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Supercritical fluid extraction of steroids from biological samples and first experience with solid-phase microextraction–liquid chromatography

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Abstract

Modern extraction techniques, supercritical fluid extraction (SFE) and solid-phase microextraction (SPME) were used for isolation of four corticosteroids from biological matrices. SFE was applied for extraction from solid matrices — hydromatrix and pig muscle. The effects of various extraction conditions were studied. Good recoveries of corticosteroids from hydromatrix were obtained under moderate extraction conditions and without modification of carbon dioxide. On the contrary, the best recoveries from spiked pig muscle were obtained with modified carbon dioxide. SPME was used for extraction from liquid samples — water and urine. The eventuality of the use of this fast solvent-free technique in steroid analysis is demonstrated. Several extraction conditions were optimized. Extracted steroids were analyzed by HPLC–UV and a special SPME–HPLC interface was used for combination with SPME.

Keywords: Supercritical fluid extraction; Solid-phase microextraction; Steroids

1. Introduction

The sample pretreatment is an essential step preceding identification of compounds of biological interest in analytical processes. Here, extraction as a sample preparation method plays a very important role in various analytical fields (toxicological, forensic, clinical, biochemical and pharmaceutical analysis). Biological materials including urine, blood, saliva, hair and tissue are much more complex than any others. They contain many endogenous compounds that can cause interferences during analysis or affect target analytes. A successful isolation of target analytes from biological matrices is needed prior to the determination of biologically active compounds and this presents a big challenge. Selective analysis of drugs from a variety of biological matrices is often performed today by solid-phase extraction [1], while classical methods have employed liquid–liquid extraction. Ideally, a sample preparation method should be fast, easy to perform and solvent-free. Modern extraction techniques such as SFE and SPME are attractive alternatives to time- and solvent-consuming classical extraction methods.

Supercritical fluid extraction [2,3] can be defined as the technique using the supercritical fluid ‘SF’ (substance above its critical temperature and pressure) to remove analytes from various matrices. We take advantage of significant properties of the supercritical fluid for the extraction. The most important

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of them are (1) good solvating power (which is related to density), (2) high diffusivity, (3) low viscosity, (4) minimal surface tension. Carbon dioxide is the most frequently used supercritical fluid. It has been the choice for most analytical applications because of its moderate critical parameters [3] (critical pressure p_c=7.29 MPa, critical temperature t_c=31.0 °C) and other suitable properties. It is relatively non-toxic, is available in high purity, has low reactivity and is environmentally compatible.

SFE is conceptually simple to perform and relatively rapid. However, several experimental conditions have to be considered and optimized prior to an effective routine use of SFE, including temperature and pressure of the extraction, extraction time (or flow-rate of SF), trapping method of analytes, modification of CO_2, type of extraction mode (dynamic or static) and some equipment parameters (size of extraction vessel, size and type of restrictor etc.). Not only successful isolation of analytes is needed for an effective extraction but also their trapping is important. SFE can be connected directly to an analytical instrument in 'on-line' mode or analytes can be collected in a device independent of the analytical system. There are various types of trapping [3]: into an empty bottle (inert material), into a liquid solvent, onto an active solid sorbent or recently, into overheated organic solvent vapor [4]. Modification (addition of an organic solvent) of supercritical CO_2 is necessary for the extraction of polar analytes (most of the biologically active compounds). Modifiers can also significantly increase the extraction yield in influencing the matrix effects. The modifiers can be introduced directly into the stream of the fluid or into the extraction vessel.

Although SFE has been most frequently used for extraction of drugs from solid and semisolid samples, animal feed [5], hair [6], tissue [7–9], formulated drugs [10] in forensic science [11] and pharmaceutical [5] and clinical analysis, several applications from biological fluids were also described [12–14].

