

Time-Of-Flight Mass Spectrometry: Unification of Analytical Methods and Preventive Screening in Doping Control Analysis

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Abstract

The evolution of Time-Of-Flight (TOF) technology in the fields of electronics and hardware geometry (orthogonal acceleration, reflectron) resulted in an improved mass accuracy and good sensitivity in scan mode. In other analytical fields, like food, the use of TOF instruments is extensive, but not in doping control analysis [1, 2]. Our approach in the use of TOF technology comprises two main axes:

1. Unification of the gas and liquid chromatographic/ mass spectrometric procedures using one hydrolysis step, common extraction step, one derivatisation procedure for the aliquots that are designated to be injected in GC instruments and, finally, the underivatised aliquot of the same sample to be injected to LC instrument.
2. Preventive screening for designer steroids using CG/TOF/MS and LC/TOF/MS analysis.

Since the project is supported by an ongoing 3 years (2005-2008) grant from WADA, the present study will cover the parts of the entire task, together with data showing the combined screening of LC/TOF/MS and GC/TOF/MS for the prevention of designer steroids abuse.

Introduction

The aims of the project are the following:

- A. Unification of the WADA Accredited Doping Control Laboratories screening procedures of classes of prohibited substances usually analysed by GC/MS or LC/MS, e.g. stimulants, narcotics, anabolic agents, diuretics etc. The goal of the new method is to apply a unique sample preparation (hydrolysis, extraction and derivatisation) and analysis by GC and LC TOF/MS instruments. In the current publication, results concerning hydrolysis and GC derivatisation are presented.
- B. Preventive Screening: a new combined doping control screening analysis of anabolic steroids in human urine using LC/TOF/MS and GC/TOF/MS has been developed. Based on the validation data, we propose the use of the TOF/MS technology in a preventive way: to analyze selected by the sports authority doping control samples in high mass accuracy full scan mode and to store in a long-term basis the A and B bottles. When a new designer steroid is revealed, the laboratory would be able, without reanalyzing the stored samples, to reprocess the electronic data files acquired during the routine screening in comparison with the newly acquired reference data and reactivate the test in case of a suspect finding. This methodology, which can be used in addition to the routine screening for anabolic steroids, is based on the assumption that a combined screening with different chromatographic systems, GC and LC and with different ionization devices, electron impact and electrospray, would be able to detect “blindly” abuse of anabolic steroids with similar steroid structures with those included in the current validation.

The GC/TOF/MS and LC/TOF/MS analysis is running in the WADA Accredited Doping Control Laboratory of Athens, Greece (OAKA) and the LC/TOF/MS in the RIKILT – Institute of Food Safety, Wageningen, Holland (RIKILT).

Experimental

Unification of Sample Preparation: Hydrolysis.

In the current step of the study, the comparison of the results of hydrolysis using the enzymes E.Coli (to cleave the glucuronide esters conjugates) and H.Pomatia (to cleave the

glucuronide and sulfate esters conjugates) to the endogenous steroids was performed. Endogenous steroids were quantitated using 3-point calibration curves. The 7 endogenous steroids incorporated in the current study are presented: Testosterone, Epitestosterone, Androsterone, Etiocholanolone, Dehydroepiandrosterone, 5 α -androstane-3 α ,17 β -diol, 5 β -androstane-3 α ,17 β -diol. Materials as in [3].

Instrumentation. GC/MS Agilent 6890 Series Gas Chromatography coupled with MSD 5973, with autosampler HP-5973 and HP Chemstation software. The chromatographic column was HP Ultra 1 17m \times 0,200 mm \times 0,11 μ m.

Testing urine samples. Two types of urine samples were used in the current step of the study:

A. Urine samples from a young aged girl, in order to be spiked with the reference solutions of the endogenous steroids and used as calibration samples.

B. Urine samples from 110 male and female athletes, who had agreed in the Athlete's Sample Collection Form, that their samples can be used for research purpose. The samples had been reported as negatives for prohibited substances, were originated from males and females and stored frozen at -20 °C until their reanalysis.

Analytical Methodology. After spiking of the urine samples, both calibration and athletes', with standard solutions, they were hydrolysed [3]:

- either pH is adjusted to 5.2 with subsequent addition of the enzymatic solution of β -glucuronidase with sulfatase activity from H.Pomatia and hydrolysis for 3h at 50°C
- or pH is adjusted to 7.0 with subsequent addition of the enzymatic solution of β -glucuronidase without sulfatase activity from E.Coli and hydrolysis for 1.5h at 50°C

After hydrolysis the samples are buffered and extracted in pH 9.5 with diethylether, evaporated, derivatised and analysed as [3].

