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„Comparison of the ratios of sulpho-conjugated and glucuro-conjugated phase II metabolites of endogenous steroids in urine samples after administration of testosterone preparations via UHPLC-HRMS"

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„Sport ist Mord. Damit man sich aber dazu nicht allzu sehr anzustrengen braucht, gibt es Doping.“

© Erhard Blanck (*1942), deutscher Heilpraktiker, Schriftsteller und Maler

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1. Introduction

1.1. Testosterone

Testosterone is the most important male sex hormone and has androgenic effects, as it is involved in the formation of male reproductive tissues and secondary sex characteristics, and anabolic effects promoting muscle mass, bone density and strength and the growth of tissues with androgen receptors in general. Thus, it belongs to the endogenous anabolic androgenic steroids (EAAS) and has as all steroids a core structure that consists of 3 cyclohexane rings and one cyclopentane ring counting 17 carbons. There are two methyl groups at positions 10 and 13, one oxo group at position 3 and one 17β-hydroxyl group as shown in Figure 1. It is besides 5α-dihydrotestosterone (DHT) the most biologically active androgen[2,3].

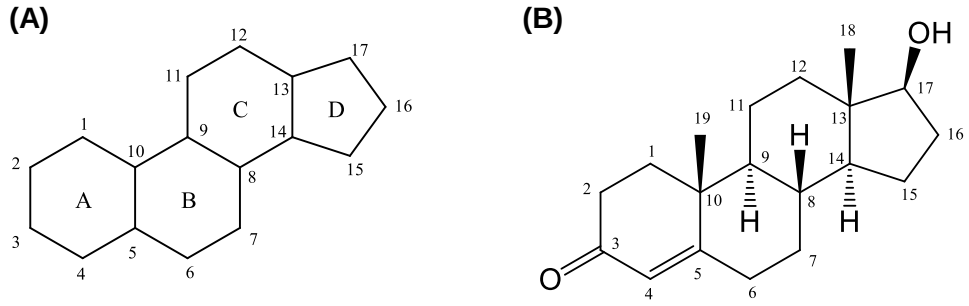

Figure 1: Steroid ring system (A) and structure of testosterone with labelled rings and carbon atoms (B).

1.1.1. Biosynthesis and regulation

Testosterone is secreted under control of luteinising hormone (LH) and follicle-stimulating hormone (FSH) in males with healthily functioning gonads to 95 % by the Leydig cells in the testes and in females to a much lesser extent by the ovaries and the adrenal cortex[3,4]. It is synthesized from cholesterol. In humans the major route for the formation of testosterone is the so-called Δ^5 pathway which starts with pregnenolone and goes on with its conversion via CYP17 to 17-hydroxypregnenolone and further to dehydroepiandrosterone (DHEA), to androstenedione via 3β-hydroxysteroid dehydrogenase/isomerase and finally to testosterone via 17β-hydroxysteroid dehydrogenase. The less favoured Δ^4 pathway is another synthetic route via progesterone[3] (Figure 2).

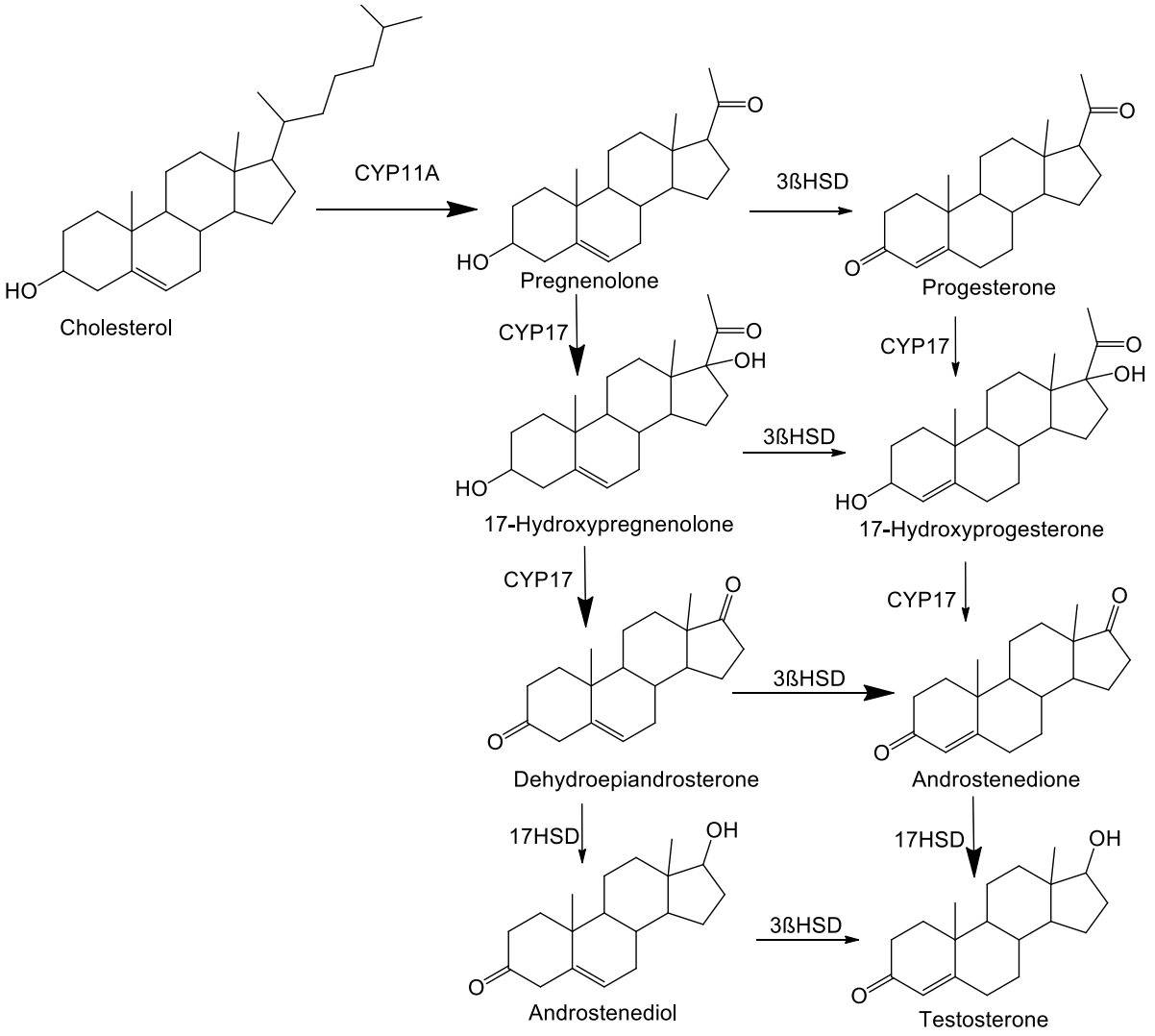

Figure 2: The biosynthetic pathway of testosterone with intermediates and enzymes involved. The larger arrows refer to the preferred Δ^5 pathway.

LH and FSH are secreted by the anterior pituitary, which presents together with the hypothalamus the function of controlling the testicular steroidogenesis. The hypothalamus secretes gonadotropin-releasing hormone (GnRH) in a pulsatile way, this stimulates LH secretion, while circulating testosterone and estradiol exert a negative feedback and act as inhibitors. The so-called hypothalamic-pituitary-testicular axis can be disturbed by several factors, such as short-term physical exercise that leads to a short rise of circulating testosterone or the administration of anabolic steroids that indicates a negative feedback loop lowering LH secretion[3].

1.1.2. Biological activity and function

Testosterone and other androgens are transported to the target tissues by the blood, where they are bound to albumin and sex-hormone binding globulin (SHBG). Only a small fraction is transported as free steroid. Albumin has a lower affinity, but a high capacity for binding steroids because its concentration in plasma is very high. SHBG, on the other hand, is present in much lower concentration, but has a high affinity with sex steroids. Because

dissociation of testosterone from albumin is easy, this part of the bound proportion is able to leave the blood vessel as unbound steroid and enter the tissue, while the biological activity of the part tightly bound to SHBG is said to be not given. Alterations in SHBG concentrations affect the bioavailability of testosterone[3].

The modulation of androgenic effects is dependent on steroid-converting enzymes and the distribution of androgen receptor coregulators within target tissue cells. In reproductive organs and skin, for instance, testosterone is converted to DHT by 5α-reductase, in adipose tissue and parts of the brain it is converted to estradiol and in skeletal muscle it is binding directly to the androgen receptor. The receptor is activated through an androgen binding at the right domain, causing an allosteric change. The activated receptor enters the nucleus and is attached to the DNA, where a transcription complex consisting of comodulators is formed. These comodulators are able to activate or repress transcriptional regulation and therefore play an important role in cell function and growth. So steroid action can be genomic, but also nongenomic as neurosteroids positively modulate the $GABA_A$ receptor, a transporter of Cl^- in the central nervous system[3].

The function of testosterone is represented as virilising and anabolic as described above. Since too little testosterone can be reason for medical issues like erectile dysfunction or osteoporosis it is used for medical treatment known as hormone replacement therapy[5]. Also, it is used for masculinization of transgender men, male hypogonadism, wasting protein diseases in association with cancer, burns, AIDS and more[6,7,8,9,10]. It can be administered as transdermal gels, films or patches, by intramuscular injection and orally as tablets and probably via even more forms[11,12].

On the other hand, an excess of anabolic steroids like testosterone can lead to severe side effects bearing the risk of lethal outcome. This is supposed since there are no clinical studies possible on such high dosages and investigations have to be carried out on individual reports of bodybuilders on side effects after certain dosages and cases of deaths with linkage to steroid abuse. But this data is neither representative nor unbiased. It is also not possible to gain much valid information about side effects of androgenic anabolic steroids from therapeutic applications because the dosages are much too low and only a few molecules structurally similar to testosterone are used. Possible side effects of anabolic androgenic steroid abuse include disturbances in the cardiovascular, musculoskeletal, endocrine and reproductive system, negative impact on liver, kidney and skin and change in psyche and behaviour[13,14,15].

1.1.3. Metabolism

Most steroid metabolic action takes place via hepatic enzymes and makes the androgens less toxic, less active and more polar for the excretion. The functionalising phase I metabolism of testosterone includes oxidation of the 17β-hydroxyl group, the reduction of the A-ring and hydroxylation, as well as conversion to androsterone and etiocholanolone and to a lesser extent to epiandrosterone, 5α- and 5β-androstane-3α.17β-androstanediols and estradiol. Phase II metabolites are the glucuronide and sulphate conjugates. Glucuro-conjugation is performed by reaction with uridine-5´-diphosphoglucuronic acid (UDPGA), which is catalysed by uridine diphosphoglucuronosyltransferases (UGTs). Sulpho-conjugation or also called sulphonation is achieved through reaction with 3´-phosphoadenosine-5´-phosphosulphate (PAPS) via sulphotransferase enzymes (SULTs) (Figure 3). The enzymes for the conjugation can be found in several tissues as kidney and intestine, but the most active site is the liver. In urine most androgens are excreted mainly as glucuronide conjugates[3,4].

