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Toward the Use of a Molecularly Imprinted Polymer in Doping Analysis: Selective Preconcentration and Analysis of Testosterone and Epitestosterone in Human Urine

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A molecularly imprinted polymer (MIP), templated with methyltestosterone, has been synthesized for the cleanup of hydrolyzed urine samples for subsequent testosterone (T) quantification by LC−MS/MS. A concentration of 2 ng/mL testosterone could be quantified after a single step extraction on the MIP. The limit of detection and quantification with the criteria of a signal-to-noise ratio of 3 and 5 were 0.3 and 2 ng/mL, respectively. These values meet the conditions set by the World Anti-Doping Agency for the minimum required performance limits for doping controls, between 2 and 10 ng/mL. Epitestosterone (E) was also separated on this polymer and could be detected at concentrations down to 0.3 ng/mL. The quantification of T and E gives access to the determination of the T/E ratio, essential in doping analysis. Hence, our polymers can offer a more specific extraction procedure, resulting in increased sensitivity with limits of detection 10 times lower than the ones achieved by the standard SPE C_{18} sorbents employed in official testing laboratories.

Testosterone (T) is the primary male sex hormone. Due to its anabolic effects that lead to increases in muscle mass and strength, it is often illegally used to enhance athletic performance in sports. From the statistics provided by the World Anti-Doping Agency (WADA) in 2004, among the 1191 positive cases reported, 33% were due to testosterone.[1] One difficulty in the detection of testosterone abuse is the fact that it is a natural hormone. The range of T concentrations observed in random urine collections is large, such that direct quantification is generally not sufficient to determine if a doping violation has occurred. For this reason, a method for doping control based on the ratio of testosterone to its isomer epitestosterone (E) has been proposed.[2] Epitestosterone, a natural component of biological fluids, is only a minor product of the metabolism of testosterone, and its concentration is not influenced by exogenous administration of T.[3] Epitestosterone is generally present in almost equal concentrations to testosterone in urine; the ratio of urinary testosterone to epitestosterone in Caucasian adults is nearly constant, around 0.9−1.6 ± 1.0 in men and women.[4] Athletes are considered to be positive for drug abuse if T/E ≥ 4, and the concentration of testosterone or epitestosterone is >200 ng/mL, as set by WADA standards.[5]

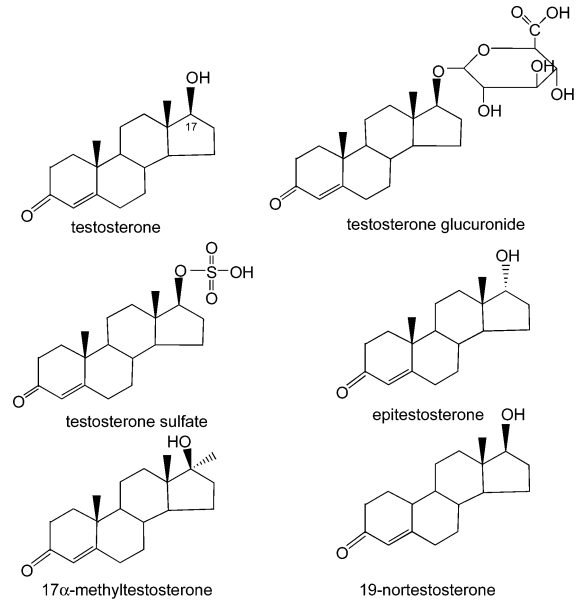

Figure 1. Structural analogues and metabolites of testosterone.

Both T and E are normally present in urine as their glucuronides (G) and sulfates (S) (Figure 1), with the free forms accounting for <3%.[6] In doping control, the most commonly used technique is based on gas chromatography coupled to mass spectrometry (GC/MS).[7−9] Determination of the concentrations of T and E is done after the conversion of the glucuronides,[6,8,9] the major urinary conjugates to their free form by enzymatic

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hydrolysis using β-glucuronidase.[7–12] Typical sample preparation methods consist of solid phase extraction (SPE) using reversed phase C18 cartridges, hydrolysis of the conjugates, liquid−liquid extraction, and derivatization to form trimethylsilyl ethers. Alternative techniques using LC−MS/MS which allow the direct determination of the conjugated analytes (TG, TS, EG, and ES) have been reported, after SPE extraction.[4,13] The measurements of the TG/EG ratio are validated only if the concentrations of free T and E are <5% of their associated glucuronides[5] due to the fact that the conjugates can undergo chemical modifications caused by thermal degradation and bacterial contamination. LC−MS/MS offers faster sample preparation and eliminates the quantitative recovery problems associated with the hydrolysis and derivatization steps in GC/MS, but the latter remains the preferred technique because it has a better resolution and sensitivity. The main reasons are that LC−MS/MS suffers from ionization and matrix effects leading to poor responses for quantification.[4,6,13] Recently, a method combining hydrolysis followed by liquid−liquid extraction and Girard P derivatization associated with LC/Q-TOF mass spectrometry has been reported to be very sensitive, this being attributed to derivatization with Girard P reagent for significantly increasing the ionization.[11] In any case, one of the possible ways to obtain a more sensitive response during mass quantification is to improve sample preparation procedures. Urine and a fortiori hydrolyzed urine are complex matrixes. Therefore, the development of new sorbents exhibiting highly selective recognition and binding of T and E will be extremely useful for sample cleanup.