Another new extraction method studied in our experiments was solid-phase microextraction developed by Arthur and Pawliszyn [15]. SPME is a process in which analytes are adsorbed onto the surface of a small fused-silica fiber coated with suitable polymeric phase and placed in a syringe-like protective holder. This is followed by the desorption of the analytes into a suitable instrument for separation and determination. SPME has been exploited most effectively when coupled to gas chromatography due to the direct and convenient sample introduction into GC. Coupling SPME with HPLC was described more recently and requires a special interface [16]. Analytes are desorbed from the fiber using a liquid solvent.

SPME is based on the distribution of analytes between extraction phase (polymer) and the matrix. Extraction can be varied and enhanced in a number of ways. SPME can be performed by placing the fibre directly into the liquid sample or as a headspace extraction (HS-SPME) [17,18]. The great advantage of SPME is also the possibility of its combination with various derivatization methods [19]. Derivatization can improve extraction efficiency, selectivity and the following detection. SPME is a complex equilibrium process and for this reason several extraction conditions have to be considered, optimized and then held constant to ensure the reproducibility of the extraction. The most important factor in SPME is the affinity of the fibre for target analytes; others are temperature, time of adsorption and desorption, pH, sample volume, addition of salt, stirring etc. SPME has been introduced only recently but it has gained much interest and popularity in the analysis of biological samples [20–23]. SPME coupled with LC–MS was also used for analysis of corticosteroids in urine [24].

This paper presents the possibility of the extraction of four corticosteroids from biological matrices. Corticosteroids are hormones of the adrenal cortex and have a great affinity to the protein binding. Corticosteroids are compounds of clinical importance and are determined for diagnosis and treatment of diseases, in pharmacokinetic studies and for identification of the abused drugs in sport. An effective isolation from the complex biological matrices is necessary prior to the determination of these compounds.

2. Experimental

2.1. Chemicals

Chemicals were obtained from the following suppliers: cortisol (98%) Sigma (St. Louis, USA), cortisone (97%) Fluka (Buchs, Switzerland), deoxy-

corticosterone (99%) Sigma (Steinheim, Germany), corticosterone (rein) Serva Feinbiochemica (Heidelberg, Germany), methanol for HPLC (gradient grade) J.T. Baker (Deventer, Holland), acetonitrile for HPLC Chromasolv® (gradient grade) Riedel-de Haën (Seelze, Germany), acetic acid (99.8%) Lachema (Neratovice, Czech Republic), trifluoracetic acid anhydrous (TFAA) (protein sequencing grade) Sigma (Steinheim, Germany), carbon dioxide (4.5) Linde AG (Höllriegelskreuth, Germany), nitrogen (4.0) Linde AG (Prague, Czech Republic). Hydromatrix was obtained from Chrompack (Middelburg, Holland), glass wool from Supelco (Bellefonte, USA), target syringe filter, PTFE 0.45 μm, 13 mm from Biotech a.s. (Prague, Czech Republic). Water was demineralized in-house on the system Ultra CLEAR UV from SG (Barsbuttel, Germany).

2.2. Instrumentation

Supercritical fluid extractions were carried out on an instrument made by SEKO-K (Brno, Czech Republic). The length of the stainless steel extraction vessel was 68 mm and its inner diameter was 12 mm.

For SPME, a system from Supelco (Bellefonte, PA, USA) was used: SPME fiber assembly 50 μm Carbowax™/TPR-100 for HPLC, SPME fiber holder and SPME–HPLC interface (with Rheodyne® Valve).

Analysis was performed with a HPLC system consisting of the following items: high pressure pump LCP 4000 Ecom (Prague, Czech Republic), gradient programmer GP 5 Ecom, security guard C_{18}, 4 mm L×3 mm I.D. Phenomenex (Torrance, CA, USA), analytical HPLC column 125×4 mm packed with LiChrospher 100 RP-18e 5 μm Merck (Darmstadt, Germany), UV detector LCD 2084 Ecom.