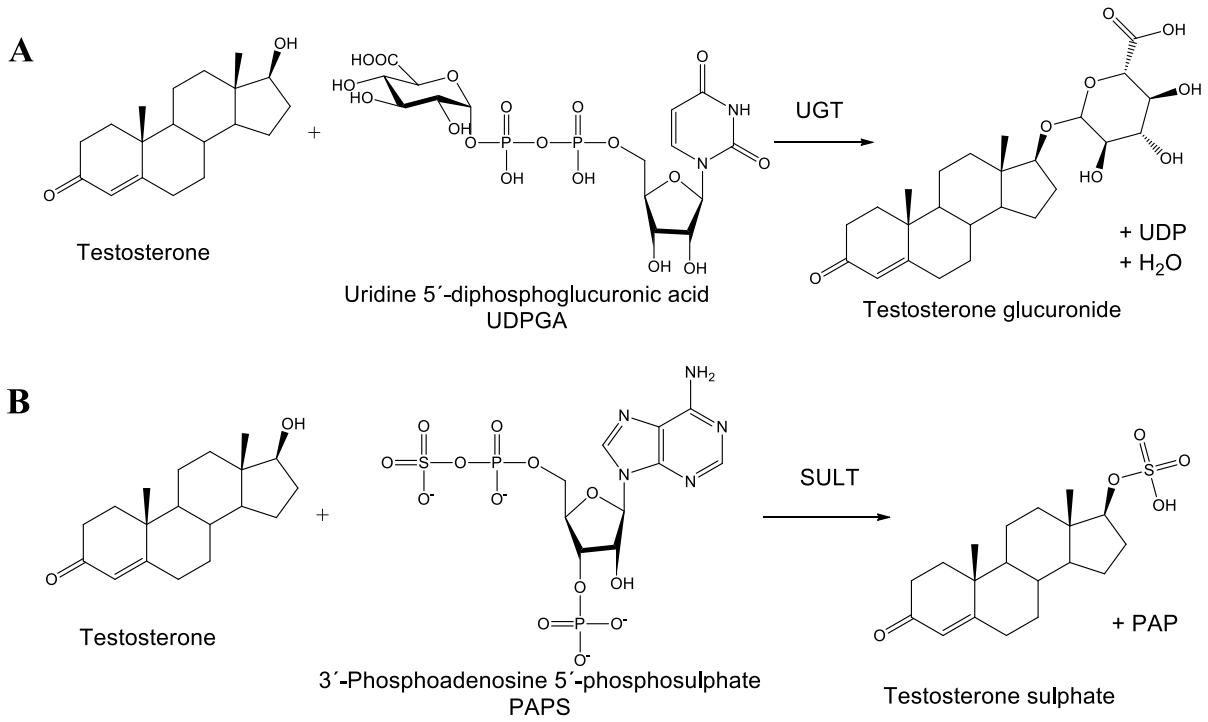

Figure 3: Conjugation reaction of testosterone with (A) UDPGA to testosterone glucuronide and with (B) PAPS to testosterone sulphate.[3]

1.1.4. Testosterone preparations

As mentioned in 1.1.2, testosterone can be introduced into the body in various forms, intramuscular injection being one of the most widely-used. Though, unmodified testosterone has only a short half-life and would have to be administered very often. Esterified testosterone at position 17 is first slowly absorbed into the general circulation system and

then hydrolysed to the active molecule. The half-life of the esterified molecule is even increased when the fatty acid chain is longer[16].

1.2. Doping and anti-doping

1.2.1. Historical development

There are several theories about the origin of the expression “doping”. The etymology may be the Dutch word “doopen”, that means “to dip” refering to a sauce of gravy or another sticky liquid[17]. Other sources say that “dop” was an extract of grape skins made by Zulu warriors to get stimulated in fights and religious ceremonies[18,19]. “Dope” had several meanings over the last centuries from drug in general or more specifically in connection with opium, marijuana and heroin, but also medicine. “To dope” once only meant to poison, then also to use stimulating or repressing substances on racehorses and later, on human athletes too[17]. Although the expression “doping” is relatively young and its definition changed sometimes slightly, the use of performance enhancing substances, drugs, plants, mixtures, beverages and other stuff is as deeply rooted in history as the will of being faster and stronger than others in men. There are early documentations in the Greek culture that state doping in the ancient Olympic games that were held from 779 $_{BC}$ to 393 $_{AD}$. Also, the gladiators in ancient Rome used substances in various forms to fight more powerfully, endure longer and recover faster. And not only in sports the physical performance had to be improved, since battles had to be fought to conquer countries and impotence had to be cured, which was said to be possible by the ingestion of testicles, for instance[20].

But there was not only endorsement on doping as physicians in ancient Greece criticized the heavy meat diet of the athletes when they reported intestinal disorders[21]. Still, until today, doping agents did not disappear but became even more and more popular as mixtures of different stimulants like cocaine, caffeine, strychnine and alcohol in the late nineteenth and early twentieth century. In 1920 the first restrictions against drugs in sports were established and in 1928 the International Amateur Athletic Federation (IAAF) prohibited doping. Nevertheless, official testing on human athletes was not performed until 1968 at the Summer Olympic Games in Mexico after the International Olympic Committee (IOC) had published its first List of Prohibited Substances. The Medical Commission of the IOC was responsible for the establishment of requirements of doping analysis and equipment of anti-doping laboratories to be accredited by the IOC. Soon, testing was performed not only in, but also outside the competitions because it became obvious that there were banned substances that could be excreted before the competition, but performance enhancement was long enough. The first big doping scandal was in 1988 when the Canadian sprinter Ben Johnson was found positive on the steroid stanazolol. Until then, the use of stimulants such as amphetamines and steroids like testosterone and derivatives was kind of normal in various

sports and not that much of public interest. During the Cold War the governments of several states that were economically inferior, as for instance the German Democratic Republic (GRD), were sponsoring and organising the doping of their athletes because they were longing for acknowledgement of the other countries. This led to numerous court trials and late health problems of affected athletes[3]. Another big scandal was when the American cyclist and multiple Tour de France winner, Lance Armstrong, was convicted of sophisticated doping for many years and stripped of his titles since 1998[22]. Nevertheless, this was not an isolated case because it seems the majority of professional cyclists takes performance-enhancing drugs[20]. The Bay Area Laboratory Co-Operative (BALCO) scandal in 2003 revealed that many well-known athletes took designer steroids, which had not been detected in urine samples until then because they were not searched for. Another case of conspiracy was exposed when 2016 Russia´s entire track and field team was banned from the Olympic Games in Rio. There was evidence that the Russian government ran the biggest doping programme in history[23].

Public awareness of doping increased not only through the violation of the Anti-Doping Code[24] by famous athletes, but if nothing else through lethal cases like the death of the Danish cyclist Knut Enemark Jensen during the Summer Olympic Games in Rome in 1960, who had taken amphetamine as the autopsy showed[3].

1.2.2. WADA

The challenges due to new doping agents and methods like designer steroids and peptide hormones like erythropoietin (EPO) grew, and this complexity plus the emerging new technical developments of analytical principles like gas and liquid chromatography coupled with mass spectrometry (GC-MS, LC-MS), high resolution mass spectrometry (HRMS) and isotope ratio mass spectrometry (IRMS) had to be strictly regulated. This regulation for an internationally harmonized fight against doping should first start with the Anti-Doping Convention of the Council of Europe in 1989, where a Monitoring Group and an Advisory Group were established. After the huge doping incident at the Tour the France 1998, the First World Conference on Doping was held in 1999 in Lausanne, Switzerland. On the basis of the Lausanne Declaration on Doping in Sport[25], the IOC, the Council of Europe, the Monitoring Group and several representatives of governments founded the World Anti-Doping Agency (WADA)[3,26].

The World Anti-Doping Code[27] entered into force in 2004 and is fundamental for the worldwide compliance on anti-doping policies and regulations. One of the tasks of WADA is to monitor the acceptance and implementation of the Code. Educational programmes on athletes, coaches and doctors are run to prevent them from health risks and legal consequences of doping. Also, scientific research, updating the list of prohibited substances

and methods[28] and accreditation of anti-doping laboratories is part of WADA´s functions. Furthermore, Therapeutic Use Exemptions (TUEs) and the Athlete Biological Passport (ABP) must be managed. This happens through the Anti-Doping Administration & Management System (ADAMS). In collaboration with National and Regional Anti-Doping Agencies (NADOs and RADOs) and athletes WADA wants to share information and raise the awareness to find solutions[26].

1.2.3. Testosterone in anti-doping analysis

1.2.3.1. Historical background

Testosterone was one of the first substances used to enhance performance in fights, battles, sports and potency since its presence in the testes of humans and animals made its consumption rather easy[18,20,21,3]. It was and is still one of the most important doping substances especially in strength sports and was used by a huge number of athletes either individually or in organised teams or on a governmental level as it was the case in the GDR and Russia[3].

Testosterone is prohibited in sport and was introduced to the list of banned substances by the International Olympic Committee in the early 1980s. Back then, the T/E value, which is the ratio of urinary testosterone glucuronide to epitestosterone glucuronide, greater than 6 was considered to result from testosterone administration[3,21].

Epitestosterone is, as testosterone, mainly produced in the testis, but the exact pathway is not yet fully known. Though, a possible precursor for its biosynthesis is epiandrostenediol, a by-product in pregnenolone reaction and it was found that the interconversion of testosterone to epitestosterone is negligible in humans. Therefore, it was early established to use the ratio of urinary testosterone to epitestosterone to detect the administration of testosterone because after its administration the ratio increases[3].

Since 1992, elevations of the T/E due to physiological or pathological conditions were taken into account, but had to be proven or disproven, depending on which side one was, by further tests. Soon it was clear that more and more naturally elevated T/E ratios were detected and that especially the Asian population had a very low natural T/E ratio that would not exceed 4 or 6 even with testosterone doping. As a result, urinary steroid profiling was established, where long term data about reference ranges and stability of several parameters was collected, as well as individual concentrations and ratios of testosterone, epitestosterone, androsterone, etiocholanolone and 5α- and 5β-androstane-3α, 17β-diol[3]. Since the late 1990s a positive finding is complemented with stable isotope analysis for the detection of metabolites, which were synthesized exogenously. This technique is a combination of gas chromatographic (GC) separation, on-line combustion (C) and isotope

ratio mass spectrometry (IRMS). The ratio of ^{13}C and ^{12}C is calculated by measuring the CO_2 resulting from the combustion of the prior separated analytes. The carbon isotopic signature of organic compounds in the human body differs depending on the diet because there are different metabolic pathways in different sorts of plants for CO_2 fixation. The carbon isotopic profile of synthetic steroids metabolised in humans is therefore differing from normal steroid metabolites[3,29].

Steroid profiling is nowadays generally performed using GC/MS instrumentation. The glucuronides are hydrolysed enzymatically, and a derivatisation step is conducted for volatility and stability for the gas chromatographic separation. The mass spectrometric analysis is performed in selected reaction monitoring (SRM)[3].

1.2.3.2. The Athlete Biological Passport

The first version of the Athlete Biological Passport (ABP) was effective in December 2009 and contained only the Haematological Module or blood module to reveal blood doping, such as blood transfusions or the use of EPO, for instance. This information is obtained from blood samples. The Steroidal Modul for the better detection of steroid doping was officially introduced in January 2014. The analytical matrix is the athlete´s urine samples. The function of the ABP is to monitor selected biological variables over time to reveal indirect effects of doping in contrast to classical direct detection of the doping agent or method. The introduction of the ABP into anti-doping analysis led to an increase of Adverse Analytical Findings (AAFs), which is defined as the identification of the presence of a prohibited substance or its metabolites or markers or evidence of the use of a prohibited method in a sample and reported by a WADA-accredited laboratory[26].

1.2.3.3. Influences on the steroid profile

Other synthetic anabolic androgenic steroids can alter the amount of steroidal metabolites excreted, for instance in a way of lowering it generally or more specifically like the 5α-reductase inhibitor finasteride, which can be seen in alteration of the 5α- to 5β-metabolite ratios[30]. Aromatase inhibitors and anti-estrogens alter the normal metabolization pattern. Substances like ketoconazole act as confounding factors as they can inhibit testosterone synthesis[31]. Masking agents like probenecid can reduce the urinary concentration of glucuronoconjugates, and diuretics are used to increase the urine volume for lower concentration of doping agents[3,32]. The intake of large amounts of alcohol can lead to very high testosterone and successive T/E levels. In this case IRMS also shows that the testosterone is endogenous. Microbial growth possibly followed by insufficient refrigeration during storage and transport can lead to hydrolysis of steroid conjugates and successive increase of free steroids[3,33].