The technique of molecular imprinting is widely employed to produce robust, stable, and cheap materials with specific binding sites.[14] This is achieved by copolymerizing functional and cross-linking monomers in the presence of a molecular template. After removal of the template, complementary cavities are obtained that allow rebinding of the template with very high specificity, comparable to that of natural receptors. These materials have been widely used as affinity matrixes for sample preparation and selective extraction of target analytes.[15−20] We report here the development of a molecularly imprinted polymer (MIP) specific for testosterone. The MIP was successfully applied for the specific extraction of testosterone and epitestosterone from hydrolyzed and nonhydrolyzed, spiked urine samples, followed by LC−MS/MS quantification.

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EXPERIMENTAL SECTION

Reagents and Materials. All chemicals were purchased from either Sigma-Aldrich (St-Quentin Fallavier, France), Acros Organics (Noisy Le Grand, France) or VWR International (Strasbourg, France), unless otherwise stated. Anhydrous acetonitrile and toluene were purchased from Sigma-Aldrich. Azo-bis-dimethylvaleronitrile (ABDV) was from DuPont Chemicals (Wilmington, USA). The monomers, methacrylic acid, and ethyleneglycoldimethacrylate were used as such. [1,2,6,7-^{3}H]Testosterone (activity: 1 mCi/mL; specific activity: 73 Ci/mmol) was from Amersham (Buckinghamshire, UK). *Attention: radiolabeled compounds represent a health hazard and must be handled with extreme care. They must be stored and disposed of according to specific legislation.* For binding assays, 3 μL was diluted in 10 mL of anhydrous acetonitrile or toluene and stored at −20 °C until further use. Deuterated standard, [16,16,17-^{2}H]testosterone solution (100 ng/μL) and lyophilized β-glucuronidase type IX-A from *E. coli* (25 kU/22 mg solid) were purchased from Sigma-Aldrich. Deionized water came from a Milli-Q water purification system (Millipore, Molsheim, France). Urine samples from two healthy European male volunteers aged 35 and 45 years were collected in sterile containers and immediately stored at −20 °C until analysis. *Attention: biological samples from human origin represent a potential health hazard and must be handled with care. They must be stored and disposed of according to specific legislation.*

Preparation of Molecularly Imprinted Polymers. The polymers were prepared in anhydrous acetonitrile or toluene. Typically, 0.2 mmol of methyltestosterone, 1.6 mmol of methacrylic acid (MAA), 5 mmol of ethyleneglycoldimethacrylate (EDMA), and 0.04 mmol of ABDV were dissolved in 1.5 mL of solvent in a glass vial fitted with an airtight septum. The mixture was then purged with nitrogen for 5 min on ice. Polymerization was done overnight at 40 °C in a water bath. The hard bulk polymers were ground manually with a mortar and pestle, transferred to 2 mL microcentrifuge tubes and milled with 2.8 mm ceramic beads in the presence of methanol in a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The polymers were then transferred to 50 mL centrifuge tubes and washed at 60 °C under agitation with 2 rounds of methanol/acetic acid (9/1), 3 rounds of ethanol/acetic acid (9/1), and 2 rounds of ethanol. They were then dried overnight under vacuum. Nonimprinted polymers (NIPs) were synthesized in the same way but without the addition of the imprinting template. The size of the polymers was determined by dynamic light scattering using a Zetasizer Nano-ZS (Malvern Instruments, Orsay, France) and confirmed by scanning electron microscopy.

Equilibrium Binding Studies. The imprinted and nonimprinted particles were suspended in the appropriate solvent in a sonicating bath. From this stock suspension, polymer concentrations ranging from 0.5 to 40 mg/mL were pipetted in separate 2 mL polypropylene microcentrifuge tubes and 0.2 or 0.4 pmol of [^{3}H]testosterone was added. The final volume was adjusted to 1 mL with solvent. The tubes were incubated overnight at ambient temperature on a tube rotator (SB2, Stuart Scientific). They were then centrifuged at 16 000*g* for 10 min, and a 500 μL aliquot of the supernatant was pipetted into a scintillation vial that contained 4 mL of scintillation liquid (Ultra Gold,

PerkinElmer). The amount of free radioligand was measured with a liquid scintillation counter (Beckman LS-6000 IC). The amount of testosterone bound to the polymers was calculated by subtracting the amount of unbound testosterone from the initial amount of testosterone added to the mixture.

Competition Studies. In order to compare the selectivity of the molecularly imprinted polymer toward testosterone and epitestosterone, they were added to the binding assays to compete with radioactive testosterone. The competition assays were performed similarly to the binding studies described above. Nonradiolabeled ligand ranging from 1 to 10 000 nM and 0.2 nM (15 nCi) radioactive testosterone mixed with 0.5 mg of MIP in 2 mL polypropylene microcentrifuge tubes were used. The final volume was adjusted to 1 mL with toluene. Competitive binding was allowed to proceed overnight at ambient temperature. The amount of bound ligand was calculated by measuring the radioactivity from 500 μL of supernatant following centrifugation at 16 000*g* for 10 min.

Enzymatic Hydrolysis of Urine. A stock solution of *E. coli* β-glucuronidase was prepared at 7.7 kU/mL in 50 mM potassium phosphate buffer pH 6.8. Urine samples, if cloudy, were centrifuged at 16 000*g* for 5 min, before hydrolysis. Two milliliters of the supernatant was diluted with 1 mL of buffer; 200 units of glucuronidase were added, and the sample was incubated at 37 °C for 21 h in a water bath. After reaction, 1 mL of the mixture (equivalent to 0.67 mL of urine) was diluted with 4 mL of 10 mM sodium citrate buffer, pH 3.0, and applied to 30 mg of MIP and NIP. The extraction was done as described below.

