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Molecularly imprinted adsorbents for preconcentration and isolation of progesterone and testosterone by solid phase extraction combined with HPLC

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Abstract The use of a novel procedure of solid-phase extraction with molecularly imprinted polymers (MISPE) has been described. A MISPE procedure relying on tailor-made, artificial-mimic materials capable of selectively rebinding target analytes (steroids) based on a combination of recognition mechanisms, such as size, shape and functionality, was custom designed for progesterone and testosterone. The combination of MISPE with LC/DAD is a simple and an efficient method for the determination and quality control of progesterone and testosterone in human urine samples.

Keywords Steroids · Molecularly imprinted adsorbents · MISPE · HPLC · DAD

Abbreviations

AIBN	2,2′-azobis(2-methylpropionitrile)
α	separation factor
BME	benzyl methyl ether
DAD	diode array detector
EDMA	ethylene glycol dimethacrylate
HPLC	high performance liquid chromatography
k	retention factor
LOQ	limit of quantification
LOD	limit of detection
MAA	methacrylic acid
MIP	molecularly imprinted polymer
MISPE	solid phase extraction with molecularly imprinted polymer
NIP	non-imprinted polymer
P1, P2, P3, P4	non-imprinted and imprinted polymers with progesterone as a template
r	correlation coefficient
RSD	relative standard deviation
T1, T2, T3, T4	non-imprinted and imprinted polymers with testosterone as a template
TRIM	trimethylolpropane trimethacrylate
BME	benzyl methyl ether
FLD	fluorescence detector
MS	mass spectrometry detector
UV-Vis	ultraviolet visible
4-VP	4-vinylpyridine
S/N	signal-to-noise ratio

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1 Introduction

Steroid hormones are a biologically active, low-molecular group of lipids based on the cyclopentanoperhydrophenanthrene skeleton of four rings. Progesterone and testosterone are grouped as a family because of their correlative act on fruitful function (Morand and Lyall 1968). Hormone synthesis starts with a cholesterol molecule by peripheral conversion, mainly in the adrenal cortex, ovary, testes and placenta (Fig. 1) (Ruggiero and Likis 2002).

Sex steroid elements are cardinal in the development of human sexual organs and secondary sexual characters, as well as in the adjustment and maintenance of the female and male reproduction system (Ričanyová et al. 2009). Steroids are required for normal functioning and native circulation in the human body at native levels. Hence, carcinogenic effects are possible at enhanced levels (Ingerslev and Halling-Sorensen 2003). They interact in the cardiovascular system,

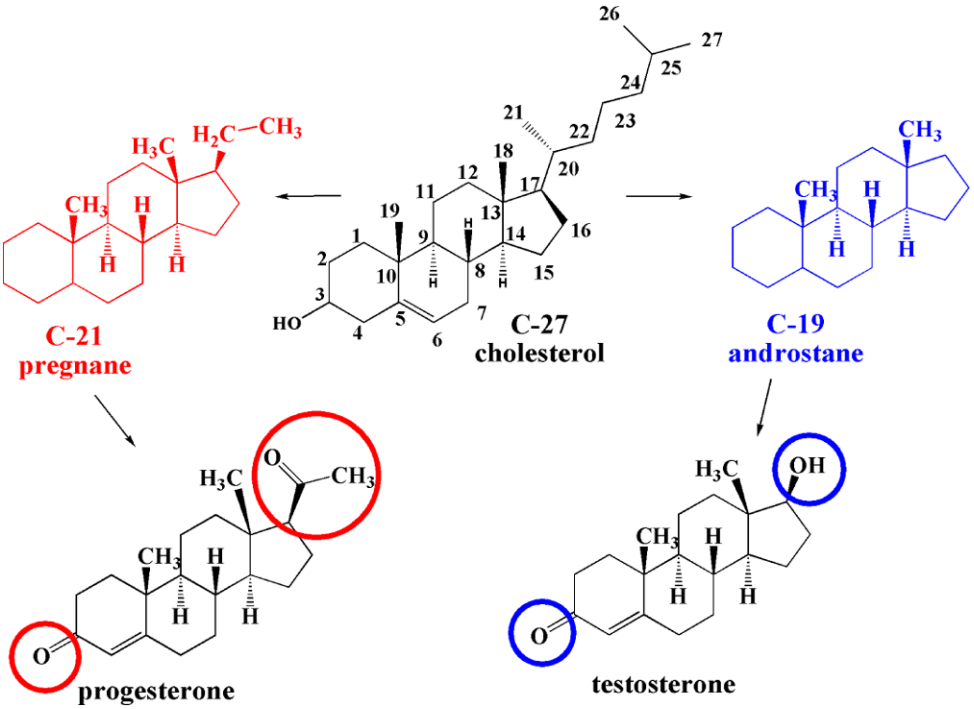

Fig. 1 The classification of steroid hormones

brain, liver, skin and muscles. Sex steroids are carcinogenic agents, therefore, starter steroids, their metabolites and convergents need to be evaluated (Ruggiero and Likis 2002).