Genetic variations in expression or activity of metabolic enzymes like UGTs and SULTs lead to inter-individual or inter-ethnic differences in metabolic pathways. In doping control, the detection of administration of exogenous testosterone or its precursors is dependent on the steroidal profile, which is affected by genetic polymorphism. In Asian populations there are much more individuals who are homozygotic carriers of a genetic deletion of UGT2B17 (del/del) than in Caucasian populations. Thus, in such individuals the reduction of the glucuronidation of testosterone may lead to lower T/E ratios despite of testosterone administration, whereas there are also individuals with naturally higher T/E ratios without administration of testosterone[3,33].

1.3. Purpose of this work

Up to date, steroid profiling is mainly concentrated on the analysis of glucuronide-conjugated steroids via GC-MS after hydrolytic separation of glucuronic acid. However, phase II metabolism also includes sulpho-conjugation, but there is no data about the endogenous steroid-sulphate-profile. The aim of this work was to evaluate the influence of testosterone preparations on the phase II metabolism and thus the endogenous steroid profile of phase II metabolites in urine, which are in this work represented by 6 analytes shown in Figure 4.

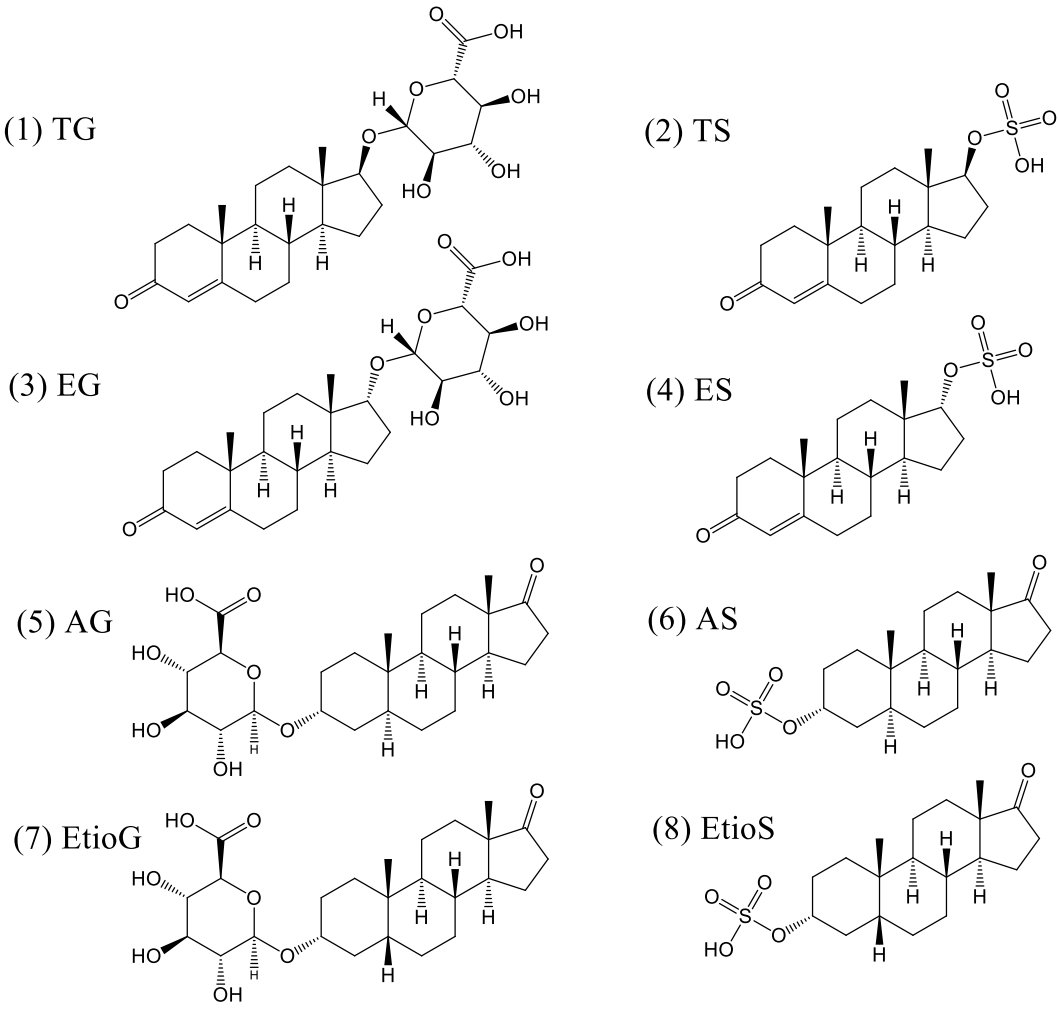

Figure 4: Molecular structures of the analytes: (1) TG=Testosterone glucuronide, (2) TS=Testosterone sulphate, (3) EG=Epitestosterone glucuronide, (4) ES=Epitestosterone sulphate, (5) AG=Androsterone glucuronide, (6) AS=Androsterone sulphate, (7) EtioG=Etiocholanolone glucuronide, (8) EtioS=Etiocholanolone sulphate.

2. Materials and methods

2.1. Chemicals and reagents

The following anabolic-androgenic steroid standards were purchased from The National Measurement Institute (NMI, Sydney, Australia).: testosterone-sulphate (TS), epitestosterone-sulphate (ES), androsterone-sulphate (AS), etiocholanolone-sulphate (EtioS), testosterone-glucuronide (TG), epitestosterone-glucuronide (EG), androsterone-glucuronide (AG) and etiocholanolone-glucuronide (EtioG). The deuterated forms of all analyte molecules (d_3-TS, d_3-ES, d_4-AS, d_5-EtioS, d_3-TG, d_4-EG, d_5-AG, d_5-EtioG) were also available and bought from the NMI (Syndey, Australia), except for d_5-AG, which was synthesized at the Vienna University of Technology (TU Wien, Austria). ULC-MS water, ULC-MS acetonitril (ACN) and ULC-MS formic acid (FA) were obtained from Biosolve Chimie (Dieuze, France). MeOH was purchased from Chem-Lab (Zedelgem, Belgium). Ultra-pure MilliQ® water was obtained by a Millipore™ system by Merck. Artificial urine consisted of 2.5 g/L potassium dihydrogen phosphate, 9.0 g/L sodium chloride, 2.5 g/L di-sodium hydrogen phosphate and 3.0 g/L ammonium chloride, which were obtained from Merck (Darmstadt, Germany), 25.0 g/L urea, which was purchased from GE Healthcare Life Sciences (Uppsala, Sweden) and 2.0 g/L creatinine from Sigma-Aldrich®.

2.2. Solid phase extraction

In solid phase extraction (SPE) there is an interaction between a solid and a liquid and the analytes in the liquid matrix are extracted when their affinity is higher to the solid phase than to the matrix. The retained analytes can be eluted with a solvent with an even higher affinity. The retention and elution mechanisms of the analyte molecules function like in liquid chromatography with intermolecular forces between the analytes and the surface molecules of the solid phase and the solvent molecules. In modern SPE systems the adsorbent is packed between two fritted disks in a polypropylene cartridge. The typical procedure starts with a condition step by passing a solvent, that activates the active sites of the packing, through the cartridge. Before the sample is loaded, the first solvent is removed by another solvent similar to the sample matrix. Then happens the retention of the analytes. Another washing step follows to remove interfering molecules. At last the analytes are eluted with another appropriate solvent[34].

In this project I used the OASIS hydrophilic-lipophilic balance (HLB) cartridges from Waters® that combine hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene. Although glucuronated and sulphonated steroids are more polar than non-conjugated steroids, the core structure with a lot of carbon-hydrogens is still non-polar enough for appropriate interaction with the reversed-phase extraction sorbent[35].

2.3. Liquid chromatography

Since isobaric compounds cannot be differentiated in the mass spectrometer, chromatographic separation is essential. Liquid chromatography is a separation technique based on the interaction of the sample analytes dissolved in a liquid with a solid stationary phase and a liquid mobile phase. Two main types of liquid chromatography are normal-phase liquid chromatography (NPLC) and reversed-phase chromatography (RPLC). In NPLC, the dissolved analytes are retained on a polar stationary phase and eluted with less polar solvent. In RPLC, the elution solvent is more polar than the stationary phase[36]. For steroid analysis, the use of RPLC seems to be a good choice because of the non-polar carbon-hydrogen ring system that forms the core structure. The retention of molecules on the stationary phase works through more or less strong interactions like dipole-dipole interactions, Van der Waals forces, hydrogen bonds and ionic strength, as well as size exclusion.

The column of choice for this project was a C18 column with trimethylsilyl (TMS) endcapping to replace the accessible silanol groups to prevent polar compounds from unwanted interaction. The silica particles consist of a solid core and a porous shell, which has the advantage of smaller pore volume in comparison to fully porous particles and leads to reduction of volume present for band broadening through longitudinal diffusion, the B term in the van Deemter equation. Also, the core-shell technology allows higher flow rates due to lower back pressure than with fully porous particles and thus faster separation[37].

2.4. Mass spectrometry

The history of mass spectrometry starts over 100 years ago and has evolved since then very fast to one of the most important analytical techniques due to its high sensitivity, low detection limits, high speed and many possibilities of application. It is used in environmental chemistry for the analysis of trace elements and compounds in air, water, soil and waste, in biomedical research for proteomics and metabolomics, in food analysis, in forensic science, determination of high molecular masses, species analysis, determination of elements, surface analysis and depth profiling[38,39,40].

The principle of mass spectrometric analysis is the measurement of the mass-to-charge ratio of ions in the gas phase. The mass is expressed in atomic mass units (1 u = 1.660 540 x 10^{-27} kg) and the charge in elementary charge (1 e = 1.602 177 x 10^{-19} C). The main parts of a mass spectrometer are the ion source, the analyser and the detector. Depending on the properties of the sample it is loaded in dry or liquid state into the mass spectrometer, either directly or after chromatographic separation. Ions are produced by reception or loss of an electron and accelerated through the mass spectrometer till they reach the detector. This

must happen under high vacuum to avoid collisions of the ions with other gaseous molecules and successive loss of their trajectory. For more analytical information there may be more than one analyser to allow fragmentation of the ions[38].

Electrospray ionisation (ESI) is a soft ionisation technique that allows studying very small sample volumes of non-volatile and thermally labile bio-molecules. Since multiply charged ions can be obtained, the analyte molecules can be large as polymers and biopolymers and still be analysed by instruments with limited mass range. Nowadays ESI is also used for the analysis of small polar molecules like drug metabolites. Coupling to high performance liquid chromatography (HPLC) enables the analysis of complex biological samples[41]. The ionisation process is characterised by applying a strong electric field under atmospheric pressure to the liquid sample coming from a capillary tube. At the liquid surface this field leads to a charge accumulation, which is followed by dispersal of a fine spray of charged droplets. Further, the solvent is evaporated, and the ions are ejected from the highly charged droplets and sampled by a skimmer cone and accelerated into the mass analyser[38,41].

The Q Exactive Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer is a high-resolution mass spectrometer that combines several features of simpler mass analysers. There are radio frequency (RF)-lenses that focus the ions in a tight beam into the mass spectrometer, a bent flatapole ion guide that prevents neutral molecules from entering the quadrupole and a quadrupole mass filter that allows for precursor selection. The orbitrap mass analyser is attached to the c-trap, a curved linear ion trap used for storing ions after the quadrupole so that they can be transferred to the orbitrap in pulses despite a continuous ESI source. The orbitrap is an ion trap mass analyser trapping the ions in an orbital motion around a spindle-like electrode which is surrounded by an outer barrel-like electrode. The frequency signal of the oscillating ions is an image current and is converted through Fourier transformation to a mass spectrum[38].