Extraction of Testosterone from Water and from Urine with Radioactivity Monitoring. One milliliter of water, spiked with 100 pmol of testosterone and 0.4 pmol (30 nCi) of radioactive testosterone, was added to 30 mg of MIP and NIP in 2 mL polypropylene microcentrifuge tubes. The contents were vortexed, left to incubate for 3 min on a tube rotator, and centrifuged at 16 000*g* for 5 min. The supernatants were removed with a pipet. The polymers were then successively washed 3 times with 1.7 mL of water and thoroughly dried under vacuum overnight. One milliliter of toluene, optionally containing a small amount (up to 0.1% (v/v) of acetic acid), was added, and the polymers were left on a tube rotator for 5 min. After centrifugation (16 000*g*, 3 min), the supernatants were collected. This was repeated four times. The bound testosterone was eluted with 3 × 1 mL of ethanol. Aliquots (500 μL) of the supernatants were pipetted into scintillation vials containing 4 mL of scintillation liquid, and the radioactivity was measured by liquid scintillation counting.

Urine Samples. One milliliter of urine, spiked with 100 pmol of testosterone, was diluted with 4 mL of 10 mM sodium citrate buffer, pH 3.0, and added to 30 mg of MIP and NIP in a 15 mL Falcon tube. The contents were vortexed, left to incubate for 3 min on a tube rotator, and centrifuged at 10 000*g* for 5 min. The supernatants were removed with a pipet. The polymers were then successively washed 2 times with 5 mL of water, suspended in 1 mL of water, and transferred to 2 mL polypropylene microcentrifuge tubes. The contents were spiked with 0.4 pmol (30 nCi) of radioactive testosterone, and the same cleanup and elution procedures, as described for water samples, were applied. Radioactivity was measured on 500 μL aliquots of the supernatants.

Extraction of Testosterone from Water and from Urine with LC−MS/MS Monitoring. The same protocol, as described above, was applied except that no radioactive testosterone was added. After drying the polymers, cleanup was done with 3 × 1 mL of toluene, optionally containing 0.1% (v/v) of acetic acid, and the elution was with 3 × 1 mL of ethanol, respectively. The recovered supernatants were evaporated to dryness by portions of 0.5 mL using a SpeedVac concentrator system (Savant). The dry residues were suspended in 100 μL of acetonitrile, vortexed vigorously, and centrifuged for 15 min at 16 000*g*. An appropriate aliquot of the supernatant was mixed with an appropriate known concentration of the internal standard testosterone-d_3. Five microliters was then injected in the LC−MS/MS.

Liquid Chromatography/Tandem Mass Spectrometry (LC−MS/MS). A Dionex Ultimate 3000 RSLC system connected to a column ACCLAIM RSLC 120 C_{18}, 100 × 2.1 mm, 2.2 μm (Dionex), was used for HPLC separation.

For the analysis of spiked urine, the solvent system was as follows: A, 0.1% formic acid in water, and B, acetonitrile. The gradient program began with 20% B, was ramped to 80% B at 5 min, held at 80% for 7 min, returned to 20% B in 2 min, and held for 1 min. The flow rate was 450 μL/min. Under these conditions, the retention time for testosterone was ∼4.71 min. Positive ESI (electrospray ionization) mass spectra were acquired using a Quantum ACCESS Thermo Fischer triple quadrupole spectrometer equipped with an ESI source, set to a voltage of 3.8 kV. The source temperature was 80 °C, and the desolvatation temperature was 110 °C. Collision energy was 21 V; tube lens was 95 V, and the collision gas was argon. The cone and the desolvatation nitrogen flow rates were 50 and 450 L/h, respectively.

For the analysis of hydrolyzed urine, the solvent system was as follows: A, 0.1% formic acid in water, and B, methanol. The gradient program began with 20% B, was ramped to 80% B at 15 min, held at 80% B for 3 min, returned to 20% B in 2 min, and held for 2 min. The flow rate was 450 μL/min. Under these conditions, the retention times for testosterone and epitestosterone were ∼12.81 and ∼13.85 min, respectively. Positive ESI mass spectra were acquired using a Waters Quatromicro triple quadrupole spectrometer equipped with an ESI source, set to a capillary voltage of 3.42 kV. The source temperature was 120 °C, and the desolvatation temperature was 350 °C. The cone voltage was maintained at 32 V; the collision energy was 20 V, and the collision gas was argon. The cone and the desolvatation nitrogen flow rates were 46 and 643 L/h, respectively.

For both spectrometers, the spectra were recorded in SRM (selected reaction monitoring) mode with a dwell time of 200 ms. Testosterone and the internal standard testosterone-d_3 were monitored using transition m/z 289 → m/z 97 and m/z 289 → m/z 109 and m/z 292 to m/z 97 and m/z 292 → m/z 109, respectively.