Unification of Sample Preparation: Derivatisation.

Materials. Trimethylchlorosilane (TMCS) was obtained from Fluka, Germany (purity \geq 98%), ethanethiol from Sigma, Germany, propanethiol from Merck, Germany. The rest reagents were originated as in [3].

Instrumentation. Anabolic steroids analysis was performed by GC/MS and HRMS [3].

Stimulants, narcotics and diuretics analysis was performed by GC/MS [3].

Control samples. Initial tests and additionally derivatization process optimization tests were performed using an initial group of twelve substances representatives in terms of volatility, polarity, molecular weight and detection limit. This group was tested at concentration level higher than MRPL as following: testosterone 500 ng/mL, androsterone 500 ng/mL, 6 β -OH-methandienone 500 ng/mL, fluoxymesterone tetrol 500 ng/mL, 3-OH-Stanozolol 500 ng/mL, aminoglutethimide 500 ng/mL, atenolol 500 ng/mL, amiloride 500 ng/mL, benzoylegonine 1000ng/mL, amphetamine 100 ng/mL, ephedrine 3000 ng/mL, ethacrynic acid 1000 ng/mL. A final group of substances included in the routine GC quality control samples at concentration level of MRPL were tested with the optimum derivatization process.

Optimization of derivatisation process. A number of factors, i.e. the reaction conditions of temperature and time, the heating device, the derivatization reagents' proportions and the solvent or catalyst addition were examined during optimization process. One or two step derivatization process were also tested. In the one-step derivatization process, TMS derivatives of tested substances were prepared by the addition of 100 μ l derivatization reagent to the test tube after evaporation of working and internal standard solution. The tube was then capped, vortexed and incubated at 80 $^{\circ}$ C for 30 min. The following derivatizations reagents were tested in the one-step procedure:

- 1) MSTFA/NH₄I/DTE 1000/2/4 (v/w/w)
- 2) MSTFA/NH₄I/Ethanethiol 1000/2/4 (v/w/v)
- 3) MSTFA/NH₄I/Propanethiol 1000/2/5 (v/w/v)

The two-step derivatization process was performed as following: TMS derivatives of tested substances were prepared by the addition of 50 μ l of first derivatization reagent (MSTFA) in the test tube after evaporation of working and internal standard solution. The tube was then capped, vortexed and heated for 20 min in a dry block heater maintained at 80 $^{\circ}$ C temperature. After that, the addition of 50 μ l of the second derivatization reagent [MSTFA/NH₄I/Propanethiol 1000/2/5 (v/w/v)], was performed and the tube was further heated at 80 $^{\circ}$ C for 20 min. The optimised derivatization process was applied on the final group of tested substances in concentration level according to WADA MRPL.

Preventive Screening with GC and LC TOF/MS

We present validation data of qualitative gas chromatography-orthogonal acceleration-time-of-flight mass spectrometry (GC/TOF/MS) and liquid chromatography-orthogonal acceleration-time-of-flight mass spectrometry (LC/TOF/MS) methods for the detection of anabolic agents and corticosteroids (together with indicative β -blockers, stimulants, narcotics

and diuretics) in athlete urine samples, proving the capability of this combined screening system to detect additional designer analytes with similar molecular characteristics [full paper in 3]. Validation data were collected after the analysis of the respective validation QC samples following the routine sample preparation procedure for anabolic steroids and corticosteroids [4].

Results and Discussion

Unification of Sample Preparation: Hydrolysis.

In the Figures 1-8, the results of the comparison of the concentration of the 7 endogenous steroids measured in the 110 athletes' testing samples following the two different hydrolysis conditions, but identical for the rest of sample preparation, are presented. The following conclusions can be inferred from the task of the unification of hydrolysis procedure:

- Except the dehydroepiandrosterone, the concentrations of the rest endogenous steroids, testosterone, epitestosterone, androsterone, etiocholanolone, 5 α -androstane-3 α -17 β -diol and 5 β -androstane-3 α -17 β -diol and the testosterone to epitestosterone ratio (T/E) do not differ substantially (screening data) between the samples prepared from E.Coli or H.Pomatia enzymatic solutions.
- Except the dehydroepiandrosterone, the rest endogenous steroids, testosterone, epitestosterone, androsterone, etiocholanolone, 5 α -androstane-3 α -17 β -diol and 5 β -androstane-3 α -17 β -diol are conjugated mainly as glucuronides. Dehydroepiandrosterone is conjugated as sulfate. This is the reason for the increased yield of dehydroepiandrosterone in the samples prepared after hydrolysis from H.Pomatia enzymatic solution compared to the respective from E.Coli shown in Figure 8. Similarly, as shown in Figure 7B, a tendency exists towards the increase of the T/E after hydrolysis from H.Pomatia enzymatic solution.
- Enzymatic solution from H.Pomatia can be applied successfully to the hydrolysis of endogenous steroids. Additional statistical evaluation will be performed to evaluate the influence of the enzymatic solution change to the steroids screening WADA criteria.