2.5. Sample preparation

Samples were prepared via solid phase extraction on OASIS HLB-cartridges from Waters (60 mg/ml, 3 ml). The cartridges were first conditioned with 2 ml methanol and washed with 2 ml MilliQ water. As internal standard (IS) a mixture of the deuterated analogues of the analyte molecules was used. The solution was prepared in methanol with following concentrations: 1.8 µg/ml d_3-TG, 1.8 µg/ml d_4-EG, 56 µg/ml d_5-AG, 51 µg/ml d_5-EtioG, 0.7 µg/ml d_3-TS, 0.7 µg/ml d_3-ES, 15 µg/ml d_4-AS and 15 µg/ml d_5-EtioS. 25 µl were added to 2 ml sample and transferred onto the cartridges followed by one washing step with 2 ml water. Elution was carried out with 2 ml methanol. The solvent was then evaporated and the extract was reconstituted in 150 µl ACN/water with 0.1 % FA (20:80, v/v). After 10 minutes on the heat block at 60 °C the samples were transferred into autosampler vials.

2.6. UHPLC-MS conditions

The analysis was performed on a Vanquish UHPLC system coupled to a Q Exactive Focus mass spectrometer from Thermo Fisher Scientific. The software used was Xcalibur™ by Thermo Fisher Scientific. Chromatographic separation was performed on a Kinetex C_{18} column (1.7 µm, 100 Å, 150x2.1 mm, Phenomenex®). The column temperature was 25 °C, the mobile phase consisted of water (A) and ACN (B) both with 0.1 % FA. The flow rate was 300 ml/min. Injection volume was 1 µl. The gradient started isocratically with 2 minutes of 25 % B, then went linearly to 39 % B in 10.5 minutes, then increased to 98 % B within 1 minute and stayed there for 1.5 minutes. Then the gradient dropped to 25 % B in 0.1 minutes and ended with 2.9 minutes of re-equilibration. The total runtime was 18 minutes. Mass spectrometric analysis was performed in ESI negative mode with a spray voltage of 2800 V and an ion source temperature of 320°C. Full MS mode was used to scan without fragmentation in a mass range from 100 m/z to 700 m/z. The resolution was 70.000.

2.7. Method validation

The validation was done according to the WADA criteria of the International Standard for Laboratories (ISL)[1].

2.7.1. Recovery

Due to the endogenous presence of the analytes in human urine, extraction recovery was tested with real urine samples which were spiked with deuterated internal standard. Therefore, ten different urine samples were split into two aliquots, where one was first spiked with internal standard and extracted as written, and one was first extracted and spiked afterwards. The areas of the internal standards were compared and the standard deviation of the recoveries was calculated.

2.7.2. Linearity

The calibration was prepared by spiking and preparing artificial urine at 7 concentration levels, each level four times, and measured in duplicates. Calibration curves were based on the ratios of the peak areas of the analytes to their corresponding deuterated molecules in the internal standard: TG to TG-d3, EG to EG-d4, AG to AG-d5, EtioG to EtioG-d5, TS to TS-d3, ES to ES-d3, AS to AS-d4 and EtioS to EtioS-d5.

2.7.3. Carry-over

Carry-over was evaluated by injection of a blank, followed by an extracted spiked artificial urine sample which was used as highest level of the calibration curve. This was followed by a second blank. When a peak was found at a suitable retention time in the second blank, its area was expressed as percentage of the area of the corresponding peak in the previous calibration standard.

2.7.4. Matrix interferences

Since biofluids as urine present complex matrices its influence on the signal had to be checked. Several points in the linear range of the analytes were prepared without extraction in mobile phase, which is ACN/water (20:80, v/v). The resulting slope was compared to the calibration slope and the deviation was expressed in percentage.

2.7.5. Intermediate precision

Two different analyte concentrations in artificial urine were prepared 10 times on 3 different days. The first concentration was the lower limit of quantification and the second was level three of the calibration curve. Intermediate precision was expressed as the relative standard deviation of the resulting values.

2.7.6. Robustness

Robustness testing was carried out by adding acetate buffer to two spiked artificial urine samples to obtain a pH of 5 and carbonate buffer to obtain two samples with pH = 9. Those plus two pH = 7 samples without addition of buffer were extracted, measured and the resulting values were compared.

2.8. Clinical study samples

The urine samples analysed in this project were collected in a clinical study conducted in a public hospital in Zagreb. All subjects were healthy male adults. Each of them received a single intra-muscular injection of one of two different testosterone ester preparations (Figure 5):

- Nebido® with 1000 mg/4 ml testosterone undecanoate, a “slow acting” testosterone ester
- Sustanon®, a mixture of 30 mg testosterone propionate, 60 mg testosterone phenylpropionate, 60 mg testosterone isocaproate and 100 mg testosterone decanoate, all in all 250 mg/ml testosterone ester

From all subjects 19 samples were taken within 2 months, 3 before and 16 after the administration at the following points: 1, 4, 8, and 12 hours and 1, 2, 3, 4, 5, 6, 8, 11, 15, 18, 22 and 60 days after the administration as shown in Table 1 and Table 2[42].

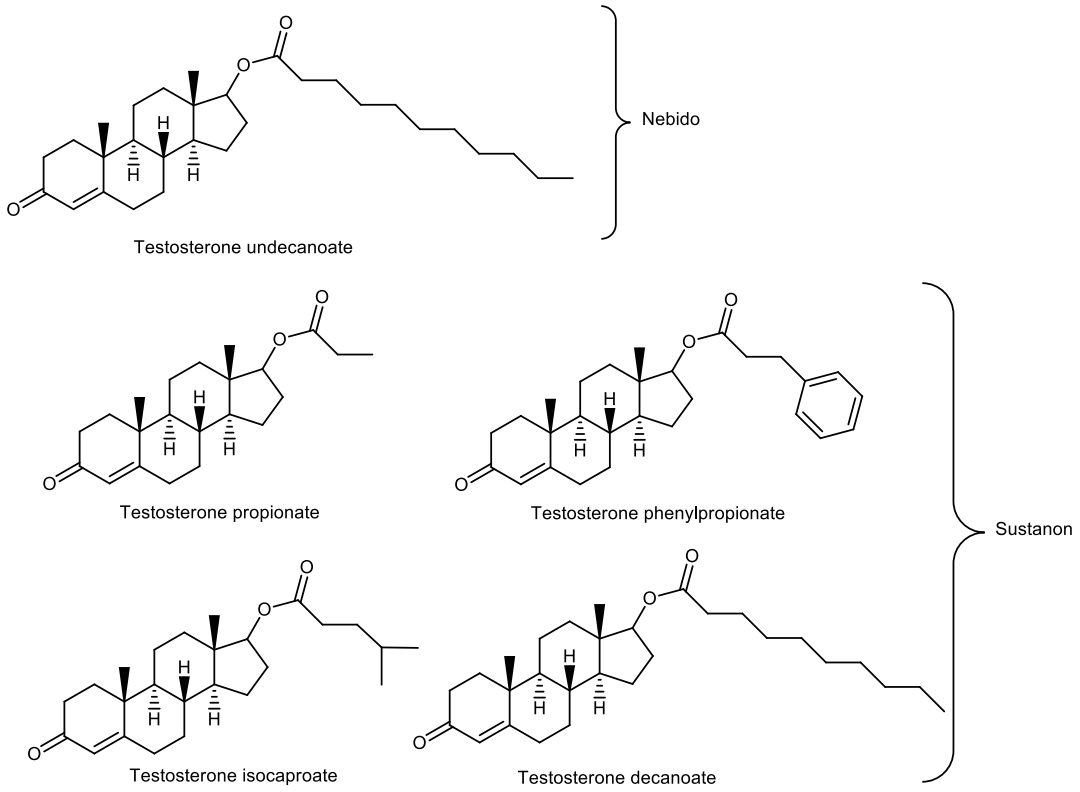

Figure 5: Testosterone esters used for the study preparations Nebido® containing testosterone undecanoate and Sustanon® containing testosterone propionate, testosterone phenylpropionate, testosterone isocaproate and testosterone decanoate.

Table 1: Study subjects and corresponding preparation.

Sample name	Subject/Subject	Applied preparation
S01	1	Substanon®
S02	2	Nebido®
S03	3	Substanon®
S04	4	Substanon®
S05	5	Nebido®
S07	7	Nebido®

Table 2: Sample number and sampling points expressed in days.

Sample number	Sampling days after application	
1	0	before application
2	0	
3	0	
4	0.04	(1 hour)
5	0.17	(4 hours)
6	0.33	(8 hours)
7	0.50	(12 hours)
8	1	
9	2	
10	3	
11	4	
12	5	
13	6	
14	8	
15	11	
16	15	
17	18	
18	22	
19	60	

3. Results

3.1. Method validation

The calibration ranged from the LOQ (limit of quantification), which was found by spiking artificial urine with low concentrations of analytes and evaluating the lowest concentration with acceptable error and repeatability, to concentrations in the range of high natural concentrations found in literature[43]. For TG it ranged from 1 to 600 ng/ml, from 0.6 to 372 ng/ml for EG, from 0.4 to 235 ng/ml for TS and ES, from 9.4 to 5660 ng/ml for AS, from 7.9 to 4710 gn/ml EtioS, from 30 to 2400 ng/ml for AG and from 29 to 2870 ng/ml for EtioG. For AG and EtioG the points for the determination of the linear working range were not the same as for the other substances due to effects of column overloading and thus non-linear correlation between concentration and signal. Therefore, to get enough points for sufficient validation the linearity of AG and EtioG was achieved with more points in lower concentrations. The correlation coefficient R was over 0.99 for all curves. The repeatability is expressed as the relative standard deviation (RSD) of the calculated concentrations of the double-measured quadruplets. The RSD was about 1 and 2 % for most points of most analytes. Highest RSD was 21 % for 60 ng/ml of AG (Table 3). Matrix interferences were expressed as percentage of deviation between the calibration curve of extracted spiked artificial urine as matrix and the curve of the diluted standards prepared only in mobile phase. A positive value means a higher steepness and thus a higher signal intensity of the extracted matrix. For instance, Figure 6 and Figure 7 present the curves and slopes of TG. The slope of the curve after extraction is 0.0362 and of the mobile phase curve without extraction it is 0.0314. So, for TG the matrix curve is 15.4 % steeper than the curve from the mobile phase, for EG it is 12.0 %, for AG 8.6 %, for EtioG the deviation is only 3.7 %, whereas for TS it is highest with 26.5 %. For ES the deviation is 10.5 %, for AS it is -0.15 % as the only analyte signal affected negatively from artificial urine matrix, and for EtioS it is 8.5 % (Table 5).

Table 3: Validation data of concentration range and correlation between calculated concentration and spiked concentration as basis of the calibration curves of all analytes.