The analysis of progesterone and testosterone in urine specimens can support the diagnosis of diseases based on irregularities in the steroid profile. Due to the ease of collection and the voluminous presence of metabolites, urine specimens constitute ideal material for human studies. These compounds are present in urine at very low concentration levels (ng mL^{-1}). Daily testosterone production rates have been estimated at 4 to 12 mg in young men and 0.5 to 2.9 mg in young women (van Anders et al. 2007; Michaud et al. 1999) About 20–25 mg of progesterone is produced daily during a woman's monthly cycle, and up to 300–400 mg is produced daily during pregnancy. The daily dose of natural progesterone in men is 10–12 mg. The actual daily quantity varies between individuals subject to several factors such as recent fluid intake (water and other water-based food/beverages), diet, temperature, blood pressure, general health (some diseases may affect urine volume/time) and mental state. It is also determined by metabolic pathways of sexual steroids in the human body, the composition and structure of human organs and tissues. The natural complexity of steroids is the reason why accurate clinical determination of progesterone and testosterone levels in biological specimens is problematic (Schöneshöfer and Weber 1983). The diversity of steroid structures and their polarity ranges also poses a problem during the separation and simultaneous analysis of steroids in biological fluids. Hormone release varies between night and day, in particular between different phases of the female menstrual cycle. In view of the above, special emphasis was placed on the collection of urine samples delivering adequate and reproducible amounts of steroids. The determination of steroids in complex matrices such as water, physiological fluids and tissues requires appropriate isolation and preconcentration methods. In qualitative analyses and laboratory practice in life chemistry (bioanalytics, clinical analysis, ecology, pharmacology and medicine), the right selection of susceptible and reproducible methods supporting final determination and validation is essential (Ričanyová et al. 2009).

In the present decade, the design, preparation and application of molecularly imprinted polymers (MIPs) play an increasingly important role in biomedical research (Ansell et al. 1996; Szumski and Buszewski 2004; Andersson et al. 1994; Sreenivasan 1998, Gadzała-Kopciuch et al. 2009) in solid phase extraction (SPE) (Buszewski 1990; Stevenson 1999). High performance liquid chromatography (HPLC) (Gomes et al. 2007; Anderson 1999; Wilson 2009; Gonzalo-Lumbreras et al. 2003), coupled with sensitive and selective detectors such as spectroscopic ultraviolet-visible (UV-Vis) (Huber et al. 1991; Ng and Yuen 2003), diode array (DAD) (Almeida and Nogueira 2006; van Elswijk and Irth 2003), electrochemical CoolArray (Shimada et al. 1981; Gatti et al. 1998; Buszewski et al. 2010), fluorescence (FLD) (Fukushima et al. 2006; Mao et al. 2004) and mass spectrometry (MS) (Xu et al. 2005; Marquet 2002) offers the most accurate and effective tool for the determination of sexual steroid levels in bio-fluids.

The aim of this study was to develop a method for the preparation of sexual steroid samples from complex human urine specimens with emphasis on molecular man-made

sorbents—molecularly imprinted polymers—for solid-phase extraction. In this respect, molecularly imprinted solid-phase extraction (MISPE) with "fingerprint" sorbents was deployed as a selective procedure for steroid analysis in human urine samples. Aided by a HPLC diode array detector, the selective MISPE procedure is a rapid and sensitive method for the selective preconcentration and determination of sexual steroids, such as progesterone and testosterone, in human urine specimens.

2 The experiment

2.1 Materials

Methacrylic acid (MAA, purity 99%), ethyleneglycol dimethacrylate (EDMA, purity 98%), trimethylolpropane trimethacrylate (TRIM, purity 98%) were supplied by Aldrich (St. Louis, MO, USA). 2,2′-Azobis(2-methylpropionitrile) (AIBN, purity 98%), progesterone and testosterone (purity >99%) were purchased from Fluka (Buchs, Switzerland). Anhydrous solvents for the synthesis of polymers (chloroform, toluene, isooctane) were obtained from POCh (Gliwice, Poland). HPLC grade acetonitrile and methanol were supplied by J.T. Baker (Mallinckrodt Baker, Deventer, The Netherlands). Water was obtained using a Milli-Q ultrapure water producing system (Millipore, Bedford MA, USA).

2.2 Instrumentation

The HPLC system comprised the Agilent 1100 Series model (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, an autosampler, a diode array detector and ChemStation software for data collection and instrument control. The chromatographic separation of urine samples was performed using a Supelcosil LC-C18-DB (250 mm × 4.6 mm, $d_p = 5$ μm) column with methanol–water (70:30%, v/v) mobile phase at a flow rate of 1 mL min^{-1}. The column was kept in the column oven (Gynkotek, Germering, Germany) at the temperature of 25 °C. The analytes were measured at setting wavelength $\lambda = 280$ nm by DAD. Solid-phase extraction was performed using a 12-port vacuum manifold supplied by Mallinckrodt Baker (Deventer, The Netherlands). MISPE columns were prepared with SPE glass columns equipped with porous PTFE disks from Mallinckrodt Baker, used at the top and at the bottom of the polymer bed.