Unification of Sample Preparation: Derivatisation.

In one-step derivatisation process, anabolic steroids derivatisation was achieved with all tested reagents. On the contrary, stimulants, narcotics and diuretics derivatisation process wasn't successful, e.g. in some cases only underivatised analytes were detected.

Application of the two-steps procedure resulted in improvement in derivatisation reaction, especially for stimulants, narcotics and diuretics. Indicatively, the amino group of amphetamine and ephedrine was only partially or remained underivatized in the one-step procedure. Amphetamine-NTMS and ephedrine-OTMS, NTMS derivatives were detected by the two-steps procedure. Anabolic steroids derivatisation was also successful. Optimization of the two-steps derivatisation process was then performed. Final optimized conditions were applied for the derivatisation of the substances at MRPL concentration level. The optimisation of the derivatisation process resulted in the following two-steps procedure :

Step 1: 50 μ l MSTFA +25 μ l ACN, 80 $^{\circ}$ C, 10 min

Step 2: 50 μ l MSTFA/NH₄I/Propanethiol 1000/2/3, 80 $^{\circ}$ C, 10 min

The two-steps procedure failed in thiazides, sydnocarb, mephentermine and phenylephrine, where alternative analysis (e.g. LC) will be proposed. Thiazides remained undetected at a concentration level of MRPL, which also happened with sydnocarb. It is well known, all later substances create problems in GC analysis. Mephentermine-TMS and phenylephrine-TMS, at MRPL concentration level, had a weak signal, which combined with background interference, resulted in low signal to noise ratio.

Preventive Screening with GC and LC TOF/MS

Five of the tested substances (16-OH-furazabol, 17-epimethandienone, 6 β -OH-turinabol, 17 α -methyl-5 α -androstane-3 α ,17 β -diol and 17 α -methyl-5 β -androstane- 3 α ,17 β -diol) did not fulfill the S/N >3 criterion for both GC and LC TOF/MS.

Efforts were made to improve the dynamic range of the GCT, since it was found to influence the chromatographic peak shape and height in case of peak coelution. In this context, various experiments were performed (injection volume, extract dilution, chromatographic run time). Optimum results were obtained from the above described experimental conditions.

Presented validation data [4], based on LC/TOF/MS and GC/TOF/MS preventive screening, support our proposal to use these technologies in combination, for the acquisition of MS signal from steroid designer drugs in a blind but accurate and generic way, which may cover a wide molecular features range: from substances with difficulty in derivatisation, but amenable to LC/ESI, to substances with difficulty in ionization, but amenable to GC conditions and/or derivatisation. This dual blind data acquisition is supported by the full scan high resolution and mass accuracy data collection in low ppm mass error level with sensitivity as shown [4]. Thus, doping control samples analyzed for a known repertoire of prohibited

substances, can blindly be analyzed, in addition, for a wide range of unknown molecules in a sensitive and accurate way.

Practically, we propose the following sequence of events for the application of this new service: a) for an ordinary doping control test, the responsible sports authority asks from the WADA Laboratory to additionally analyze the sample using the TOF/MS technology and store, in long-term basis, the A/B bottles, b) when a new designer steroid, or new steroid metabolite which improves detection, or new steroid from the internet market becomes known and reference material is available, the Laboratory compares the stored sample data file with the reference data acquired under the same chromatographic and MS methods, c) upon detection of a suspicious signal, the Laboratory, with the agreement of the sports authority, reactivates the test based on the stored A/B bottles and proceeds to the confirmation and probably to issue a complementary Certificate of Analysis.