Analyte	Range (ng/ml)	Correlation coefficient R	Spiked concentration (ng/ml)	Average calculated concentration (ng/ml)	RSD (%) (n=8)
TG	1-600	0.9992	1.00	0.81	6
			2.00	1.95	2
			20.0	19.8	1
			100	99.3	3
			200	202	2
			400	406	2
			600	594	2
EG	0.6-372	0.9992	0.62	0.44	7

Analyte	Range (ng/ml)	Correlation coefficient R	Spiked concentration (ng/ml)	Average calculated concentration (ng/ml)	RSD (%) (n=8)
			1.24	1.19	1
			12.4	12.4	1
			62.0	61.4	2
			124	126	1
			248	254	2
			372	369	1
AG	30-2400	0.9927	30.0	31.1	13
			60.0	70.9	21
			600	689	3
			1500	1610	2
			2400	2310	1
EtioG	29-2860	0.9984	28.7	26.5	9
			57.3	60.9	16
			573	639	3
			1430	1519	2
			2290	2310	1
			2860	2630	4
TS	0.4-235	0.9995	0.39	0.29	2
			0.78	0.69	2
			7.85	7.13	1
			39.2	37.0	1
			78.5	75.6	1
			157	157	2
			235	237	1
ES	0.4-235	0.9994	0.39	0.28	3
			0.78	0.68	1
			7.85	7.16	1
			39.2	37.1	1
			78.5	76.5	1
			157	159	1
			235	236	1
AS	9-5660	0.9992	9.44	9.66	1
			18.9	20.4	2
			189	188	1
			944	935	2
			1890	1910	1
			3780	3880	2
			5660	5600	2
EtioS	8-4710	0.9982	7.86	5.45	2

Analyte	Range (ng/ml)	Correlation coefficient R	Spiked concentration (ng/ml)	Average calculated concentration (ng/ml)	RSD (%) (n=8)
			15.7	13.2	1
			157	137	1
			786	693	1
			1570	1440	1
			3140	3130	2
			4710	4790	1

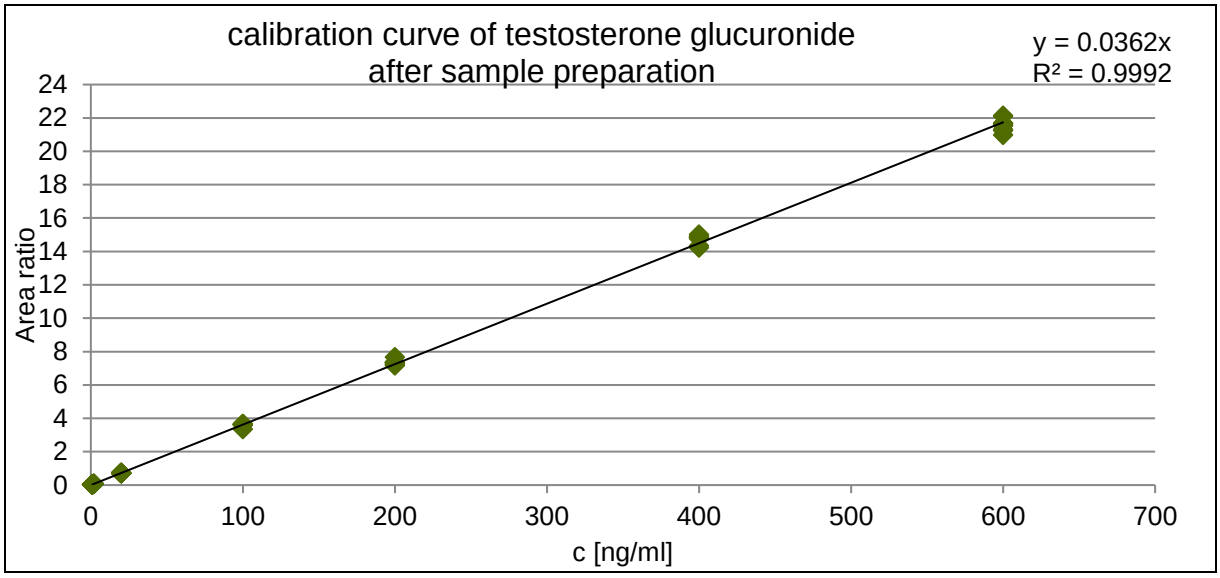

Figure 6: Calibration curve of testosterone glucuronide extracted from spiked artificial urine.

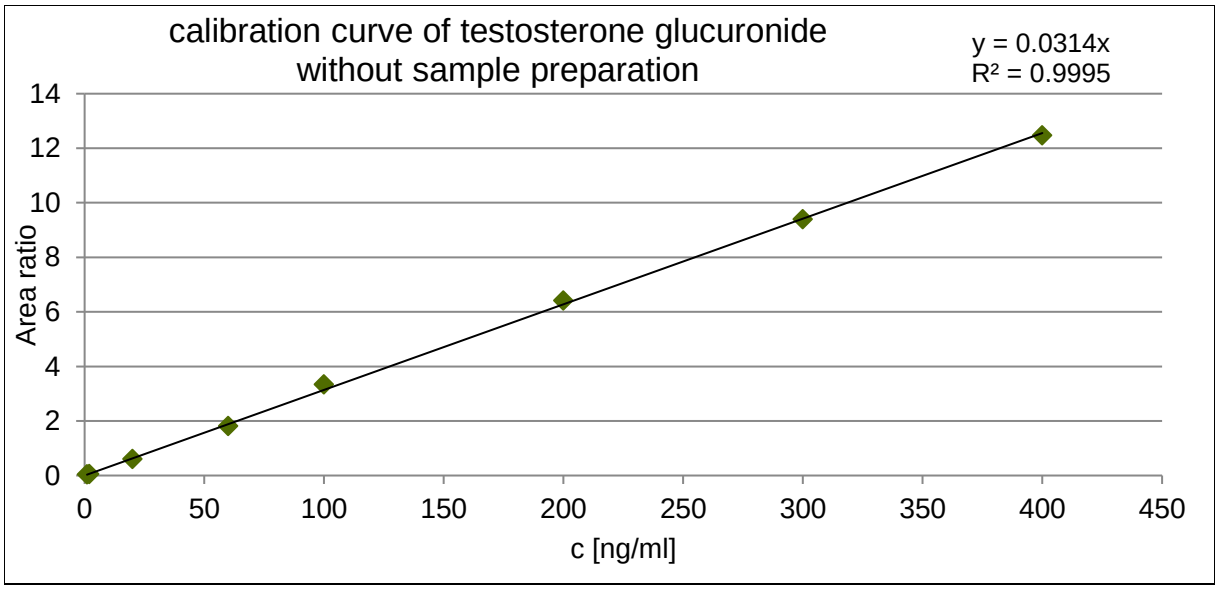

Figure 7: Calibration curve of testosterone glucuronide diluted in mobile phase.

Carry-over was between 0.0 % (EG and ES) and 0.45 % (AS) for all analytes after injection of a spike at the highest concentration of the linear working range. So, contamination of samples following samples with high analyte concentration was negligible. The intermediate precision for the preparation of 10 spikes in 3 different days was higher for the higher concentration as one can see that the RSD is lower than for the lower concentration. Here it was between about 7 and 11 %, whereas for the higher concentrated spikes it was about 3 % for every analyte (Table 4). The average recovery for the deuterated analyte molecules spiked in urine samples was over 97 %. Therefore, one can assume that the extraction yield is very high with this method and only low percentage is lost during preparation (Table 5).

Table 4: Validation data of carry over and intermediate precision of all analytes.

Analyte	Carry over (%)	Intermediate precision			
		Average calculated concentration at LOQ (ng/ml)	RSD (%)	Average calculated concentration at Level 3 (ng/ml)	RSD (%)
TG	0.004	1.1	9.5	19.9	3.2
EG	0	0.6	10.6	12.3	3.8
AG	0.004	38.1	10.8	605	3.9
EtioG	0.002	33.6	8.8	581	3.4
TS	0.002	0.4	9.6	7.6	3.1
ES	0	0.4	9.4	7.6	3.3
AS	0.05	12.5	7.1	194	3.2
EtioS	0.006	8.4	8.0	158	2.4

Table 5: Validation data of recovery and matrix interference of all analytes.

Analyte	Average recovery (%)	RSD (%) (n=10)	Analyte	Matrix interference (%)
d3 TG	98.9	3.0	TG	15.4
d4 EG	98.5	4.2	EG	12.0
d5 AG	98.7	4.2	AG	8.6
d5 EtioG	98.4	4.0	EtioG	3.7
d3 TS	98.6	4.6	TS	26.5
d3 ES	98.4	4.0	ES	10.5
d4 AS	97.1	4.6	AS	-0.15
d5 EtioS	97.7	3.9	EtioS	8.5

The robustness test showed that a change of the sample pH by +/- 2 did not remarkably change the measured analyte concentration and retention time. The RSD was smaller than 3 % for the calculated concentrations and smaller than 0.1 % for the retention times as shown in Table 6.

Table 6: Validation data of robustness of measured concentration and retention times of all analytes at different sample pH.

Analyte	Calculated concentration at Level 3 (ng/ml)				Retention time (min)			
	pH 5.0	pH 7.0	pH 9.0	RSD (%)	pH 5.0	pH 7.0	pH 9.0	RSD (%)
TG	21.1	21.2	20.7	1.5	7.17	7.17	7.18	0.08
EG	21.1	21.7	21.5	1.8	9.92	9.92	9.92	0.04
AG	891	880	841	2.7	12.0	12.0	12.0	0.04
EtioG	720	725	738	1.3	11.6	11.6	11.6	0.03
TS	8.07	8.10	8.02	0.58	7.23	7.24	7.22	0.09
ES	7.95	8.07	7.98	1.0	7.79	7.80	7.80	0.06
AS	206	206	206	0.84	10.9	10.9	10.9	0.06
EtioS	165	164	166	0.71	11.2	11.2	11.2	0.03

3.2. Study samples

The samples were prepared and measured as written above. The specific gravity of each urine sample of all 6 subjects was measured in a previous study (2015), where the common steroidal profile was determined through a routine screening method. Since urine can be diluted depending on the hydration of the test person, analyte concentrations can be overestimated and underestimated. The calculated concentrations were therefore corrected to a specific gravity of 1.020 with the following formula, where $c_{1.020}$ is the corrected concentration[44]:

$$c_{1.020} = \frac{1.020 - 1}{Specific\ gravity\ sample - 1} \times c_{sample}$$

Due to the smaller linear working range of AG and EtioG, in 77 of the 114 samples (6 subjects x 19 samples/subject) the calculated concentrations of AG and EtioG were too high to be quantified properly in the first analytical run. Therefore, those urine samples were diluted 1:5 by taking 400 µl of urine and adding 1600 µl of water to extract and measure them one more time. The concentrations of the analytes plus ratios of the sulphates and glucuronides over time were determined. They are discussed in detail and presented in the following figures. For better structure and overview, the time axis was set in a way that all sample-taking points are equispaced to each other. Table 7 shows the mean initial T/E values from the former study and the mean initial urinary concentrations of all analytes in this project. The subjects who were given the slow-acting Nebido® preparation had a lower T/E before the application than those who were given the Sustanon® preparation, but especially S05 had a very low T/E ratio with about 0.07 before and a maximum of 3.7 after the application of Nebido, while S03 had a relatively high initial T/E ratio at about 2.6 and a maximum of 47. In Figure 8 these values are sorted by the initial T/E ratio and encoloured for better overview.

Table 7: Mean concentration of all analytes in urine of all subjects before application of the preparations.