2.3 Methods

2.3.1 Preparation of standard solutions

Approximately 1 mg of each reference compound was accurately weighed in 10 mL volumetric flasks. Methanol was added, and the resulting solution was sonicated. These stock solutions were stored at −4 °C. The stock solutions of progesterone, testosterone were accurately dissolved into a volumetric flask and filled up with mobile phase methanol-water (70:30%, v/v) to produce the required concentration (10–100 μg mL^{-1}).

2.3.2 Preparation of urine samples

Urine samples were collected in large containers without preservatives from healthy volunteers (male and female) aged between 8 and 40 years. The total daily urinary excretion of testosterone and progesterone with emphasis on 12 h nocturnal urine was determined by weight in grams. The aliquots were transferred to universal tubes to create three separate samples. All urine samples were freeze stored at −21 °C until assaying.

2.3.3 Synthesis of imprinted and non-imprinted polymers

The non-covalent imprinting "bulk" polymerization method was chosen as the general procedure for polymer synthesis. The chemical structure of chemicals used in the polymerization process is illustrated in Fig. 2.

For the preparation of steroid imprinted polymers, progesterone, testosterone (0.125 mmol) as the imprint template, functional monomer (MAA), the cross-linking monomer (TRIM, EDMA), and the initiator (AIBN—2 wt.% total monomer) were dissolved in the porogen (acetonitrile or isooctane–toluene 1:99% v/v) in a volumetric flask (10 mL). AIBN was purified by recrystallization from ethanol. Polymerization was performed at different temperatures (−4, 0, 4 °C) for 2–4 h by UV irradiation ($\lambda = 365$ nm). After the addition of the initiator, the solutions were deoxygenated with nitrogen and polymerized by the UV-initiated polymerization method. After polymerization, the completed polymer was crushed. The template was extracted by washing the polymers in the Soxhlet apparatus with acetic acid–methanol (5:95%, v/v) for 24 h. Finally, the polymers were dried in a vacuum oven for 24 h. The obtained polymers were ground in a mortar and passed through a sieve. The examined MIPs particle fractions were in the range of 63 ÷ 90 μm, 90 ÷ 250 μm and ≥250 μm. The investigation was performed in a 3 mL MISPE column with 150 mg sorbent mass and 90 ÷ 250 μm particle size. The sorbent was packed into an empty SPE glass column for subsequent evaluation. Non-imprinted polymers (NIP) were prepared as control samples under the same conditions, except for the addition of template molecules. The diagram of the general polymer preparation procedure is illustrated in Fig. 3.

2.3.4 Molecularly imprinted solid-phase extraction

Various quantities of the analyzed polymers were packed into SPE glass columns equipped with porous PTFE disks

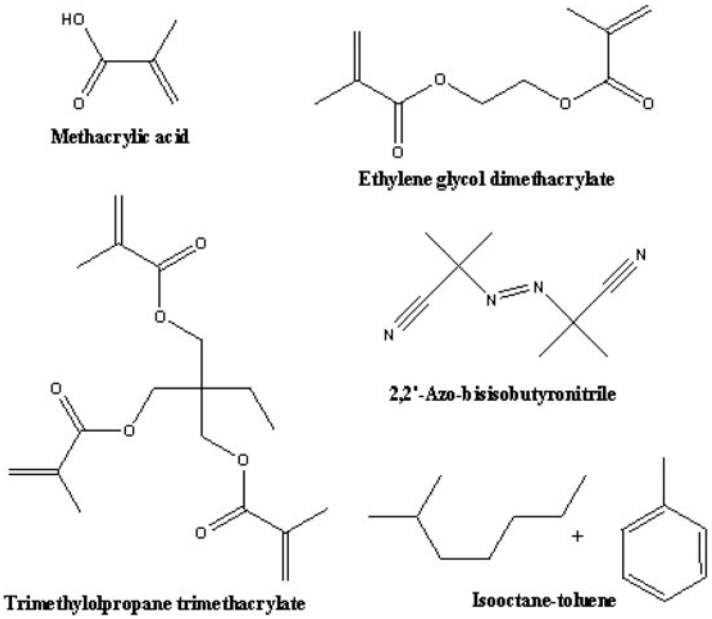

Fig. 2 Structures of chemicals used in MIPs synthesis

at the top and at the bottom of the polymer bed. The MISPE protocol accounted for the conditioning (3 mL methanol; 3 mL redistilled water) of the prepared MIPs cartridges before sample application. A spiked urine sample of 1 mL was then loaded. The cartridge was washed with 2 mL redistilled water. The analytes were eluted in 3 mL with different solvents (methanol, acetonitrile, chloroform or dichloromethane–methanol; 20:80%, v/v). The extracts were evaporated to dryness, the residue was dissolved in 200 µL mobile phase and injected into the HPLC system.