RESULTS AND DISCUSSION

Preparation and Evaluation of Molecularly Imprinted Polymers. At the beginning, we synthesized a testosterone receptor binding mimic by molecular imprinting using the same protocol as we had previously described for another steroid,

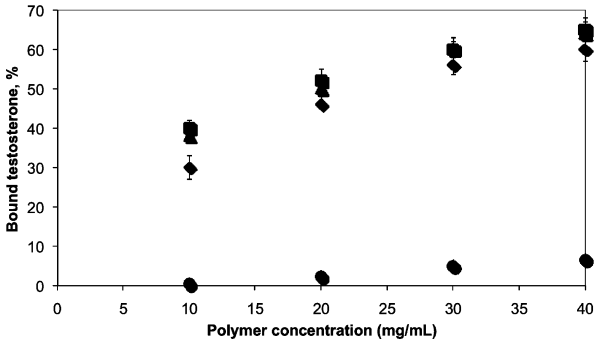

Figure 2. Equilibrium binding isotherms for radiolabeled testosterone (0.4 pmol, 30 nCi) on NIP (circle), MIP−MT (triangle), MIP−testosterone (square), and MIP−NOR (rhombus) in acetonitrile. Data are means from five independent experiments with three different batches of polymers. The error bars represent standard deviations.

β-estradiol,[21] but with testosterone as the template. We confirmed in preliminary experiments that, as for estradiol imprinting, a MIP composition of 1:8:25 (template/MAA/EDMA) was optimal for testosterone. The resulting polymer was very selective toward testosterone and showed virtually no cross-reactivities with other steroids like β-estradiol, estrone, and α-ethynylestradiol (not shown). However, it proved to be unsuitable for microanalytical applications because traces of remaining testosterone template were released into the sample during the extraction process from biological fluids (urine, plasma, or saliva), yielding false results during quantification. This template bleeding could not be detected by UV absorbance at 238 nm but was observed when LC−MS/MS, a far more sensitive method, was used for detection. We assume that the same problem was at the origin of testosterone overestimation in biological samples in a recent publication.[22] To circumvent this problem, Andersson has suggested the use of a template structurally slightly different from the analyte for molecular imprinting,[23] an approach that Takeuchi later termed the "dummy" template.[24] We adopted the same strategy and subsequently synthesized MIPs using 17α-methyltestosterone (MT) and 19-nortestosterone (NOR) as dummy templates (Figure 1). Compared to testosterone, MT has an additional methyl group on C17 and NOR has one methyl group less. Thus, both have masses different from testosterone, and even if template bleeding occurs during extraction, its mass spectrum will be different from that of testosterone. Hence, it will not interfere with the quantification of the latter. For this approach to be successful, it is important that the target analyte is specifically recognized by the MIP despite the structural difference with the template. The binding isotherms of the MIPs templated with MT, NOR, and testosterone and that of a corresponding nonimprinted control polymer are shown in Figure 2. Both MIP−MT and MIP−NOR retained T nearly as well as MIP−testosterone, with very good imprinting effects. MIP−MT, for its slightly higher binding capacity, was chosen for further studies.

Guided by the work of Karube's group, we initially used acetonitrile for the preparation of the polymers.[25] Besides acetonitrile, commonly used solvents in the molecular imprinting technique are toluene and chloroform for their aprotic and nonhydrogen bond donor character.[16] Previously, chloroform has been employed resulting in polymers with lower affinities than those prepared in acetonitrile.[26] When we tested these other solvents, and in particular toluene, for MIP preparation, we found that the MIP prepared and evaluated in toluene (Figure 3A) had a much higher binding capacity than a MIP prepared and evaluated in acetonitrile (Figure 2). Only 500 μg of polymer, rather than several milligrams, are necessary to bind 50% of the added radioligand. The NIP prepared in toluene also binds testosterone to a larger extent, indicating higher nonspecific binding. The latter can be reduced by adding a low concentration of acetic acid as a competitor to suppress nonspecific binding to the carboxyl groups in the polymers (Figure 3B).[27] Indeed, a more favorable imprinting factor (binding to MIP/binding to NIP) was obtained, increasing from 4 (no acetic acid) to 14 (0.5% acetic acid). However, in the course of our experiments, we observed that the MIP prepared in toluene showed a far better performance in extraction experiments from water and urine than the MIP prepared in acetonitrile. Toluene was, therefore, used for all further experiments.

In order to determine the relative selectivities of epitestosterone and testosterone for the MIP, competition studies at equilibrium were performed. E differs from testosterone only in the configuration of the OH on C17 (Figure 1). Fixed amounts of 0.5 mg of MIP and 0.2 pmol of radioactive T and variable amounts of competing T and E from 1 to 10 000 nM were tested. The results are shown in Figure 3C. The values of IC_{50} (the concentrations of competing ligands required to displace 50% of the specifically bound radioligand) for testosterone and epitestosterone, determined from a nonlinear regression fit, were 0.4 and 7.4 μM, respectively, which means that the cross-reactivity of E with T is 5.4%. This indicates that the MIP is rather specific for T.

Extraction of Testosterone from Water and from Urine. The polymers were evaluated as affinity sorbents for the extraction of testosterone from water and urine samples. Due to the small size (1 to 2 μm) of our polymer particles, they passed through the 20 μm frits of standard SPE cartridges (Sigma-Aldrich). These were, therefore, not suitable for our extraction experiments, and a different procedure using 2 mL polypropylene centrifuge tubes was developed. The polymers were mixed with the appropriate solvents on a tube rotator for 5 min, which is the approximate time for the solvent to percolate through an SPE column, followed by a rapid centrifugation. The supernanant was easily removed with a pipet.

