the means for the samples fortified at the 12.5 ng/ml level ranged from 91–94%. The RSDs were 6.4% or below for each steroid at both fortification levels, which demonstrated the high level of repeatability obtainable when employing the in-line SPE column trapping technique.

4. Conclusions

The results obtained in the present study indicate the potential applicability of SFE as a sample preparation technique for monitoring trace levels of anabolic steroids in bovine urine, an issue of importance to regulatory agencies in the EU. The procedure described allows the analyst to collect extracted steroids free of co-extracted solutes on standard SPE columns contained in the extraction vessel, thus accomplishing a selective one-step extraction and collection and eliminating the need for further post-SFE clean-up operations. In addition, the procedure requires only 2 ml of organic solvent/sample. This simple, straightforward combination of SFE–SPE in conjunction with GC–MS makes it possible to easily extract and analyze 10–15 urine samples/day at or below the 12 ng/ml level. Used as a screening method (>50 ng/ml), the procedure is even more rapid since the eluate may be analyzed directly by HPLC–UV without the need for the additional derivatization step required for GC–MS.

5. Disclaimer

Reference to brand of firm names does not constitute an endorsement by the US Department of

Agriculture over others of a similar nature not mentioned.

Acknowledgements

We thank Marlice A. Sipoli Marques from the UFRJ-Ladetec-R.J. Brazil for performing some of the experimental studies.

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