Subject		S01, Sustanon®	S03, Sustanon®	S04, Sustanon®	S02, Nebido®	S05, Nebido®	S07, Nebido®
Mean T/E		1.7	2.6	1.8	0.84	0.07	1.3
Mean initial urinary concentrations [ng/ml]	TG	33.3	65	23.7	42.1	3.4	26.4
	EG	43.1	56.2	35.4	84.6	68.6	32.4
	AG	7230	8240	13800	4970	3520	5010
	EtioG	6250	5200	6960	3410	3370	4200
	TS	18.2	11.8	13.6	2	3.4	16.7
	ES	34.7	50.3	36.8	14.8	23.1	31.7
	AS	1700	1910	1910	1650	1230	1370
	EtioS	392	65	110	380	488	276

	T/E	TG	EG	AG	EtioG	TS	ES	AS	EtioS
S05, Nebido	0.07	3.4	68.6	3520	3370	3.4	23.1	1230	488
S02, Nebido	0.84	42.1	84.6	4970	3410	2.0	14.8	1650	380
S07, Nebido	1.3	26.4	32.4	5010	4200	16.6	31.7	1370	276
S01, Sustanon	1.7	33.3	43.1	7230	6250	18.2	34.7	1700	392
S04, Sustanon	1.8	23.7	35.4	13800	6960	13.6	36.8	1910	110
S03, Sustanon	2.6	65.0	56.2	8240	5200	11.8	50.3	1910	65

Figure 8: Mean concentration of all analytes in urine of all subjects before application of the preparations sorted by mean initial T/E.

3.2.1. Glucuronide conjugates

The excretion of TG in the urine of the 6 subjects is shown in Figure 9. The diagram indicated with (A) shows the absolute concentrations, whereas the diagram indicated with (B) shows the relative change compared to the base concentrations, which were normalized to the mean value of the 3 initial values before the application. The mean initial urinary TG levels of the subjects are presented in Figure 8. The range was between 3 and 77 ng/ml. In the samples of the subjects who were given the Sustanon® preparation (S01, S03, S04) the concentration of TG increases dramatically already on the application day and reaches its maximum on the day after. In case of S01 it is then 5 times higher than in the base concentration, in S03 it is with 455 ng/ml – the highest concentration of all – 7 times higher and in S04, which had the lowest initial mean value with 24 ng/ml, it is 5.5 times higher at this point. A decrease is detectable in concentrations until a second maximum occurs at day 5 after the application, which is not as high as the first, though. The two maxima probably result from different kinetics in metabolism of the different substances in the Sustanon® mixture. After this, the concentration decreases again until it reaches the initial values in S03 and S04 after 11 days and in S01 after 22 – 60 days. In the Nebido® subjects (S02, S05, S07) the TG concentration increases slower and reaches its maximum value after 6 days in S02, which is 4.2 times higher than the initial value, after 5 days in S05 with a value 5.5 higher than the initial concentration and after 11 days in S07, where it is increased by a factor of 3.5. This change is consistent with the slow-acting preparation. Also, 60 days after the application all concentrations are as low as the initial concentrations. The TG concentrations in Nebido® samples were generally a bit lower than in Sustanon® samples, but especially S05 had very low values, its maximum with 18 ng/ml being lower than the initial values of the other subjects.

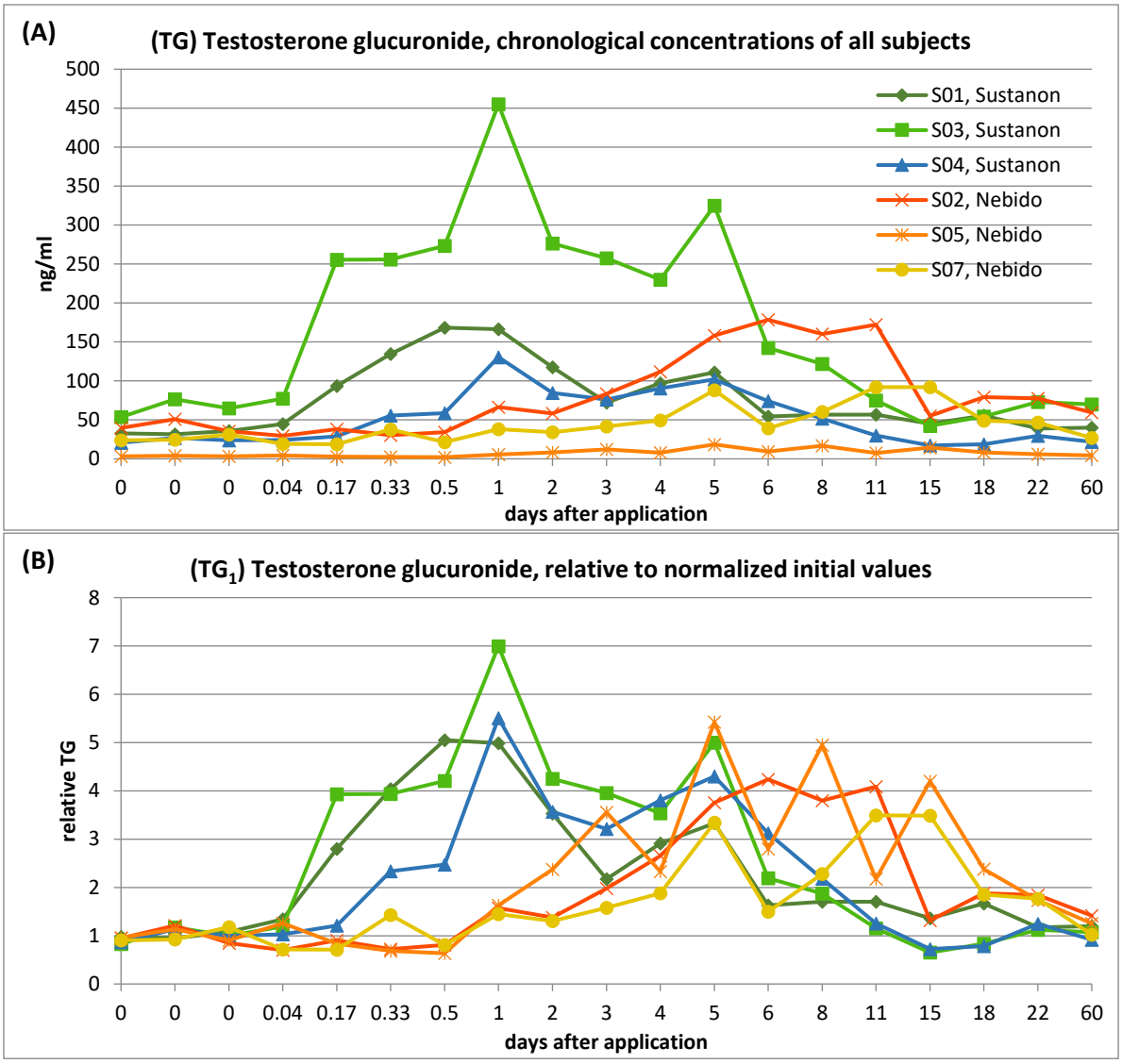

Figure 9: Excreted testosterone glucuronide concentrations (A) and relative values referring to normalized mean initial urinary TG before application (B) of all subjects over all 19 sample points.

The concentrations of EG and its normalized view is shown in Figure 10. The initial values were between 21 and 139 ng/ml and the two highest mean initial values were of the subjects with the lowest initial T/E values (S02: 85 ng/ml, S05: 69 ng/ml). Though, the lowest mean initial concentration of EG was 32 ng/ml in S07, which had a middle high T/E of 1.3. Over all values, a decrease over a time of several days to weeks after the application can be detected. The lowest concentration of all samples was 2.6 ng/ml also in S07 6 days after the application, which is just 8 % of the mean initial concentration. The biggest decrease occurred in Nebido® subject S02, were the minimum was 4 %. Generally, in the samples of the Nebido® subjects the concentration minima were about 10 % of the concentrations before the application, whereas in Sustanon® samples they were about 20 %. Thus, with Nebido® a higher relative change is noticeable. Except this, the concentrations in Sustanon® samples increase again and come close to the mean initial values before the end of the sample taking, but not those of the Nebido® samples, which again confirms a slower metabolism of the slow-acting preparation.

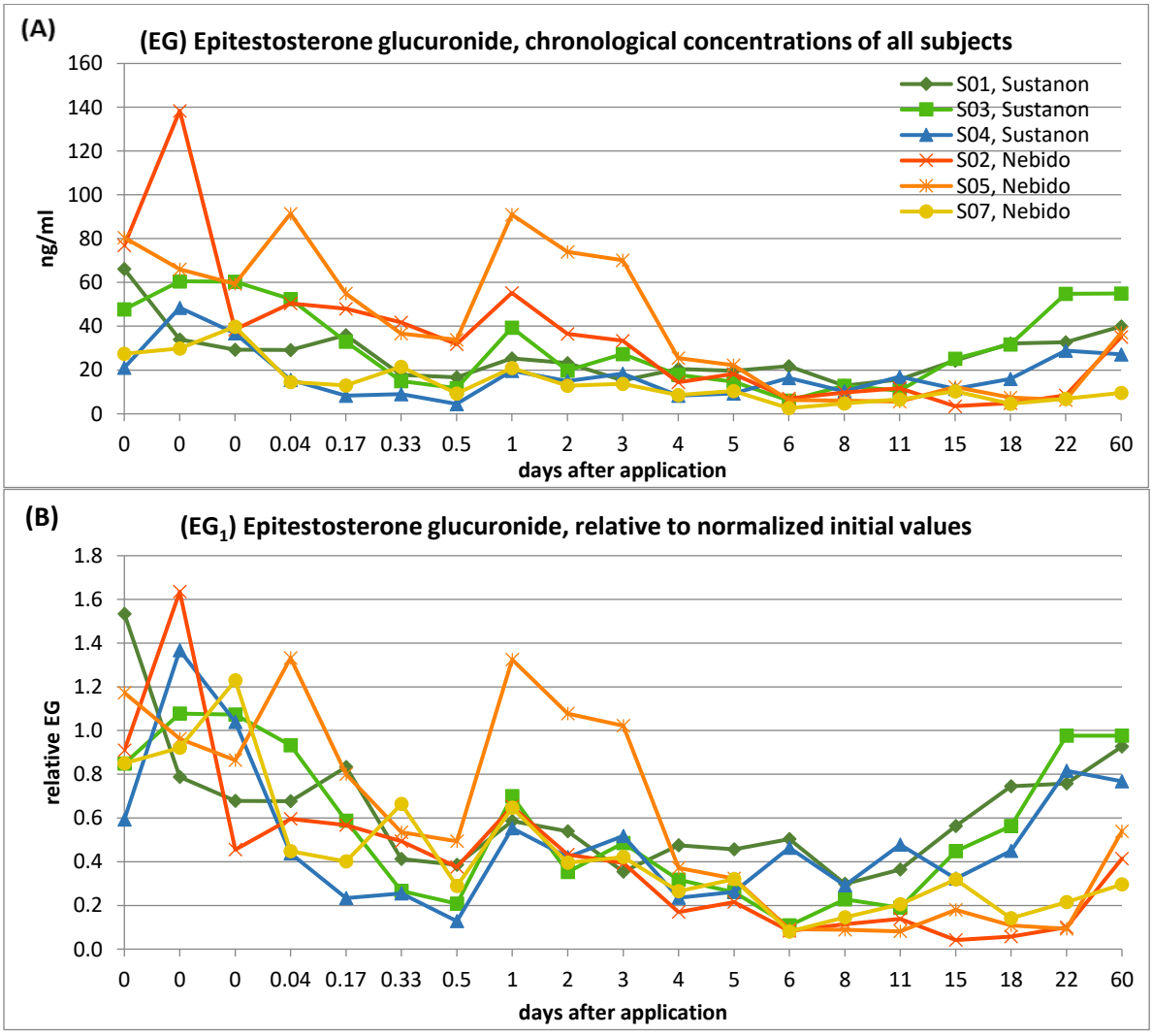

Figure 10: Excreted epitestosterone glucuronide concentrations (A) and relative values referring to normalized mean initial urinary EG before application (B) of all subjects over all 19 sample points.

AG concentrations did not vary as much as those of TG or EG (Figure 11). There was a small decrease with the lowest urinary concentrations 12 hours after the application in all subject samples except in those of S01, where no trend is visible. The minima were at about 35 % of the initial concentrations. The mean initial values were higher in subjects with higher T/E, the highest with about 13.8 µg/ml in S04 and the lowest with about 3.5 µg/ml in S05 samples.