Radioactivity Monitoring in Water. As outlined in the Experimental Section, 100 pmol of testosterone and 0.4 pmol of radioactive testosterone as tracer were extracted from 1 mL of water with 30 mg of MIP or NIP. Under these conditions, 94−96% of the spiked amount was bound to both NIP and MIP due to nonspecific hydrophobic interactions. After drying, the polymers were suspended in toluene containing 0.05% acetic acid to transform the nonspecific binding of testosterone to specific

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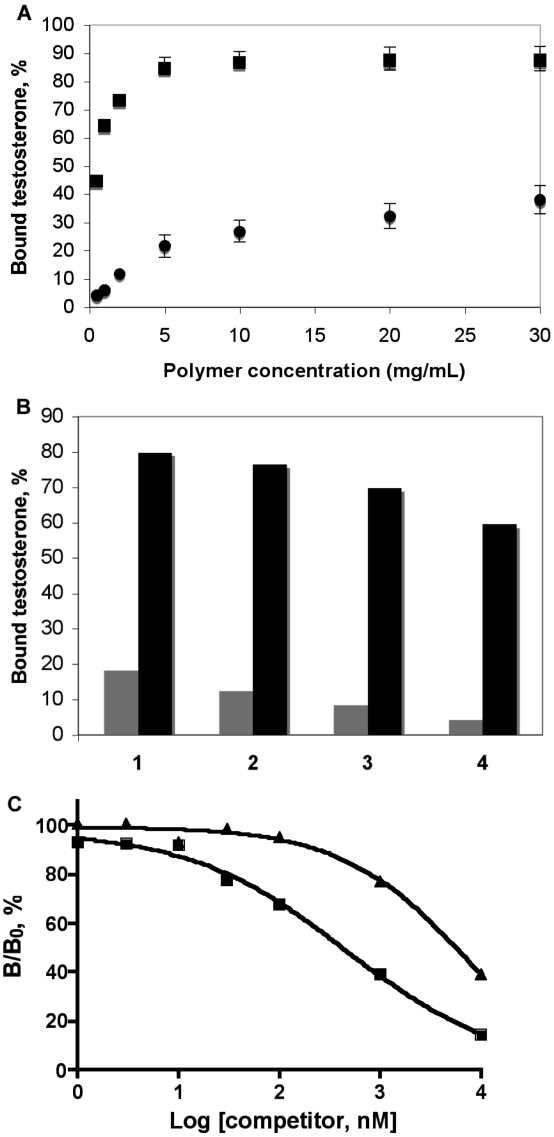

Figure 3. (A) Equilibrium binding isotherms for radiolabeled testosterone (0.2 pmol, 15 nCi) on NIP−MT (circle) and MIP−MT (square) in toluene. Data are means from five independent experiments from three different batches of MIP. The error bars represent standard deviations. (B) Effect of increasing concentrations of acetic acid (0.05% (1), 0.1% (2), 0.2% (3), and 0.5% (4)) on the binding of radiolabeled testosterone (0.2 pmol, 15 nCi) at equilibrium of 10 mg/mL NIP (gray bars) and MIP (black bars) in toluene. Data are means of duplicate experiments. (C) Inhibition of radioactive testosterone binding (0.2 nM, 15 nCi) to 0.5 mg of MIP−MT by testosterone (square) and epitestosterone (triangle) in toluene. B/B_0 is the ratio of the amounts of radioactive testosterone bound in the presence and absence of displacing ligand. Data are means of duplicate experiments.

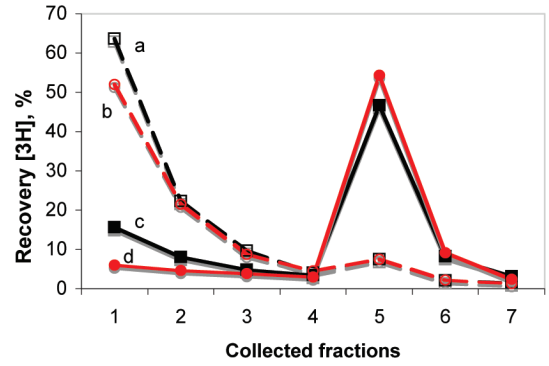

Figure 4. Elution profiles of radioactive testosterone on MIP (filled symbols) and NIP (empty symbols) during extraction of 1 mL of water, spiked with 100 pmol of testosterone and 0.4 pmol of [^{3}H]testosterone (30 nCi). Fractions 1−4 represent incubation with toluene + 0.05% acetic acid (traces b and d) and toluene + 0.1% acetic acid (traces a and c). The bound testosterone was eluted with ethanol (fractions 5−7).

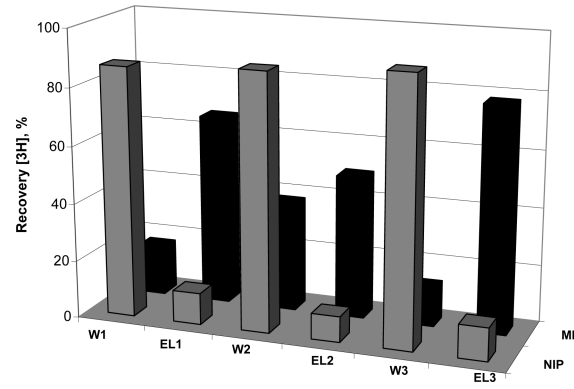

Figure 5. Extraction recoveries on 30 mg of NIP and MIP of 1 mL of water (W1 and EL1), 1 mL of urine (W2 and EL2), and 1 mL of urine, diluted with 4 mL of sodium citrate buffer pH 3.0 (W3 and EL3). All solutions were spiked with 100 pmol of testosterone and 0.4 pmol of (30 nCi) [^{3}H]testosterone. W: wash; EL: elution.