3 Results and discussion

3.1 Preparation and characterization of MIPs

The main goal of this study was to prepare polymer adsorbents marked by high selectivity in steroid recognition. Due to the physical properties (lipophilic molecules with a phenol aromatic ring and functional groups oriented in H-bonding interactions) of templates, progesterone and testosterone were used in the bulk free radical non-covalent imprinting approach. The successful assembling of the template-monomer complex was achieved by increasing the strength and the number of interaction sites, and by minimizing the remaining non-specific binding sites. The fundamental factors which influence these properties include the selection of functional monomers and cross-linkers, which were determined. The template-functional monomer and the functional monomer-crosslinker ratios were specified (Sellergren et al. 1988; Haupt et al. 1998; Buszewski et al. 2010). The progesterone and testosterone binding sites were assembled with MAA and copolymerized with EDMA or TRIM. The functional groups of progesterone and testosterone form hydrogen bonds and ionic interactions with the carboxylic groups of MAA, resulting in non-covalent imprinting sites in progesterone or testosterone-methacrylic acid pre-polymerization assembly. In view of molecular modeling results (HyperChem) cited for the same functional monomers (MMA), cross-linking monomers (EDMA, TRIM) and 17β-estradiol (Buszewski et al. 2010), as well as the work of Mosbach et al. (Sellergren et al. 1988; Haupt et al. 1998; Yilmaz et al. 1999) and the findings of other leading researchers in the investigated area, the authors of this study had assumed that molecules with similar stereogeometry would theoretically form similar polymerization complexes (Fig. 3). The noted results clearly suggest that template-MAA polymerization complexes are highly capable of separating estrogen from biological samples.

Ethylene glycol dimethacrylate and trimethylolpropane trimethacrylate were used as cross-linking agents for copolymerizing the pre-polymerization assembly. EDMA was used as a typical crosslinker for producing the optimal flexibility/rigidity and length in the polymer supramolecule. The addition of TRIM with a bond longer by one carbon was considered. The reaction mixture of the template, functional monomers, the cross-linker, the initiator and the solvent were added to a test tube at a different molar ratio; the mixture was purged with nitrogen to remove oxygen, and the test tube was sealed under vacuum. The MIPs were prepared by the UV-initiated polymerization process under different temperatures (4, 0 and −4 °C) for 2–4 h to achieve MIPs with higher load capacity, selectivity and a larger micropore population. Under the required conditions, the MIPs were sieved to produce particles (63 ÷ 90, 90 ÷ 250 and above 250 µm). The highest recovery was obtained for 90 ÷ 250 µm particle

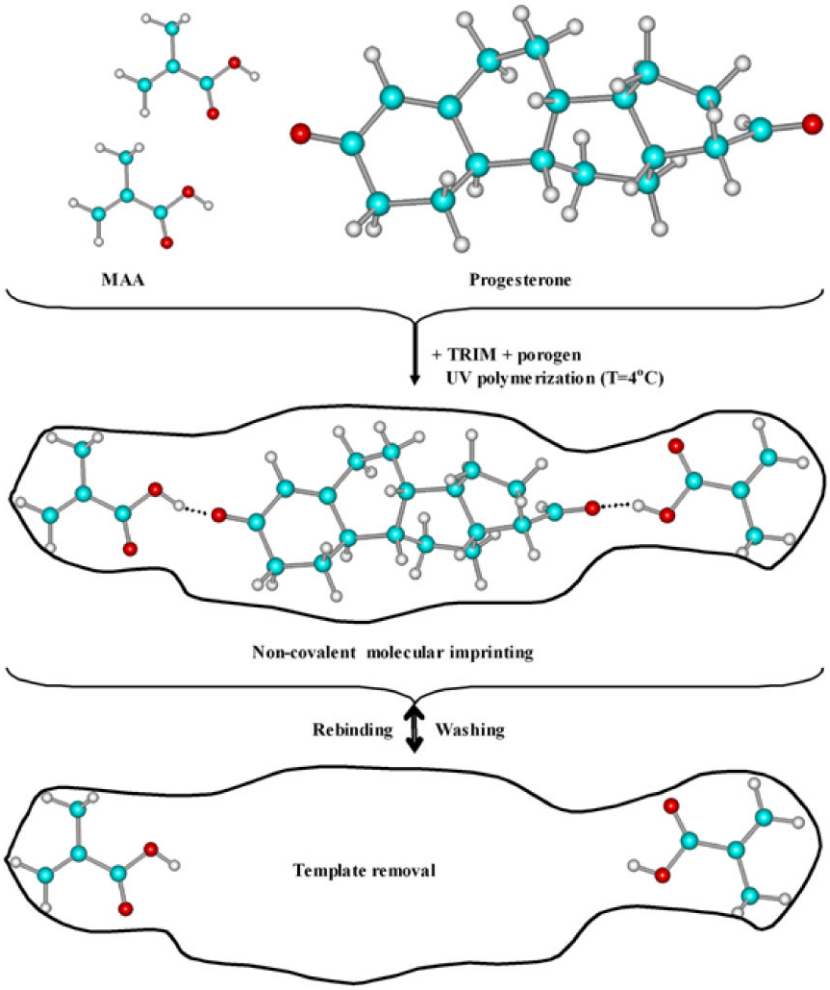

Fig. 3 Synthesis of molecular imprinted polymers and its selective recognition to target molecule

Table 1 Composition of synthesized molecularly imprinted polymers

Polymer	Template	Functional monomer	Crosslinker	Molar ratio[a]	Porogen
P1	progesterone	MAA	EDMA	0.125:5:20	ACN
T1	testosterone				
P2	progesterone				Isooctane–toluene (1:99% v/v)
T2	testosterone				
P3[b]	progesterone		TRIM	0.125:8:32	ACN
T3[b]	testosterone				
P4[b]	progesterone				Isooctane–toluene (1:99% v/v)
T4[b]	testosterone				