The main advantage of the TOF/MS screening is the possibility of targeting the reactivation of testing to suspicious only samples, saving cost and urine volume for the negative samples. It is worthwhile reminding that the Prohibited List¹ contains an exhaustive list, not of prohibited substances, but of prohibited pharmacological classes of substances, particularly referring to examples of prohibited substances and “related compounds”. Consequently, the legal prerequisites for the detection of “designer” drugs are met. This new antidoping service, based on TOF/MS technology, can be considered as preventive screening for retrospective use by the antidoping WADA Accredited laboratories. Apart from the proposed methodology, TOF technology can replace mass spectrometric instrumentation and combine several instruments to one, thus freeing laboratory resources dedicated to routine screening.

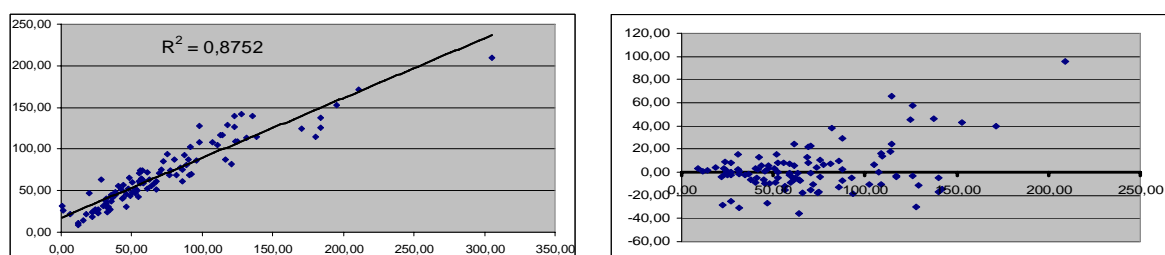
Both LC/TOF/MS and GC/TOF/MS screening methods have been introduced to the ISO/IEC 17025:2005 Scope of Accreditation of the Athens WADA Laboratory.

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References

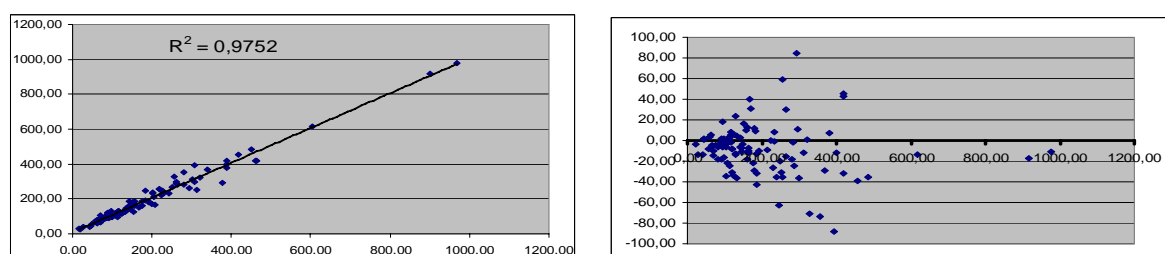
1. Kolmonen, M., Leinonen, A., Pelander, A., Ojanpera, I. (2007) A general screening method for doping agents in human urine by solid phase extraction and liquid chromatography/time-of-flight mass spectrometry, *Analytica Chimica Acta* **585**, 94-102.
2. Borges, C., Miller, N., Shelby, M., Hansen, M., White, C., Slawson, M., Monti, K., Crouch, D. (2007) Analysis of a Challenging Subset of World Anti-Doping Agency-Banned Steroids and Antiestrogens by LC-MS-MS, *J.Anal.Tox.* **31**, 125-131.
3. Tsiyou, M., Kioukia-Fougia, N., Lyris, E., Aggelis, Y., Fragakaki, A., Kiouisi, X., Simitsek, Ph., Dimopoulou, H., Leontiou, I.P., Stamou, M., Spyridaki, M.H., Georgakopoulos, C. (2006) An overview of the doping control analysis during the Olympic Games of 2004 in Athens, Greece, *Analytica Chimica Acta* **555**, 1-13.
4. Georgakopoulos, C.G., Vonaparti, A., Stamou M., Kiouisi P., Lyris E., Angelis Y.S., Tsoupras G., Wuest B., Nielen M.W.F., Panderi I., Koupparis M. (2007) Preventive doping control analysis: liquid and gas chromatography time-of-flight mass spectrometry for detection of designer steroids. *Rapid Communications in Mass Spectrometry* **21**, 2439-2446



A

B

Figure 1. A. Correlation of the urine screening concentration of 5α -androstan- $3\alpha,17\beta$ diol after H.Pomatia β -glucuronidase hydrolysis (y-axis, mean concentration 69 ng/mL) vs. E.Coli β -glucuronidase hydrolysis (x-axis, mean concentration 72 ng/mL). B. Comparison between the difference in urine screening concentration of 5α -androstan- $3\alpha,17\beta$ diol after E.Coli β -glucuronidase hydrolysis minus H.Pomatia β -glucuronidase hydrolysis (y-axis) vs. urine screening concentration after H.Pomatia β -glucuronidase hydrolysis (x-axis).