2.3. SFE procedure

2.3.1. Sample preparation

Standard solutions of steroids (cortisone, 0.722 mg/ml; cortisol, 0.696 mg/ml; corticosterone, 0.564 mg/ml; deoxycorticosterone, 0.384 mg/ml) in methanol were prepared. Ten μl of standard steroid solutions were applied directly onto the hydromatrix (4 g for analysis) for the extraction from inert matrices. For the extraction from tissue, 2 g of homogenized pig muscle were fortified with equal volumes of standard steroid solutions and held at room temperature for at least 10 min. The spiked tissue was mixed with 2 g of hydromatrix and filled into the extraction vessel.

2.3.2. Packing of the extraction vessel

The bottom end of the extraction vessel was sealed with the cap and frit, this was followed by a flock of glass wool and a 0.5-cm layer of small glass balls. The sample, the hydromatrix or fortified tissue–hydromatrix mixture was placed on this layer. The sample was covered with another flock of glass wool and glass ball layer and sealed with a frit and upper cap. In this manner, the prepared extraction vessel was put into the heating block of the instrument.

2.3.3. Extraction process

Extraction was performed either in dynamic mode or in dynamic mode followed after a 15-min static step. All modifiers (methanol, acetonitrile, methanol–acetic acid (9:1 v/v), acetonitrile–acetic acid (9:1 v/v)) were added through the six-port valve into the stream of CO_2; except for the trifluoracetic acid (TFAA) that was applied (1 ml of 1% vol. solution in acetonitrile) directly into the extraction vessel. The use of the TFAA was combined with the modification of acetonitrile added into the stream of CO_2 during the dynamic step. The trapping was carried out into the 20-ml glass vial with 8 ml of methanol at a temperature of 5 °C. The restrictor length was 20 cm and inner diameter ($I.D._{restr.}$) was at first 30 μm and later 50 μm. The restrictor was heated to 100 °C. Other extraction parameters were the subject of optimization.

2.3.4. Post-SFE procedure

The extracts were filtered through 13-mm teflon syringe filters for clean-up and the solvent was evaporated to dryness in the stream of nitrogen. Ten μl of standard solution of deoxycorticosterone (internal standard) and 0.2 ml of methanol were added to the evaporated extracts. Extracts prepared in this manner were analyzed by HPLC.

2.3.5. HPLC analysis

Twenty μl of sample were injected. Flow-rate of the mobile phase was 0.7 ml/min and the separations were achieved using the following gradient se-

quence: the solvents were methanol–water and the composition was programmed from 45:55 (v/v) to 85:15 (v/v) within 15 min. The steroids were detected at a wavelength of 242 nm.

2.4. SPME procedure

2.4.1. Sample preparation and extraction

One hundred μl of the standard solutions (the same as in the SFE procedure) of steroids were added to 4 ml water (urine) in a 4-ml glass vial. Samples were thermostated to the adequate temperature and the fiber was immersed into the liquid for a certain extraction time. No stirring was provided. Several extraction parameters were optimized — adsorption time, desorption time and extraction temperature.

2.4.2. SPME–HPLC procedure

The fiber was put into the desorption chamber of the SPME–HPLC interface filled with 45% vol. methanol after the absorption. The mobile phase solvents were the same as in the SFE procedure and the composition was programmed from 45:55 (v/v) to 85:15 (v/v) within 10 min and then kept isocratic for the next 2 min. The detection was also at a wavelength of 242 nm. The fiber was exposed to the mobile phase for 5 min and then taken out of the chamber and allowed to dry in the air. No carryovers were observed.

3. Results and discussion

Optimization of the extraction conditions is a necessary step preceding the routine use of extraction methods described above. Thus, the main purpose of this study was to find suitable experimental conditions for the isolation (and analysis) of four corticosteroids from biological matrices. SFE was used for the extraction of cortisone, cortisol and corticosterone from solid matrices — hydromatrix and pig muscle (deoxycorticosterone was used as the internal standard for HPLC analysis). SPME was used for the isolation of cortisol, corticosterone and deoxycorticosterone from liquid matrices — water and urine.

