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Letter to the Editor

Comment on 'Isolation and detection of steroids from human urine by molecularly imprinted solid-phase extraction and liquid chromatography' by Gadzala-Kopciuch et al., J. Chromatogr. B 877 (2009), 1177–1184

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The paper by Gadzala-Kopciuch et al. reports the synthesis of molecularly imprinted polymers (MIPs) for three naturally occurring steroids: testosterone, progesterone and β-estradiol. These polymers were then applied as sorbents in solid-phase extraction (SPE) to isolate the corresponding steroids from human urine. Based on HPLC analysis with peaks eluting at the same retention time as the corresponding steroid standards, the authors conclude that their MIP-SPE procedures can be used for sample clean-up and pre-concentration of the steroids in question from biological fluids, especially human urine.

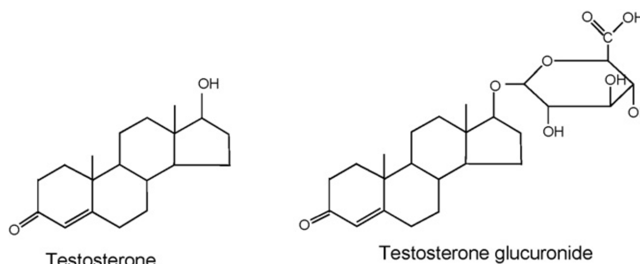
Unfortunately, closer inspection of the paper raises doubts about the validity of the results and the conclusions drawn. The authors, for instance, recovered mean amounts of 100.095 ng/mL and 219.99 ng/mL progesterone and β-estradiol, respectively, in urine samples of *normal* healthy women, and 113.01 ng/mL of testosterone in urine samples of *normal* healthy men (Table 6). These amounts are exceedingly high when compared to levels quoted in the literature (see below).

In fact, steroids are present in human urine mainly as water-soluble conjugates, i.e. glucuronides and sulfates, and not as free compounds [1–5]. Indeed, as stated in the authors' Ref. [8], about 99.7% are conjugated and 0.3% are free [2]. This means that the free steroids occur at extremely low levels. In order to quantify the total amount of steroids, these have to be deconjugated, which is usually done by enzymatic or acidic hydrolysis [1–5]. Alternatively, it is possible to quantify the conjugated forms if the appropriate standards (steroid-glucuronides and steroid-sulfates) are employed [3,4]. Since there is neither mention of a hydrolysis step in the paper, nor have glucuronide or sulfate derivatives been used as standards, we conclude that the authors have measured the endogenous free steroids contained in 1 mL of urine (see Section 2.5).

For testosterone, literature data quote the mean amount in urine after deconjugation to be 30.4 ng/mL [6]. Consistent levels of testosterone glucuronides (the major form in urine) of 32 ng/mL [7], 46.8 ng/mL [4] and 34.6 ng/mL [8] have also been published. This means that the level of free testosterone in urine before deconjugation is typically below 1 ng/mL. Similarly, the amount of urinary β-estradiol in normal women, after enzymatic hydrolysis, was found to vary from 7 ng/mL to 16 ng/mL during the menstrual cycle [9]. Lower levels of β-estradiol, 0.74 ng/mL together with 0.49 ng/mL progesterone, after hydrolysis of urine of healthy women have been found in one paper [5]. This again implies that the levels of the free, unconjugated hormones are typically below 1 ng/mL.

In the light of the above, the high levels of free steroids reported by Gadzala-Kopciuch et al. are most probably an artifact. In any case, it is unlikely that the urine matrix could sustain the solubility of such high concentrations of free steroids. The values obtained may be explained by the fact that the peaks in Figs. 8 and 9 that the authors attribute to pre-concentrated steroids from the urine sample correspond in reality to the steroids eluted from the MIP due to bleeding of residual template, which remained in the polymer after processing (template extraction) of the MIP. This greatly overestimates the amounts of steroid present in the samples. It is well-known from the literature that bleeding of the template molecule can be encountered during MIP-SPE [10–12], a fact that was also confirmed by our own experience. To avoid this interference during trace analysis, structural analogues are often employed as templates [11,12]. Incidentally, there is no mention in the paper about the analysis of the MIP for residual template. In any case, simply quantifying the template in the washing solutions would not be sufficient in this respect. Another potential problem is the presence of impurities eluting at the same time as the steroids. The testosterone peak in Fig. 8A is particularly intriguing as it is not well separated from the preceding impurities. The above problems could have been assessed, but no control chromatograms of the blank urine (without passage over the polymers) and of urine extracted with the non-imprinted polymers (NIPs) are shown.

One might argue that the MIPs could as well concentrate the conjugated steroids but their sizes are much larger compared to the free forms (see figure below for testosterone) and it is difficult to imagine that the cavity destined for the free steroids in the rigid bulk polymer could accommodate, for instance, the glucuronides. In addition, even if the conjugates were co-extracted with the free steroids, they would not co-elute with them on the analytical HPLC column.



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The authors' choice of eluents for the extraction of the steroids is also questionable in the context of the results reported. The authors present no elution profiles of the MIPs and the NIPs, so that one cannot evaluate the selectivity of the imprinted polymers versus the non-imprinted ones. An optimal selectivity requires the presence of both specific cavities and a proper choice of solvents favoring the development of specific interactions with these cavities and limiting non-specific interactions [10]. After the application of urine on the column, methanol, acetonitrile, chloroform, dichloromethane/methanol (20/80) were employed to elute the analytes (Section 2.5). Percolating methanol immediately after applying urine seems an odd choice, since this solvent is not polar enough to favor non-specific adsorption of the steroids like in water, and at the same time it will disrupt the hydrogen bonds that are at the origin of the specific binding of the steroids to the imprinted binding sites. Thus, all the steroids will elute from the column together with most of the impurities. Therefore, what is the rationale of applying acetonitrile and chloroform as eluents, which are non-hydrogen bond donor solvents, after methanol?

In conclusion, the lack of some important control experiments makes us believe that the extraction results for trace analysis of steroid hormones in urine as reported by Gadzala-Kopciuch et al. are artifacts, greatly overestimating the amount of steroids in the sample, the reason probably being bleeding of residual template from the MIP.

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