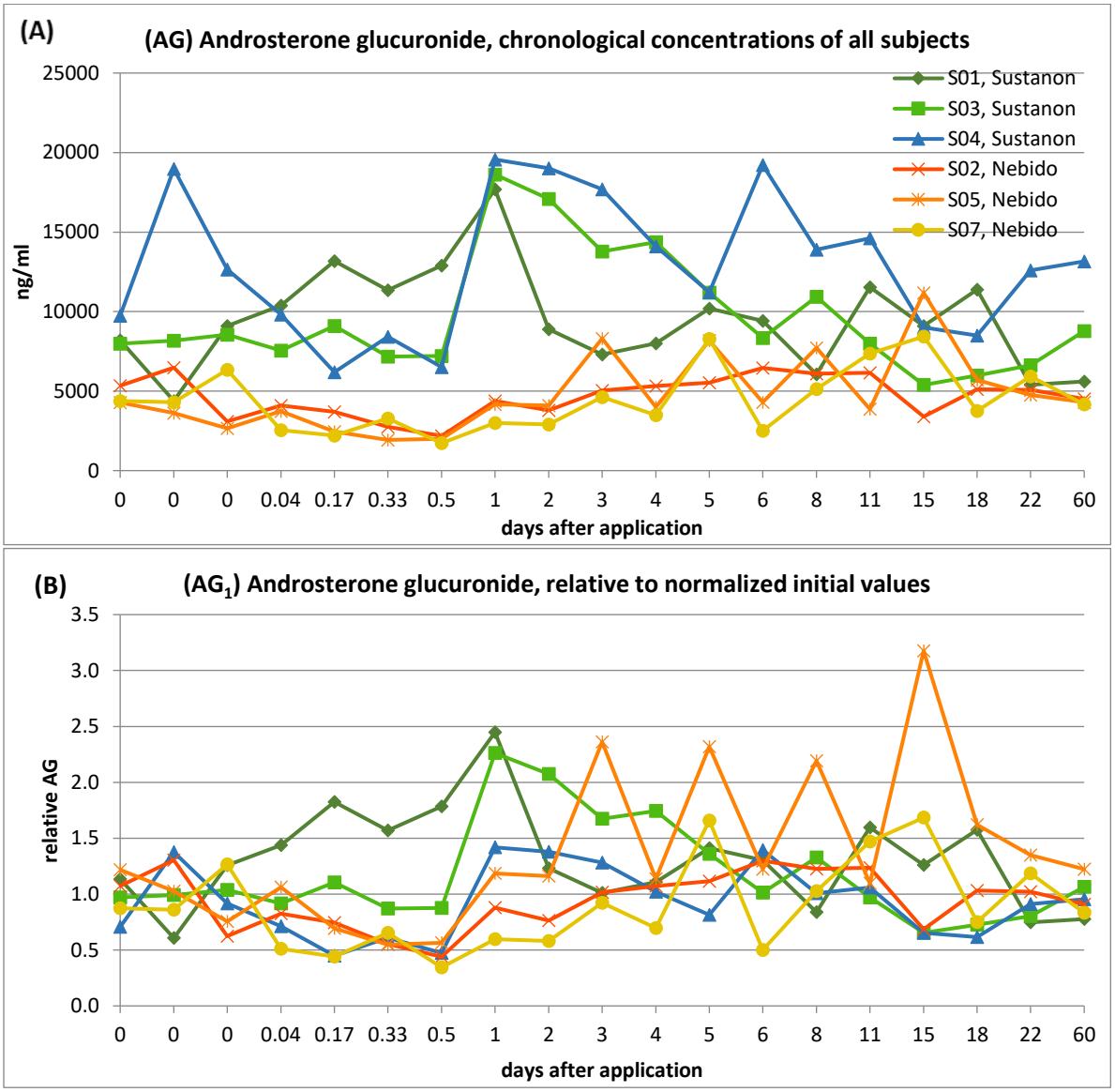

Figure 11: Excreted androsterone glucuronide concentrations (A) and relative values referring to normalized mean initial urinary AG before application (B) of all subjects over all 19 sample points.

EtioG concentrations behave similar to AG concentrations (Figure 12). Subjects with lower initial T/E have lower mean initial EtioG levels with 3.4 µg/ml as the lowest in S05 samples. Subjects with higher T/E have higher initial EtioG with 7.0 µg/ml as the highest mean initial concentration in the samples of S04. These concentrations also decrease in the first few hours after the application, except in the samples of S01, and reach their minimum at the sample taking point at 12 hours (0.5 days) after the application where they are about 30 % on average of the initial concentrations. Then they seem to rise again to the original levels and already 12 hours later no further change is visible.

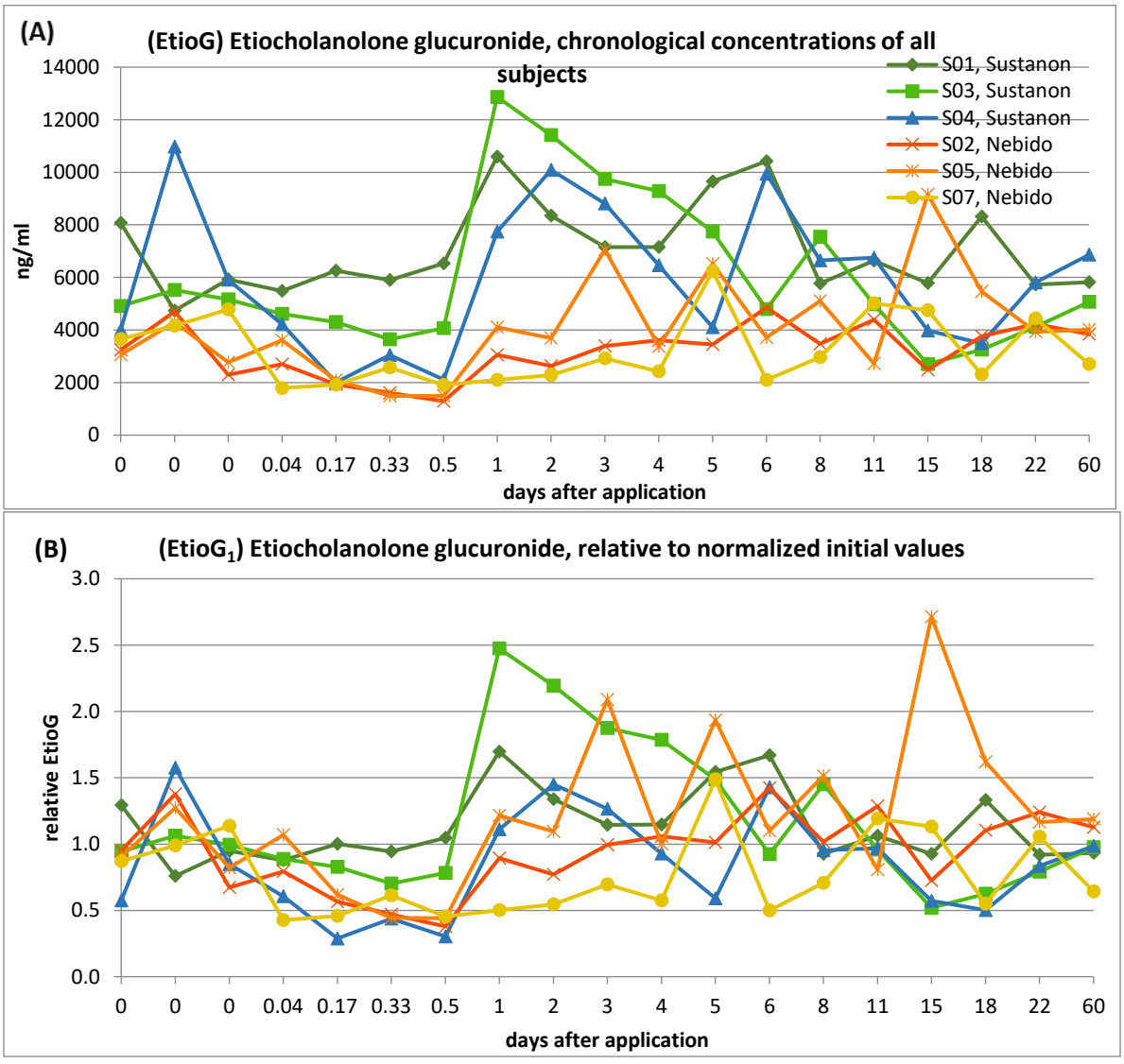

Figure 12: Excreted etiocholanolone glucuronide concentrations (A) and relative values referring to normalized mean initial urinary ETioG before application (B) of all subjects over all 19 sample points.

3.2.2. Sulphate conjugates

Figure 13 shows the concentration and its relative change of TS. The two subjects with the lowest T/E (S05 and S02) have also comparably low initial TS values below 4 ng/ml, while the other mean initial TS concentrations are between 11 and 19 ng/ml. The TS is decreasing in all samples after the application. In Sustanon® samples the minimum was reached 8 days after the application and was about 12 % of the mean initial value in S01, 6% in S03 and 4 % in S04. After that, the concentrations increased until they were at the initial levels 60 days after the application. In Nebido® subject S02 the lowest concentration of TS was found 15 days after the application. It was 20 % of the initial value. In S05 the minimum was 12 % of the initial value after 18 days and in S07 it was below 3 % at day 22 after the preparation was applied. After that, in those samples the TS concentration did not reach the initial levels during the study.