3.1. SFE

At first we investigated suitable conditions for successful extraction of steroids from the inert matrix—the hydromatrix. The optimal extraction pressure was 40 MPa and the time of the extraction (up from 20 min) had no significant effect on the extraction recovery but the increasing temperature (T_e) caused considerable decrease of the extraction recovery (see Table 1). Thus, the experiments with hydromatrix showed that the convenient conditions for the supercritical fluid extraction of corticosteroids can be p_e=40 MPa, T_e=40 °C, I.D.$_{restr.}$=30 μm and CO_2 without modification. However, the recoveries of corticosteroids from pig muscle under the same conditions were under 1%. These extremely low recoveries were perhaps due to the polarity of corticosteroids and the strong interactions between corticosteroids and tissue proteins, cells, organelles and membranes. Afterwards we studied the effects of different modifiers on the extraction recovery. The recoveries increased only slightly and we also observed plugging of the restrictor during the extraction. The restrictor was thus replaced and all other extractions were performed with restrictor with I.D.$_{restr.}$=50 μm. The 15-min static step was employed because of the use of the trifluoracetic acid (TFAA). Results are shown in Table 2. The recoveries of steroids using SFE (40 MPa 40 °C for 60 min) were from 10% to 60% without modification of the supercritical carbon dioxide. Better recoveries of steroids were obtained by modification of acetonitrile than CO_2 without modifier. The best recoveries were

Table 1
SFE recoveries of corticosteroids from hydromatrix at different extraction temperatures. Fortification level: cortisone 1.81 μg/g hydromatrix, cortisol 1.74 μg/g hydromatrix, corticosterone 1.41 μg/g hydromatrix. Extraction conditions: 30 min, 40 MPa, I.D.$_{restr.}$=30 μm, pure CO_2

Temperature (°C)	Mean (%)±SD (n=8)		
	Cortisone	Cortisol	Corticosterone
40	64.4±5.3	75.8±4.9	83.8±4.5
50	48.3±6.2	54.1±6.7	64.8±6.1
60	41.8±5.8	49.2±6.1	63.1±6.8
80	28.2±4.6	23.7±5.3	24.9±7.9

Table 2
Effects of various modifiers on the recovery of corticosteroids from pig muscle spiked at the level of 3.48 μg/g tissue (cortisol), 3.61 μg/g tissue (cortisone), 2.82 μg/g tissue (corticosterone). Extraction conditions: 60 min including 15 min static step, 40 MPa, 40 °C, $I.D._{restr.}$=50 μm

Modifiers	Mean (%)±SD (n=8)		
	Cortisone	Cortisol	Corticosterone
Without	33.2±4.3	10.8±4.7	60.3±6.3
Methanol	25.9±5.1	14.4±4.4	43.3±4.1
Methanol–acetic acid	26.2±3.3	24.7±6.1	41.0±5.9
Acetonitrile	50.7±5.7	29.7±5.0	64.2±6.0
Acetonitrile–acetic acid	42.8±4.2	30.5±2.4	71.1±5.5
Trifluoracetic acid+acetonitrile	53.9±3.9	47.1±2.5	83.5±3.9

achieved by using the trifluoracetic acid with acetonitrile as a modifier.

3.2. SPME

With the first practical experience we verified the possibility to extract corticosteroids from water samples. Initially, we searched for the best experimental conditions for an effective extraction and analysis. The fiber was chosen according to the literature [24]. The adsorption and desorption time and the temperature were the first optimized parameters. Any change of the desorption time had no significant effect. Five minutes static desorption was chosen for further experiments. The results of the experiments dealing with optimization of the extraction temperature and time are shown in Figs. 1 and 2. Fig. 1 shows the extraction yield increases with the increasing temperature up to 40 °C and above this value the extraction yield decreases. The