[a]molar ratio describes the proportion between template, functional monomer and crosslinker (T:M:C). Polymerization were performed at 4 °C (2 h. $\lambda = 365$ nm) AIBN (2 wt.% total monomer) was dissolved in the porogenic solvent

[b]Porous polymers

size. The UV-initiated process was selected as the optimal method for MIP and NIP synthesis. To provide strong, selective binding, it is vital that the template molecule preorganizes the functional monomer in a stable temperature prior to polymerization. The temperature of 0 °C produced poor globule size or an irregular formation of globules in polymers. When synthesis was performed at 0 °C, polymerization did not occur or it resulted in non-homogeneous polymeric material. The time of exposition was also changed, and it ranged from 2 h (4 °C), through 3–4 h (0 °C) up to 4 h (−4 °C). Taking into account only eight molecularly imprinted polymers (T1, T2, T3, T4, P1, P2, P3, P4) prepared under modified conditions (Table 1) were used as adsorbents in the MISPE procedure. As demonstrated by Table 1, the

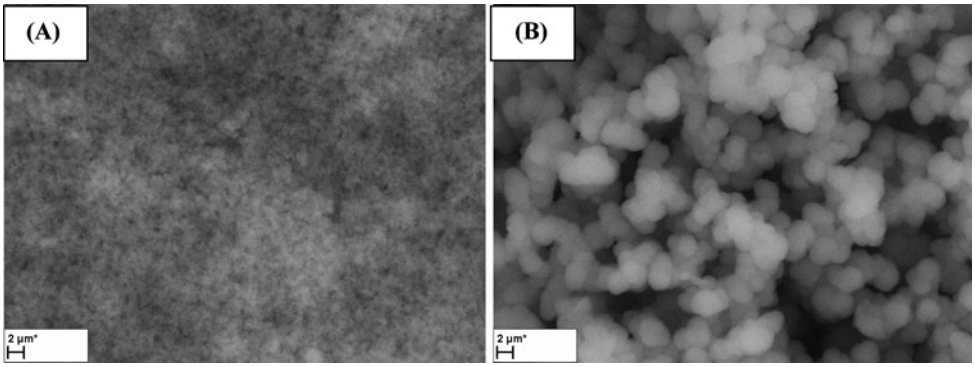

Fig. 4 Scanning electron micrographs of non-imprinted (**A**) and imprinted (**B**) polymer for testosterone (T4)

Table 2 The effect of porogen properties on particle size distribution investigated by SEM

Template	MIP	Porogen	Particle size[a] (µm)
Progesterone	P3	Acetonitrile	2.78
Testosterone	T3		2.91
Progesterone	P4	Isooctane–toluene (1:99% v/v)	4.81
Testosterone	T4		5.16

[a]Number-average particle size

MAA was chosen as a functional monomer and EDMA was selected as the cross-linker in the synthesis of T1, T2, P1, P2 progesterone and testosterone imprinted polymers. The monomer : crosslinker ratio of 1:4 was applied to obtain a polymer with 80% crosslinker. The subsequent polymers—T3, T4, P3, P4—were also synthesized with MAA as the functional monomer, but TRIM was used as the crosslinking agent. The porogens were chosen carefully to simultaneously maximize the complex-forming interactions between progesterone, testosterone and MAA (Sellergren et al. 1988; Haupt et al. 1998; Sellergren and Hall 2001). The porogenic solvent, acetonitrile, and the porogenic mixture comprising isooctane–toluene (1:99%, v/v) were chosen in view of their ability to dissolve progesterone and testosterone. Normally, non-polar, aprotic solvents which stabilize hydrogen bonds, e.g. toluene, isooctane, chloroform, are preferred during the preparation of MIPs. Both acetonitrile and the isooctane–toluene mixture were used to secure non-covalent H-bonding interactions between monomers and steroids. The above supports template and monomer (MAA–steroid complex) solubility prior to polymerization, and it has a positive effect on pore formation. The solubility of steroid molecules and the stability of the MAA–steroid complex were the limited factors in the choice of porogens. Consequently, acetonitrile and isooctane–toluene (1:99%, v/v) affected the number of binding sites and pore size distribution of the imprinted materials. The SEM microscopic technique confirmed that the application of isooctane–toluene (1:99%, v/v) gave rise to a higher number of specific interactions between steroid templates (progesterone, testosterone) and monomers (MAA), resulting in compact and more porous morphology of the imprinted polymers. Steroid binding with MAA during MIP synthesis produced larger globules and pores in comparison with NIP molecular imprinting without steroids. The non-imprinted polymer had very small surface globules. Figure 4 presents the morphology of the resulting T4 imprinted and non-imprinted polymer synthesized with MAA as the functional monomer, with TRIM as the crosslinker, with testosterone as the template and with isooctane–toluene (1:99%, v/v) as the porogenic mixture.