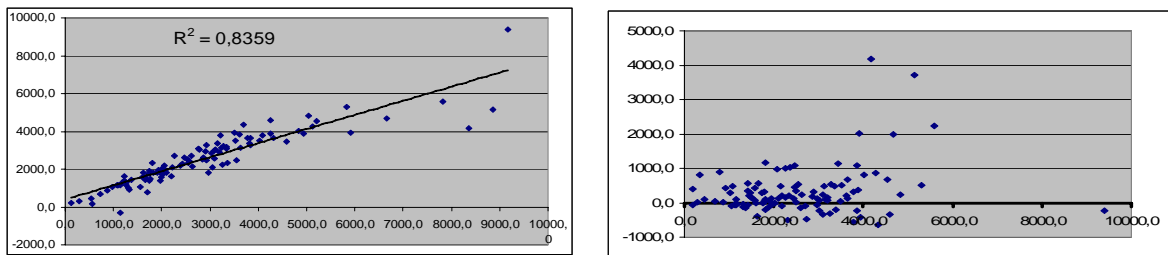


A

B

Figure 2. A. Correlation of the urine screening concentration of 5β -androstan- $3\alpha,17\beta$ diol after H.Pomatia β -glucuronidase hydrolysis (y-axis, mean concentration 197 ng/mL) vs.

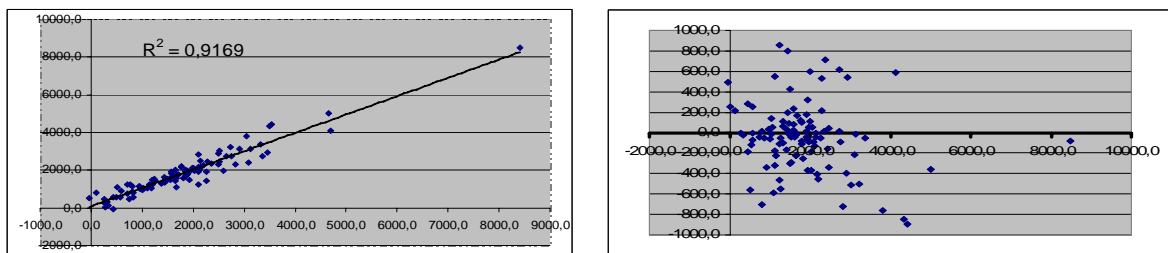
E.Coli β -glucuronidase hydrolysis (x-axis, mean concentration 189 ng/mL). B. As Figure 1B for 5β -androstan- $3\alpha,17\beta$ diol.



A

B

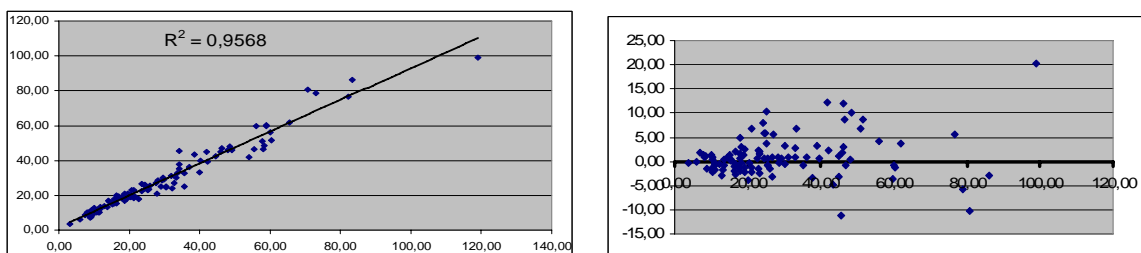
Figure 3. A. Correlation of the urine screening concentration of androsterone after H.Pomatia β -glucuronidase hydrolysis (y-axis, mean concentration 2523 ng/mL) vs. E.Coli β -glucuronidase hydrolysis (x-axis, mean concentration 2840 ng/mL). B. As Figure 1B for androsterone.