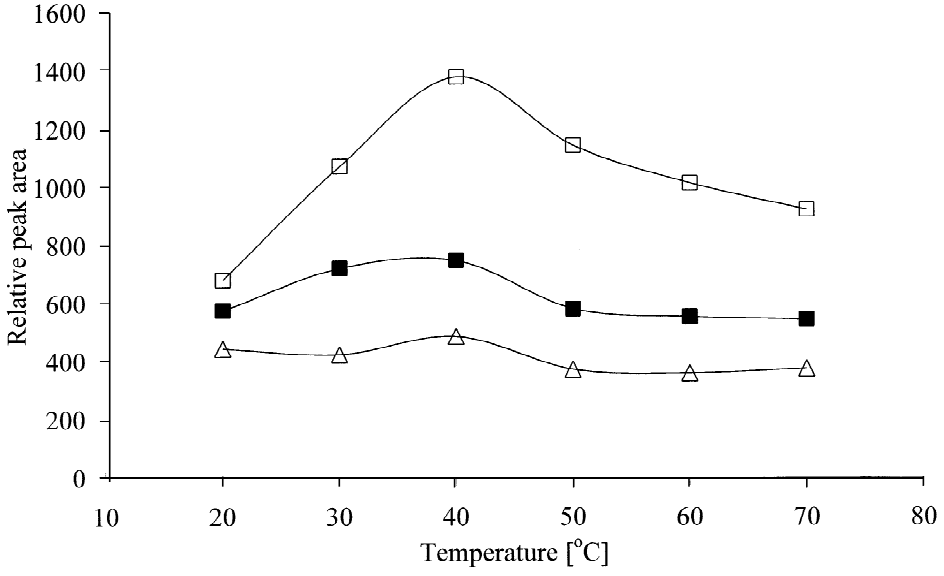

Fig. 1. Extraction efficiency versus extraction temperature curves for corticosteroids in water on the 50 μm CW/TPR fiber, the time of adsorption was 20 min, the time of desorption 5 min. The fortification level was 17.4 μg/ml for cortisol, 14.1 μg/ml for corticosterone and 9.6 μg/ml for deoxycorticosterone. Curve assignment: □, deoxycorticosterone; ■, corticosterone; △, cortisol.

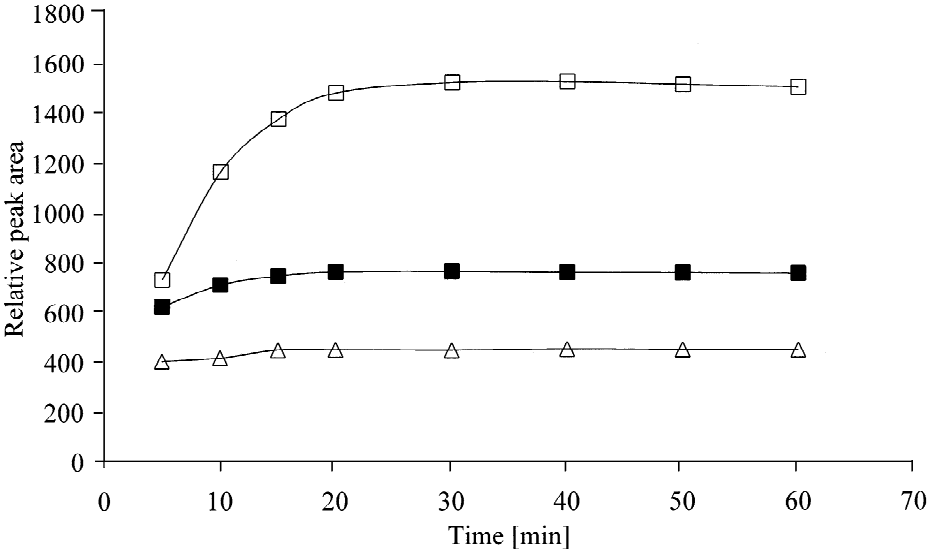

Fig. 2. Extraction efficiency versus adsorption time curves for corticosteroids in water on the 50 μm CW/TPR fiber at 40 °C, desorption time 5 min. The fortification level was 17.4 μg/ml for cortisol, 14.1 μg/ml for corticosterone and 9.6 μg/ml for deoxycorticosterone. Curve assignment: □, deoxycorticosterone; ■, corticosterone; △, cortisol.