The use of TRIM for fixing the pre-polymerization assembly of steroids and MAA was a good choice. Even in the solution and prior to polymerization, TRIM produces higher quality associates with progesterone or testosterone than EDMA. The above most probably results from a lower number of methacrylate groups in the EDMA molecule (Fig. 2). TRIM with a more developed structure may orderly copolymerize the pre-polymerization complex. Polymers T4 and P4 were characterized by the highest molecular recognition ability. A comparison between P3, T3 and P4, T4 imprinted polymers, prepared under identical synthesis conditions with different porogens (isooctane–toluene (1:99%, v/v), acetonitrile), was carried out by means of a SEM analysis (Table 2). In view of the above results, the synthesis was performed using TRIM as the optimal method for copolymerizing the polymer network. A possible explanation is that TRIM is one carbon bond longer than EDMA, and it may more effectively copolymerize the polymer network created in the pre-polymerization of the MAA-steroid complex. The above has been validated by Shea et al. (1990), Sellergren and Hall (2001) and Haupt et al. (1998) and Sellergren et al. (1988) who reported that the use of TRIM resulted in polymers capable of higher loading in comparison with EDMA supports.

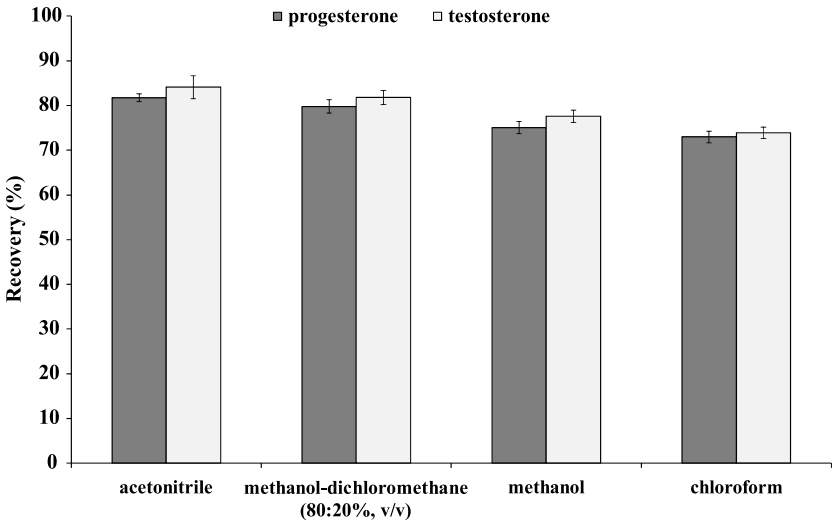

Fig. 5 The effect of various eluents on the recovery of testosterone and progesterone

3.2 Molecularly imprinted solid-phase extraction of steroids

The MIPs were used as sorbent cartridge in SPE extraction for the selection and preconcentration of progesterone and testosterone trace levels with the aim of obtaining the highest possible level of sensitivity, precision and efficiency. Various solvents, such as acetonitrile, methanol, chloroform and dichlormethane-methanol (20:80%, v/v), were compared and used for steroid elution (Fig. 5).

The best results were noted in respect of acetonitrile, whereas progesterone and testosterone were retained by MIP cartridges. Chloroform was also relatively successful in eluting progesterone and testosterone from the MIP cartridge, however, the specific interactions between the steroid and the MAA were stronger than the interactions between the steroid and the solvent, and recovery values were satisfactory due to non-polar aprotic properties. The polar methanol and the methanol containing dichloromethane (80:20%, v/v) also directly disrupted the recognition ability of the MIPs, leading to a loss in the elution step for both MIP and NIP sorbents. The addition of non-polar dichloromethane increased recovery values. In view of the above, acetonitrile was selected as the elution solvent. In order to estimate the selectivity and efficacy of MIP sorbents, various sorbent quantities were prepared and examined. MIP sorbents with the mass of 50, 100, 150 and 200 mg were compared. An increase in polymer quantity (200 mg) did not lead to a rise in testosterone and progesterone recovery values. The highest selectivity and efficacy was achieved by using 150 mg of the MIP sorbent (Fig. 6). As demonstrated by Fig. 6, progesterone recovery was in excess of 81%, while testosterone recovery approximated 84%. The relative standard deviation (RSD) was below 3%.

3.3 Clinical analysis of steroids from urine specimens by HPLC-DAD

The clinical measurement of sexual steroid levels in body fluids supports the evaluation of the androgenic and progestagenic characteristics of the investigated samples. Reproductive disorders in men are mainly represented by hypogonadism, which is commonly associated with abnormally low levels of androgens (testosterone). The opposite occurs in females, in whom androgen abundance is responsible for a large number of abnormalities which contribute to the development of the "hyperandrogenic syndrome". It is coupled with aberrantly high levels of androgens (testosterone) and low progestin levels (progesterone).

The HPLC-DAD conditions, including UV-Vis spectral data for the detection of each hormone, and retention time characteristics were evaluated. It was observed that the wavelength of $\lambda = 245$ nm supported testosterone and progesterone absorption. Instrumental calibration was performed using standard solutions with concentrations in the range of (9.5; 19; 190; 450; 950 ng mL^{-1}). The linear regression equation was $y = 49.137x - 3.1242$ for progesterone and $y = 40.837x - 20.964$ for testosterone. The correlation coefficients (r) ranged from 0.9993 ± 0.0008 to 0.9993 ± 0.0005. A satisfactory degree of correlation was observed for each compound. The limit of quantification (LOQ) and the limit of detection (LOD) was defined as the analyte signal-to-background noise (S/N) ratio. The LOQ and LOD of steroids and other chromatographic characteristics are described in Table 3.