binding to the imprinted sites of the MIP (Figure 4 trace d, fractions 1−4 and Figure 5, W1). During this step, very little testosterone (13%) was retained on the NIP (Figure 4 trace b, fractions 1−4 and Figure 5, W1). This solvent system was the best compromise for maximum recovery; with the larger (0.1%) acetic acid content in toluene, testosterone was not well retained (32% nonretained) on the MIP (Figure 4 trace c, fractions 1−4). It should be noted that binding here probably occurs under nonequilibrium conditions since the contact of testosterone with the polymers lasts only for 5 min, which may explain the differences with the values of the equilibrium binding isotherms of Figure 3A. The bound testosterone was then eluted with ethanol (Figure 4 traces a−d, fractions 5−7 and Figure 5, EL1).

Radioactivity Monitoring in Urine. The same protocol was applied to the extraction from 1 mL of normal male urine (pH ∼ 7) spiked with 100 pmol of testosterone. Urine samples in general contain predominantly the soluble forms which are testosterone glucuronide, at concentrations between 120 and 163 pmol/mL[4,7] (approximately 80% of total T), and sulfates (approximately 20% of total T).[12] We assume that our MIP will only concentrate the free steroid and not the conjugates because they are much larger in size (Figure 1) and would not fit the cavities destined for testosterone in the rigid bulk polymer. Contrarily to what was observed in water (Figure 5, W1 and EL1), the testosterone in urine was not well retained on the MIP (Figure 5, W2 and EL2). Diluting the urine sample 10 times with water, as reported by others,[28] did not improve the retention. According to Möller et al.,[29] the matrix effects of urine, especially NaCl, interfere extensively with the retention on MIPs. These authors

(28) Theodoridis, G.; Zacharis, C. K.; Tzanavaras, P. D.; Themelis, D. G.; Economou, A. *J. Chromatogr., A* **2004**, *1030*, 69–76.

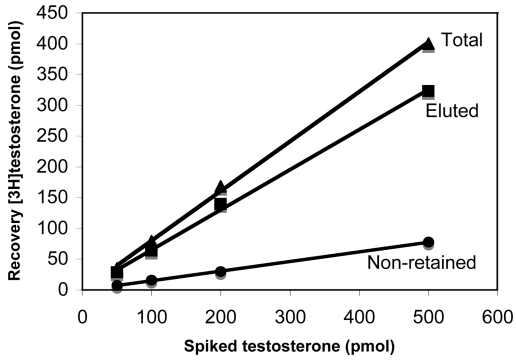

Figure 6. Capacity curves of 30 mg of MIP for 1 mL of urine, diluted with 4 mL of sodium citrate buffer, pH 3.0, spiked with 50−500 pmol of testosterone and 0.4 pmol (30 nCi) of [^{3}H]testosterone.

reported that these effects could be partially suppressed by diluting the sample with 50 mM citrate buffer, pH 3.0. In our hands, diluting the urine containing spiked testosterone 5 times with 10 mM sodium citrate buffer, pH 3.0, before application to the polymers resulted in a 10% increase in retention, which was still not as good as the retention in water. However, by suspending the polymers in anhydrous toluene instead of toluene + 0.05% acetic acid, the retention was optimal on the MIP (Figure 5, W3 and EL3). Taken together, the above results show that our polymers are able to selectively extract testosterone from aqueous samples.

To determine the retention capacity of the MIP in urine, the latter was diluted with sodium citrate buffer, spiked with increasing concentrations of testosterone (50−500 pmol), and applied to the polymer. Radioactive testosterone was employed as tracer. The results show that, up to 500 pmol, a constant amount of 85% of testosterone was retained (Figure 6).

Monitoring with LC−MS/MS: Spiked Samples. Radioactive monitoring provides a rapid and very sensitive tool to follow and optimize the extraction process. However, the radioactivity counts allow only the calculation of the relative amount of testosterone. Therefore, in order to quantify accurately the absolute amount of testosterone, the fractions were analyzed by liquid chromatography−tandem mass spectrometry (LC−MS/MS). The protocol as described in the Experimental Section was employed to extract 100 pmol of testosterone spiked in urine samples; no radioactive testosterone was added. After extraction, the recovered fractions were concentrated to dryness in a SpeedVac concentrator and resuspended in acetonitrile. A T-d_3 internal standard was added to an aliquot, which was then analyzed by LC−MS/MS. Both transitions for T (m/z 289 → 97 and m/z 289 → 109) and T-d_3 (m/z 292 → 97 and m/z 292 → 109) were employed for qualitative and quantitative analysis since almost similar testosterone values were calculated from both chromatograms, which presented no interference from matrix components. Figure 7 (A−D) shows the normalized chromatograms of the recovered fractions from the NIP and the MIP. As expected,[30] and in contrast to the findings reported by Gadzala-Kopciuch et al.,[22] a nonspiked urine sample treated in the same manner presented no detectable testosterone (Figure 7, E). For validation purposes, this sample was spiked with testosterone and T-d_3 at concentrations of 0.25−100

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(30) Tse Sum Bui, B.; Haupt, K. *J. Chromatogr., B* **2009**, *877*, 4180–4181.