decrease in yield can be caused by the decrease of the distribution constant with increasing temperature or by the decomposition of the steroids above this temperature. Equilibrium time for the investigated compounds ranges from ca. 15 to 60 min as can be seen in Fig. 2. From this profile, we have chosen the following conditions for the extraction from urine: adsorption time of 20 min (compromise between speed and extraction efficiency) and temperature of 40 °C. Fig. 3 illustrates the typical SPME–HPLC chromatogram of corticosteroids from urine. We obtained a reproducibility of about 5–10% relative standard deviation. Linear ranges were found for samples with concentrations of 1–10 μg/ml in urine.

4. Conclusion

SFE and SPME are modern extraction techniques which are, as reported, suitable for isolation of corticosteroids from biological matrices. Problems with extraction of corticosteroids from pig muscle in the case of SFE was solved by modification of supercritical carbon dioxide. The results obtained in the present study indicate the applicability of SFE as a sample preparation technique for monitoring trace levels of corticosteroids in animal tissue. There are also other attractive aspects to modify this technique; the use of another modifier, to try another type of trapping (trapping into overheated organic solvent vapor). Also shown is the great potential of SPME in the isolation of corticosteroids from water and from urine. SPME is suitable for rapid screening of field analysis of urine. Comparing in-line coupling of the SPME–LC method with LC methods utilizing traditional sample preparation techniques such as SPE or liquid–liquid extraction, the former offers similar performance in terms of precision, linearity, but it is clearly easier to use and faster to perform. SFE and SPME are convenient sample preparation methods for biological samples. Both procedures are rapid, simple to perform and require minimal amount of organic solvents. Their potential can indeed be useful in analysis of other biologically active compounds.

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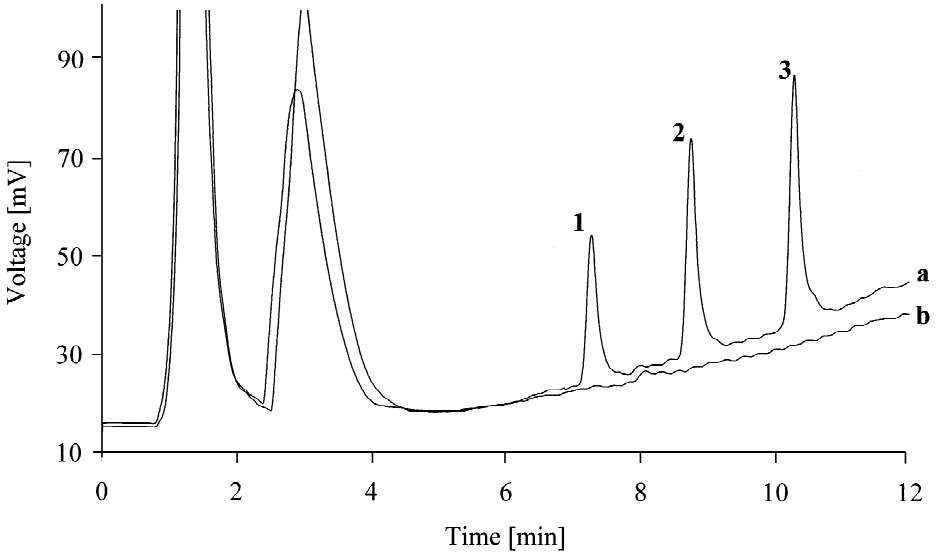

Fig. 3. HPLC–UV chromatogram of the SPME–HPLC analysis of several corticosteroids from urine spiked with cortisol (17.4 μg/ml), peak 1; corticosterone (14.1 μg/ml), peak 2; deoxycorticosterone (9.6 μg/ml), peak 3; line a, spiked urine; line b, unspiked urine. SPME conditions: T_e 40 °C, time of adsorption 20 min and desorption for 5 min.

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