Total free urinary progesterone and testosterone levels were rated in urine samples from human volunteers. The recovery and concentrations of the analyzed sexual hormones were measured in men (aged 20–40), women (aged 20–30), women with hormonal disorders (aged 20–30), girls (aged 8–12) and boys (aged 8–12). The obtained results testify

Table 3 Chromatographic characteristics of steroids

Hormones	k[a]	α[b]	LOD[c] (ng mL^{-1})	LOQ[d] (ng mL^{-1})	r
Testosterone	0.77	3.06	0.47	1.15	0.9993
Progesterone	2.36		0.47	1.15	0.9995

[a]k—retention factor—$k = (t_R - t_0)/t_0 = t_R/t_0$; t_R—retention time; t_0—hold-up time (for uracil)

[b]α—separation factor ($\alpha = k_2/k_1$)

[c]Limit of detection (3S/N)

[d]Limit of quantification (10S/N)

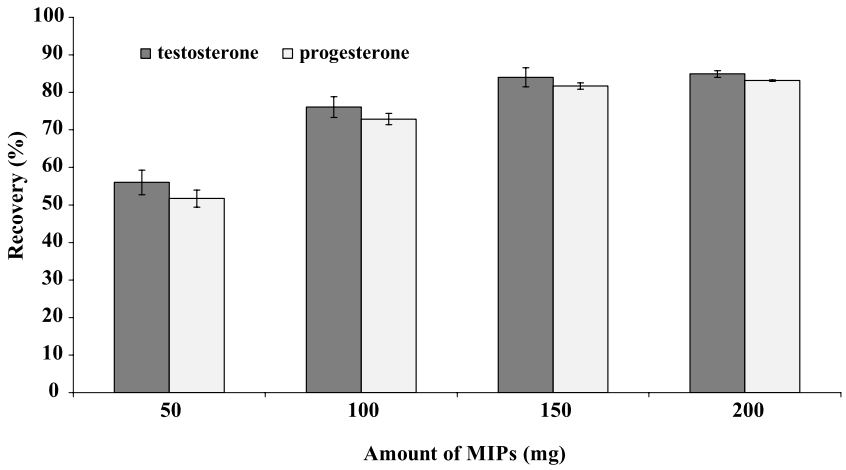

Fig. 6 The effect of change in polymer amount on recoveries of isolation steroids

to the supreme capacity of the disposable MISPE technology involving the HPLC-DAD method for progesterone and testosterone quantification. The MIP, used as a facultative sorbent for the preconcentration and the determination of progesterone and testosterone levels in human urine samples, also delivered a remarkable degree of correctness. The use of acetonitrile as the eluting solvent increased the recovery of both steroids, compared with NIP. The imprinted and non-imprinted polymers were capacitated with regard to their ability to bind steroid molecules (Fig. 7).

The efficacy of imprinted polymers P4, T4 (Table 1) was compared to non-imprinted polymers based on recovery values calculated with the involvement of the MISPE procedure for selecting steroids from urine samples. Steroid recovery was quantified by comparing the analyte/internal standard in spiked urine samples. As shown in Fig. 6, the recovery of imprinted polymers reached 81.7% (RSD = 0.86%) for progesterone and 84% (RSD = 2.55%) for testosterone, testifying to the structural and facultative, conformational memory of polymer to steroid binding. The recovery results for NIP polymers were 55.42% (RSD = 3.26%) for progesterone and 56.09% (RSD = 3.68%) for testosterone. In comparison with traditional SPE adsorbents (physical and chemical stability, storage time, imprint memory and binding strength), the above results and the recognition properties of the MIPs constitute adequate material for selective, accurate and efficient preconcentration, purification and extraction of progesterone and testosterone form human urine samples.

In healthy women, progesterone levels were in the range of $c_p = 14.67$–22.5 ng mL^{-1}, and testosterone concentrations were determined at $c_t = 0.18$–6.65 ng mL^{-1}. Steroid levels were also investigated in women with the "hyperandrogenic syndrome" (manifested by high testosterone levels). The reported values were $c_t = 1.49$–7.82 ng mL^{-1} for testosterone and $c_p = 19.71$–34.02 ng mL^{-1} for progesterone. In comparison with healthy women, elevated progesterone levels in women affected by hormonal disorders were not related to high testosterone concentrations.