A

B

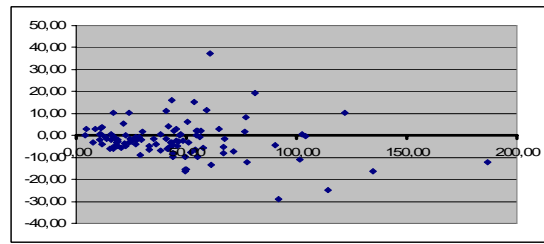
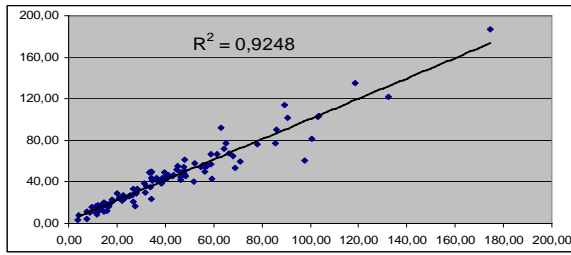
Figure 4. A. Correlation of the urine screening concentration of etiocholanolone after H.Pomatia β -glucuronidase hydrolysis (y-axis, mean concentration 1818 ng/mL) vs. E.Coli β -glucuronidase hydrolysis (x-axis, mean concentration 1777 ng/mL). B. As Figure 1B for etiocholanolone.



A

B

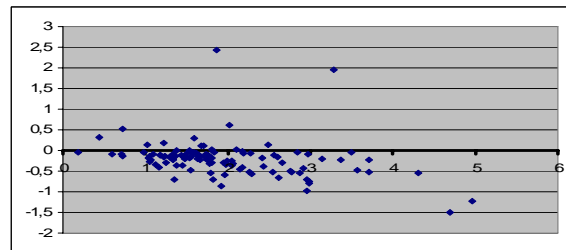
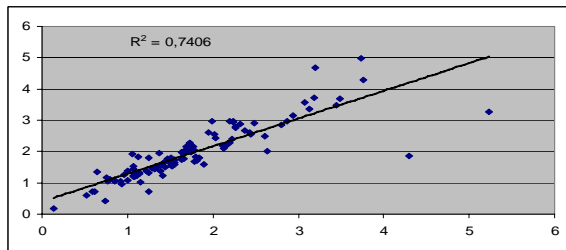
Figure 5. A. Correlation of the urine screening concentration of epitestosterone after H.Pomatia β -glucuronidase hydrolysis (y-axis, mean concentration 29 ng/mL) vs. E.Coli β -glucuronidase hydrolysis (x-axis, mean concentration 30 ng/mL). B. As Figure 1B for epitestosterone.



A

B

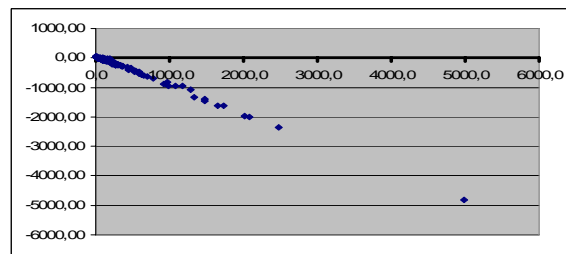
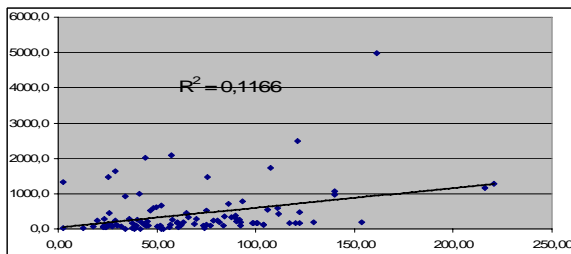
Figure 6. A. Correlation of the urine screening concentration of testosterone after H.Pomatia β -glucuronidase hydrolysis (y-axis, mean concentration 29 ng/mL) vs. E.Coli β -glucuronidase hydrolysis (x-axis, mean concentration 30 ng/mL). B. As Figure 1B for testosterone.



A

B

Figure 7. A. Correlation of the urine screening of testosterone to epitestosterone after H.Pomatia β -glucuronidase hydrolysis (y-axis, mean ratio 1.9) vs. E.Coli β -glucuronidase hydrolysis (x-axis, mean ratio 1.7). B. As Figure 1B for testosterone to epitestosterone.



A

B

Figure 8. A. Correlation of the urine screening concentration of dehydroepiandrosterone after H.Pomatia β -glucuronidase hydrolysis (y-axis, mean concentration 402 ng/mL) vs. E.Coli β -glucuronidase hydrolysis (x-axis, mean concentration 66 ng/mL). B. As Figure 1B for dehydroepiandrosterone.