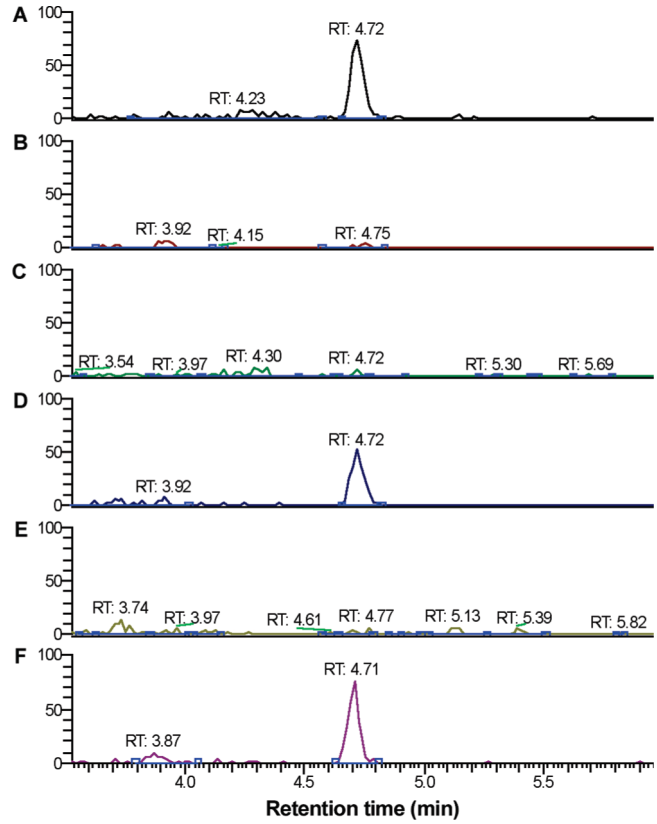

Figure 7. Chromatograms (transition m/z 97) of the fractions extracted from 1 mL of urine spiked with 100 pmol of testosterone. (A) NIP (wash with toluene + 0.1% acetic acid), (B) NIP (elution with ethanol), (C) MIP (wash with toluene), (D) MIP (elution with ethanol), (E) MIP (elution with ethanol of extraction from nonspiked urine), and (F) internal standard (T-d_3) added to D. The chromatograms were normalized to the intensity of the peak in chromatogram A.

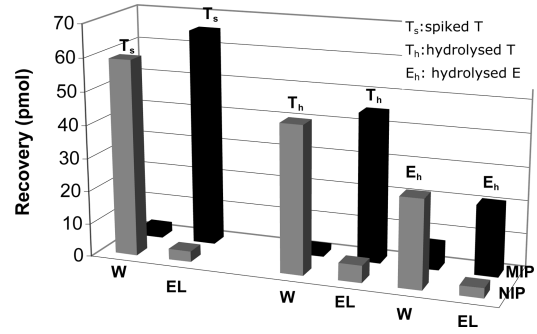

Figure 8. Extraction recoveries on NIP and MIP of 1 mL of urine spiked with 100 pmol of testosterone and of 0.67 mL of urine hydrolyzed with β-glucuronidase, as analyzed by LC−MS/MS. Transitions for T (m/z 289 → 97 and m/z 289 → 109) and T-d_3 (m/z 292 → 97 and m/z 292 → 109) were employed for quantification. W: wash; EL: elution.

ng/mL for the determination of linearity. The limit of detection (LOD) and quantification (LOQ) with the criteria of signal-to-noise ratio of 3 and 5, respectively, were 0.3 and 2 ng/mL, respectively (see Supporting Information, Figure S1).

The recoveries (Figure 8) were determined by comparing the testosterone/internal standard peak area ratios. The results confirm that our MIP is very efficient to specifically extract testosterone from urine samples, affording clean chromatograms free from matrix interference. Apparently, a better retention was observed with LC−MS/MS than with radioactivity detection

(Figure 5). However, this is most certainly due to the radioligand having partly exchanged its radioisotope with the solvent.

Hydrolyzed Urine Samples. In doping analysis, standard methods are applied to glucuronidase treated samples only; the T excreted as sulfate is usually neglected.[6,12] Moreover, no efficient and consistent sulfate hydrolysis protocol is available to date,[6,12] so we focused our effort only on the deconjugation of the glucuronides. Different protocols were experimented with *E. coli* β-glucuronidase. This was done by trial and error because no consensual literature data was available. For example, the amount of enzyme could vary from 10 U[7] to 5000 U,[9] but most of the time, it was not stated.[8,10,31] The time of incubation could vary from 1 h[7,31] to 22 h[12] and the temperature from 37 °C[12] to 55 °C.[7–9,31] For our part, 1 mL of 50 mM potassium phosphate buffer, pH 6.8, was added to 2 mL of urine, and the sample was hydrolyzed according to the following conditions: 10 units, 55 °C, 1 h; 27 units, 55 °C, 1 h; 27 units, 37 °C, 2 h; 200 units, 37 °C, 21 h. Only the last condition, derived from Hauser et al.,[12] was successful since testosterone was detected on the LC−MS/MS after extraction on our polymers. Quantification yielded 45 pmol of testosterone, but the extent of hydrolysis is unknown. Surprisingly, a peak migrating at the same retention time, t_R = 13.85 min as for an epitestosterone standard, was detected. This peak was present in all the recovered fractions and could be easily quantified (based on the area of T-d_3). The chromatograms issued from NIP wash and MIP elution, which represent most of the T and E recoveries, are shown in Figure 9. This finding suggests that our MIP can also retain E, which is baseline separated from T on LC−MS/MS. Although E displayed only 5.4% of cross-reactivity with respect to T (Figure 3C), it should be noted that the amount of polymer per volume used in the extraction experiments (30 mg) is much higher than that of the cross-reactivity tests (0.5 mg). This shifts the equilibrium toward complex formation (MIP−analyte), and it is, therefore, not surprising that the more weakly adsorbed E is also extracted by the MIP.