The noted results were determined in the range of $c_t = 13.68$–23.07 ng mL^{-1} for testosterone and $c_p = 0.49$–1.62 ng mL^{-1} for progesterone. Steroid concentrations were also investigated in children, producing $c_p = 2.49$–4.03 ng mL^{-1} of progesterone in girls and $c_t = 5.99$–16.12 ng mL^{-1} of testosterone in boys. The resulting chromatogram of progesterone and testosterone levels in spiked urine samples from girls is presented in Fig. 8. The variation in steroid concentrations is subjective. The urinary excretion of testosterone and progesterone is considerably higher in urine samples than in serum specimens. The results of this study do not support the conclusion that urine specimens, demonstrating the incorporated secretion of hor-

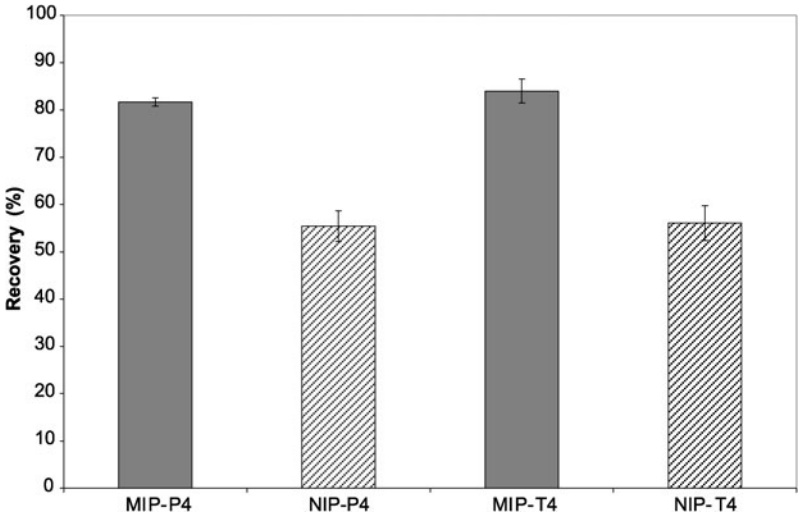

Fig. 7 The comparison of recovery values between MIP and NIP in elution step of MISPE analysis

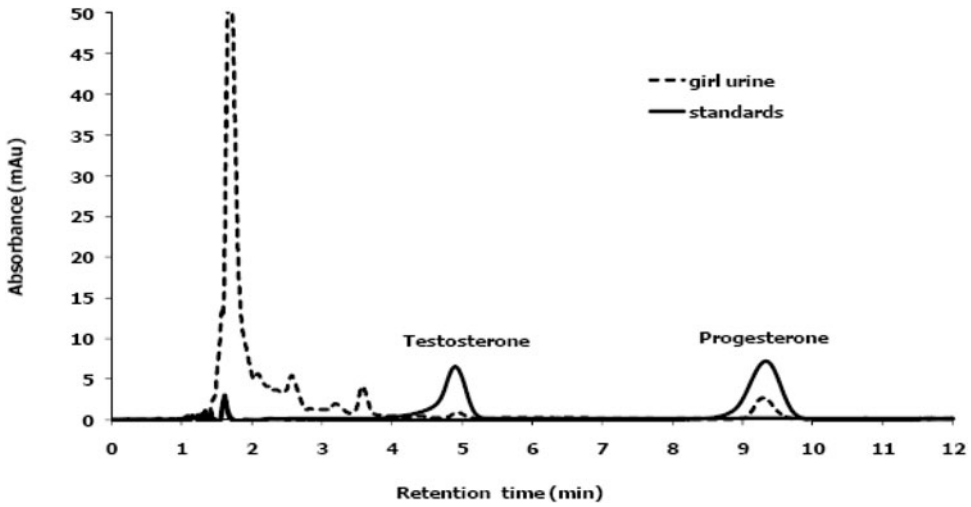

Fig. 8 Representative chromatogram obtained from extract of the urine girl with (the *dashed line*) and standard mixture of testosterone and progesterone (the *solid line*)

mones, show deviations in free hormone concentrations (the hormones are produced by several organs in the human body and their levels are determined by subjective variables). As noted by other authors, the resulting differences could be attributed to additional factors, such as the volume of collected urine, levels of physical activity and sympathetic nervous activity, as well as other dietary and nutritional factors affecting urinary excretion of hormones (Ooi et al. 1995; van Anders and Watson 2007; Saad and Vongas 2009; Andrada et al. 2007).

4 Conclusions

The MISPE technique emerged with synthesized MIPs using progesterone and testosterone as template molecules. The functional monomer MAA and the crosslinker TRIM or EDMA were dissolved in acetonitrile as porogen and isooctane/toluene (1:99%, v/v). MIPs were prepared using the UV-initiated polymerization method under the conditions described. The use of TRIM as a crosslinking agent ensured complete copolymerization. The authors concluded that structural and conformational memory is merged with the impact of the porogenic solvent. The MISPE procedure involving polymer P4, T4 as sorbents, prepared with the use of MAA, TRIM and isooctane–toluene (1:99%, v/v), delivered notably better results than other methods. As demonstrated by the results of this study, MISPE is a highly precise and adequate technique for the isolation and preconcentration of steroids. This experiment validates the usefulness of the isocratic reversed-phase HPLC method combined with DAD detection for the accurate separation of progesterone and testosterone from human urine specimens. The discussed HPLC-DAD method was highly sensitive and capable of quantifying low circulating levels of progesterone and testosterone in urine specimens. The results of this study point to the complimentary roles of the two easy, reliable and sensitive techniques—MISPE and HPLC with diode array spectroscopic detection—in clinical analyses of steroids.

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