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Optimization of the hydrolysis stage for sample preparation for the steroid profile and xenobiotics analysis

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Introduction

Comprehensive method for the detection of steroids in urine is very important for the evaluation of individual atypical results as well as their distinguishing from doping abuse cases. For that purpose we have developed a reliable procedure for the simultaneous hydrolysis of glucuronides and sulfates of endogenous and anabolic steroids in human urine. Combination of two enzymes in an appropriate buffer system is proposed as a simple and innovative approach for the preparation of urine samples.

Materials and Methods

Buffer preparation and experimental conditions

Four buffers were prepared for the experiment:

a) Phosphate buffer (0.8 M, pH 6.4); b) Imidazole buffer (0.2 M, pH 6.2, 6.4, 6.6, 6.7); c) Acetate buffer (0.2 M, pH 5.5; 5.7; 6.0; 6.4); d) Citrate buffer (0.1 M, pH 5.7, 6.0, 6.3, 6.5)

Phosphate buffer was used only at pH 6.4 designated as the standard hydrolysis (SH) because the phosphate ions inhibit the arylsulfatase activity. The control of the glucuronides hydrolysis was performed using D4-androsterone-glucuronide and D5-ethiocholanolone as described [1]. The control of the sulfate hydrolysis was not performed because solvolysis could lead to the degradation of several compounds [2], and the usage of deuterated standards was not studied yet.

Studied enzymes

Four enzymes were investigated: a) β-glucuronidase from *E. Coli* (Roche, 03 707 601 001); b) β-glucuronidase / arylsulfatase from *Helix Pomatia* (Sigma, G7017); c) β-glucuronidase / arylsulfatase from Helix pomatia (Sigma, S9751); d) arylsulfatase from *Helix Pomatia* (Roche, 10 102 890 001)

Standard hydrolysis was carried out with β-glucuronidase from *E. Coli*, while the other experiments included the mixture of enzymes, as shown in the table below. For the hydrolysis study, each enzyme mixture was tested in each buffer at four pH listed above.

Name	Enzymes	V, µl
SH	β-glucuronidase from *E. Coli* (Roche)	30
HP	β-glucuronidase from *E. Coli* (Roche)	30
	β-glucuronidase/arylsulfatase from *Helix Pomatia*, (Sigma)	10
ARS	β-glucuronidase from *E. Coli* (Roche)	30
	β-glucuronidase/arylsulfatase from *Helix Pomatia* (Sigma)	10
ARR	β-glucuronidase from *E. Coli* (Roche)	30
	arylsulfatase from *Helix Pomatia* (Roche)	10

Sample collection

One administration trial of DHEA to male volunteer was conducted after approval of the Human Ethics Committee and the informed consent of the subject. Subject has orally administered 150 mg of DHEA. A total of six urine samples were collected at regular intervals up to 24 hours after administration. All six samples were analyzed by GC/MS, and three of them, which gave the highest DHEA concentration, were combined into one urine pool for the further experiments.

Sample preparation

To 3 mL of urine 1 mL of the buffer (each at four pH values), 30 µL methyltestosterone internal standard solution (50 ng/µL in methanol), and exact amount of the enzyme (or enzyme mixture) were added. The mixture was briefly vortexed and incubated at 55ºC for 70 min. After hydrolysis 1 mL of carbonate buffer (pH 10.4) and 5 mL of diethyl ether were added and the samples were extracted for 5 min by rigorous vortexing. The organic layer was separated after centrifugation and evaporated to dryness at 60˚C for 40 min. Finally, 50-µL MSTFA/NH_4I/DTT (1000:3:2) reaction mixture was added to the test tube and heated at 70˚C for 30 min, then transferred to a 200-µL conical vial and analyzed by GC-MS.

Results and Discussion

Three buffer systems were tested under the same conditions at selected pH. Imidazole and acetic buffers were not active enough for the full hydrolysis of androsterone and etiocholanolone (data not shown), so the main ratios and concentrations of the investigated steroids in imidazole and acetic buffers were incorrect. The ratios of all steroids in citrate buffer were higher then in SH (**Fig. 1A**) because the concentrations of investigated steroids increased disproportionately (**Fig. 1B**). Higher yield of steroids compared to SH (100% completeness of hydrolysis) could be explained by the additional contribution of the steroids

present in the sulfate fraction. The most noticeable increase was achieved with the use of mixture ARR (β-glucuronidase from *E. Coli* and arylsulfatase from *H. Pomatia*, both manufactured by Roche). These enzymes are also preferable because of the absence of the hydrolysis side products and a comparative simplicity of the sample preparation. The developed method of hydrolysis in citrate buffer was used for the analysis of 100 routine urine samples, declared negative after doping control analysis using validated methods. The data obtained was compared with that produced under the conditions of the SH. All the data were processed in Statistica software, and no significant difference in concentrations and ratios of the investigated steroids was detected.