The recoveries are presented in Figure 8. Thirty milligrams of MIP was used for the extraction process. T was completely retained, and E was partially retained. By increasing the amount of polymer, E would also be quantitatively retained. In any case, the total amount of E, which is the sum of the recovered values from the wash and the eluted fractions, can be deduced: ~29 pmol from either the MIP or the NIP, giving access to the T/E ratio. Figure 9B represents chromatograms corresponding to 45 ng/mL of T and 19 ng/mL of E eluted from the MIP. When the sample was diluted 20 times, 2.2 ng/mL of T and 0.8 ng/mL of E were determined (see Supporting Information. Figure S2). This means that the minimum sensitivity required in doping analysis, between 2 and 10 ng/mL,[32] can be achieved after a single extraction step on the MIP. Nonspecific extraction sorbents like C_{18} cartridges coextract a large number of other compounds that are also ionized and generate a strong background in MS, compromising sensitivity.[13] Due to the specificity of extraction provided by the MIP, very clean chromatograms were obtained, thus allowing for a lower detection limit. The above results also imply that the T and E in women (TG: 6 ng/mL)[4] and children's urine would be quantifiable.

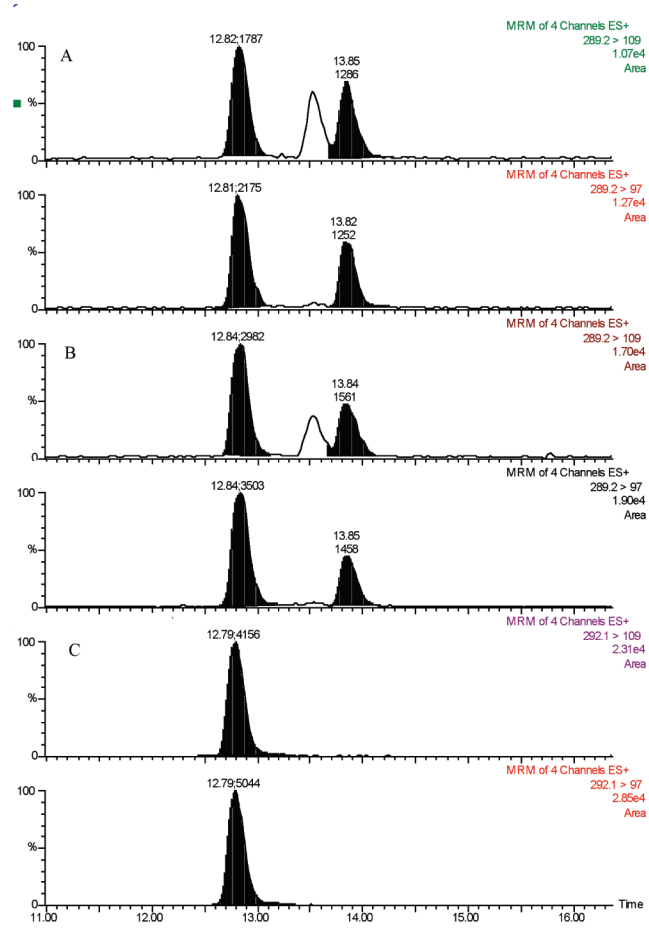

Figure 9. Chromatograms of the fractions extracted on 30 mg of NIP and MIP from 0.67 mL of hydrolyzed urine. (A) NIP (wash with toluene + 0.1% acetic acid), (B) MIP (elution with ethanol), and (C) internal standard (T-d_3) added to B. Retention time for T and T-d_3 is ~12.8 min and for E is ~13.8 min.

CONCLUSION

A molecularly imprinted polymer, templated with methyltestosterone, has been synthesized for the cleanup of hydrolyzed and nonhydrolyzed spiked urine samples for testosterone quantification. Epitestosterone was also separated on this polymer, giving access to doping analysis. Since the affinity for T is greater than E, the extent of retention of the latter is probably not optimal under the conditions used in this work. A solution could be to either increase the amount of polymer used for extraction or use a specific MIP for E. We also considered the synthesis of MIPs specific for the glucuronide or sulfate conjugates of T. However, although the deconjugation step would be avoided in this way, due to the high cost of the template and, therefore, of the resulting MIP, it was, for the time being, not considered an economically viable solution. The limit of detection with a signal-to-noise ratio of three was 0.3 ng/mL for both T and E. Official testing laboratories, using different nonspecific extraction methods, have reported LODs of 2 ng/mL and 0.5 ng/mL for T and E[11] and 3 ng/mL for TG and EG.[4,13] Hence, our polymers can offer more specific extraction requiring only one extraction step, permitting a higher sensitivity. Preliminary experiments show that they could

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also serve for the selective extraction of testosterone in other biological fluids like plasma or serum.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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