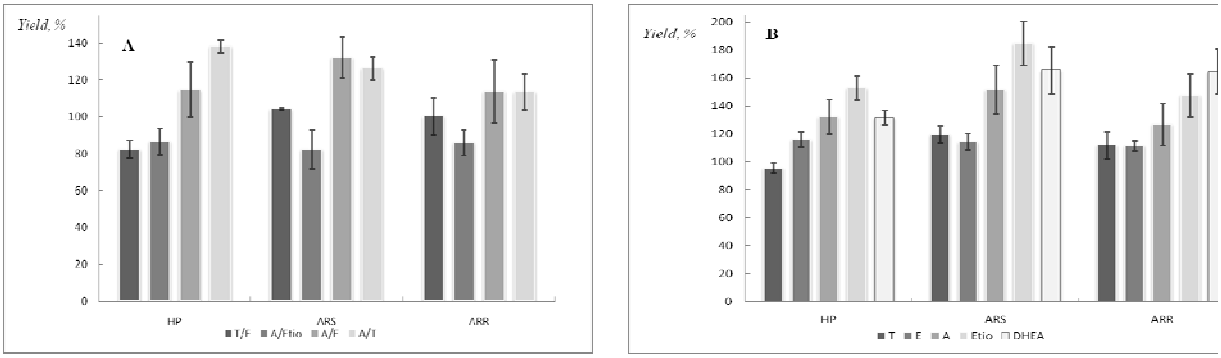

Fig. 1. Main ratios (***A***) and concentrations (***B***) of endogenous steroids in citrate buffer (% relative to SH). The data obtained under optimal pH conditions for each enzyme system are presented.

It was shown that the proposed method of hydrolysis could be used as a routine method because it provides the same yield of hydrolysis as the SH when the prohibited substances were not used. In cases of prohormone administration (especially DHEA) the increase in concentrations of the most steroids was noted.

Hydrolysis technique in citrate buffer was tested on 100 real urine samples that did not contain any prohibited substances. Also these samples were tested by SH technique. The data were processed in program "Statistica" and it was found that there are no significant differences between concentrations of investigated endogenous steroids obtained using SH and hydrolysis in citrate buffer. However the control of side enzymatic activity of *H. Pomatia* preparations related to the presence of 3ß-hydroxy-steroid-Δ5-steroiddehydrogenase and steroid Δ4-isomerase, described in the literature [3], was not performed.

The proposed method of hydrolysis was used for the analysis of two substances, excreted mostly as sulfates: the first contained terbutaline, and the second – fenoterol. The obtained data (SIM mode) are presented in **Fig. 2**. The concentrations of both investigated substances increased twice compared to the routine hydrolysis.

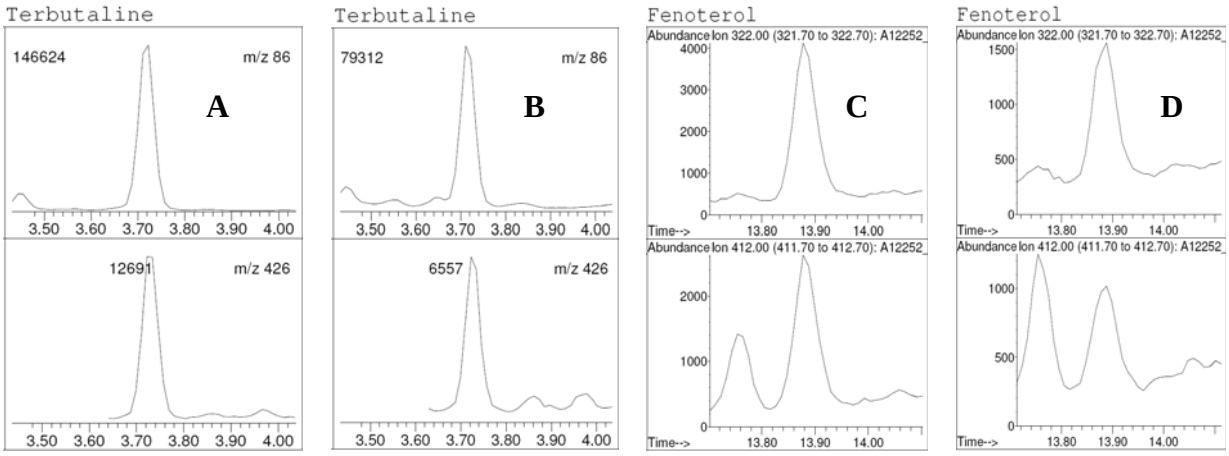

Fig. 2. The usage of proposed hydrolysis for the analysis of terbutaline and fenoterol. ***A***, ***C*** – combined hydrolysis of sulfates and glucuronides; ***B***, ***D*** – routine hydrolysis.

Conclusion

A procedure for the simultaneous hydrolysis of glucuronides and sulfates of urinary endogenous and exogenous steroids has been developed. The combined usage of two enzymes: β-glucuronidase *E. Coli* (Roche) and arylsulfatase *H. Pomatia* (Roche) in citrate buffer offered the best results for the sample preparation. This approach could be proposed as a routine method for analysis of steroid by GC-MS, as well as to identify the long-term metabolites which are eliminated in urine as sulfates, and hydrolysis of other xenobiotics to increase the sensitivity of their determination. But there are two disadvantages of this approach: 1) the side activities of the *H. Pomatia* enzyme could lead to wrong values in steroid profiling and should be studied more carefully; 2) WADA protocol for the steroid profile analysis applied only to the glucuronide fraction of steroids.

References

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[3] Gomes RL, Meredith W, Snape CE, Sephton MA. (2009) Analysis of conjugated steroid androgens: Deconjugation, derivatisation and associated issues. J. Pharm. Biomed. An., 49, 1133–1140.