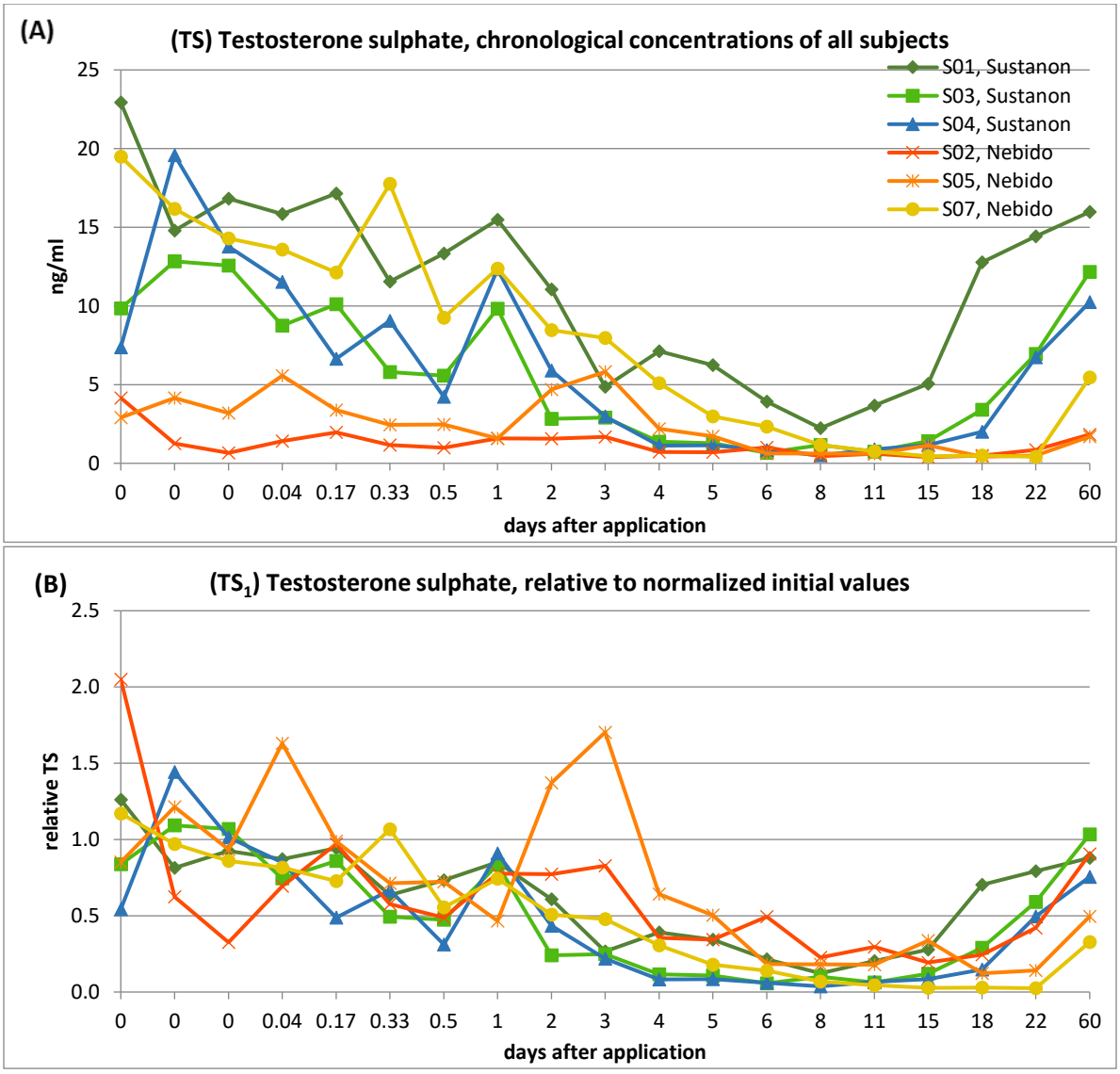

Figure 13: Excreted testosterone sulphate concentrations (A) and relative values referring to normalized mean initial urinary TS before application (B) of all subjects over all 19 sample points.

The change of the ES concentration is shown in Figure 14. The mean initial concentrations are a bit lower in samples of subjects with lower T/E. After the application of the preparations a decrease in ES concentration is visible until a first minimum after 12 hours. This is followed by a light increase till the next sampling point and then again a slow decrease. In the Sustanon® subject S01 the lowest concentration could be measured 8 days after the application, where it was about 40 % of the mean initial value. At day 60 it returned to the initial level. In S03 samples the minimum was at day 6 with 25 % and at the last sampling point it was at the original level. ES in S04 samples was lowest at day 5 with 25 % too, but in the end it was still at only 60 %. The urinary concentration of ES in the samples of Nebido® subject S02 was lowest with 25 % of his mean initial value 15 days after the application and at 80 % at the last point. In S05 the minimum was 20 % at day 8 and 50 % at day 60. In S07 the minimum was at day 18 with 20 % and about 40 % at day 60. Thus, subjects who were given the Sustanon® preparation needed less time to get back to their initial ES concentration than the Nebido® subjects.

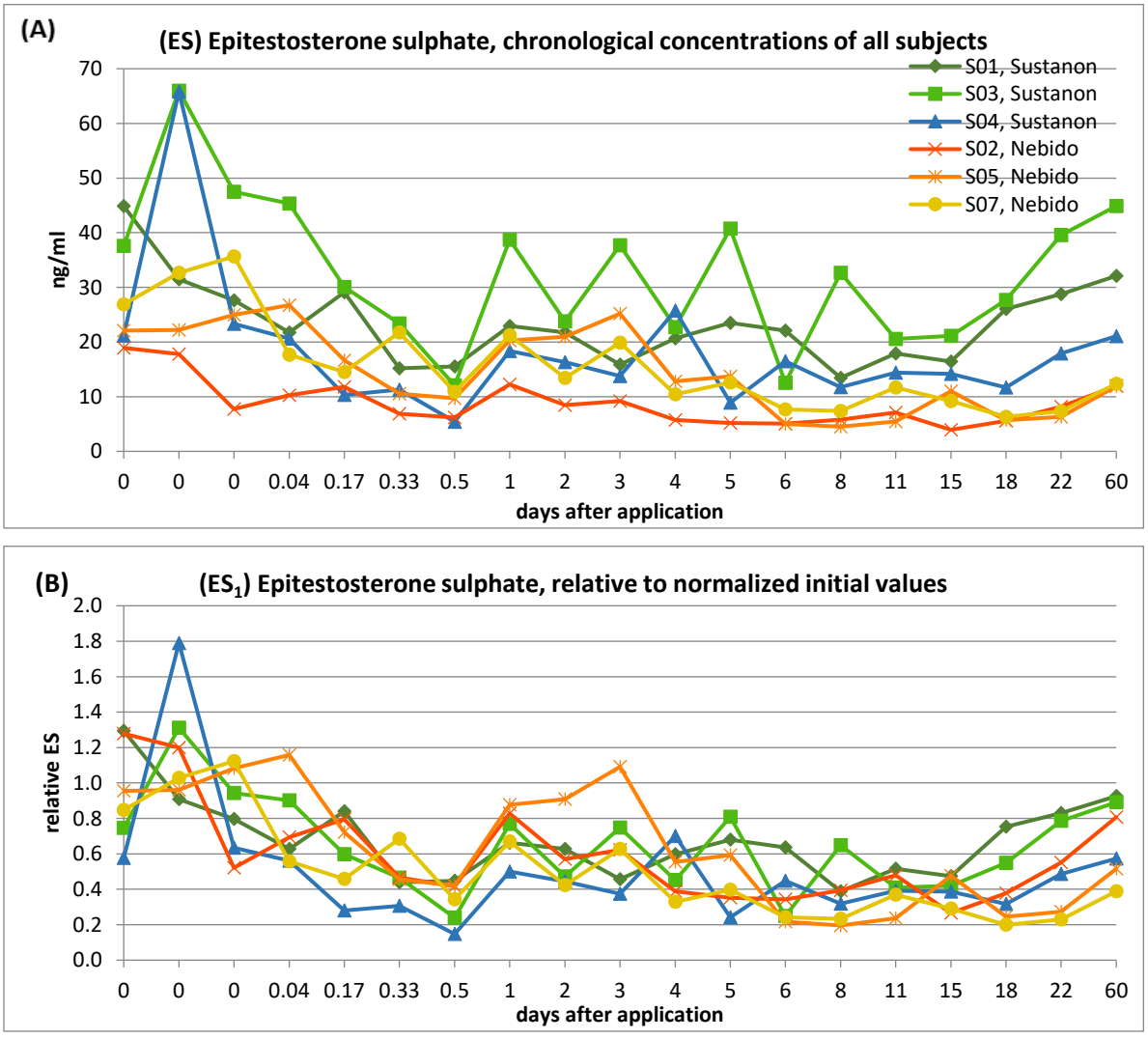

Figure 14: Excreted epitestosterone sulphate concentrations (A) and relative values referring to normalized mean initial urinary ES before application (B) of all subjects over all 19 sample points.

The mean initial concentrations of AS are between 1220 and 1910 ng/ml. The lower ones are in samples of subjects with lower T/E, the higher ones from those with higher T/E. The change of AS concentration is shown in Figure 15 and is very similar to that of AG. There seems to be a slight decrease to 50 % of the mean initial concentrations until 12 hours after the application and after this a slight increase in the samples of most subjects to twice and three times the initial value, either before one week concerning the Sustanon® subjects or after one to two weeks concerning the Nebido® subjects.

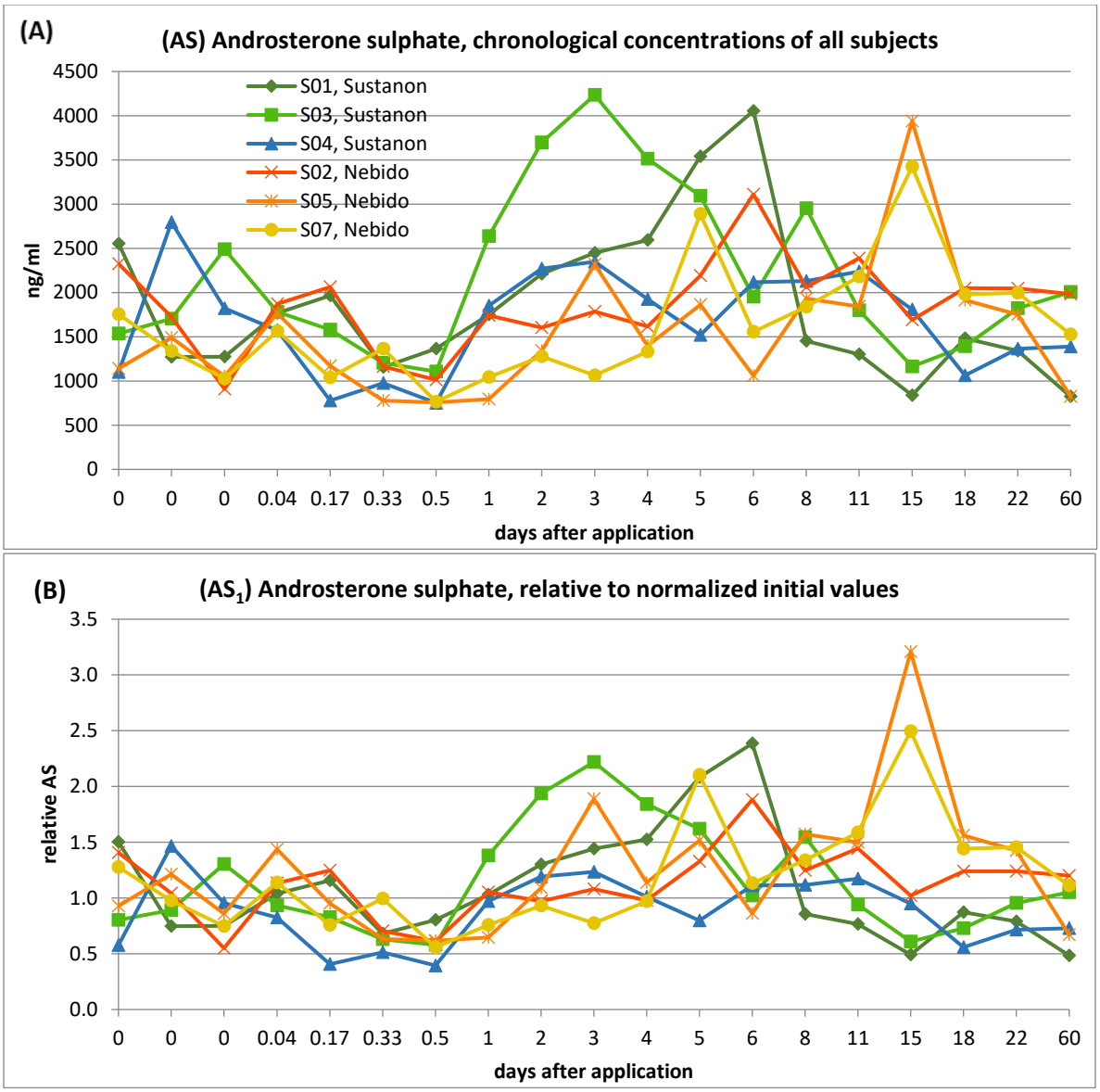

Figure 15: Excreted androsterone sulphate concentrations (A) and relative values referring to normalized mean initial urinary AS before application (B) of all subjects over all 19 sample points.

Figure 16 shows the urinary concentration and relative change of EtioS in the time frame of the study. The mean initial concentrations are between 60 and 490 ng/ml and the subjects with lower initial T/E ratios have the higher initial EtioS concentrations. The relative changes are comparable to those of AG, EtioG and AS with a minimum in concentration 12 hours after the application and a slight increase over one to two weeks before the initial levels are reached again.

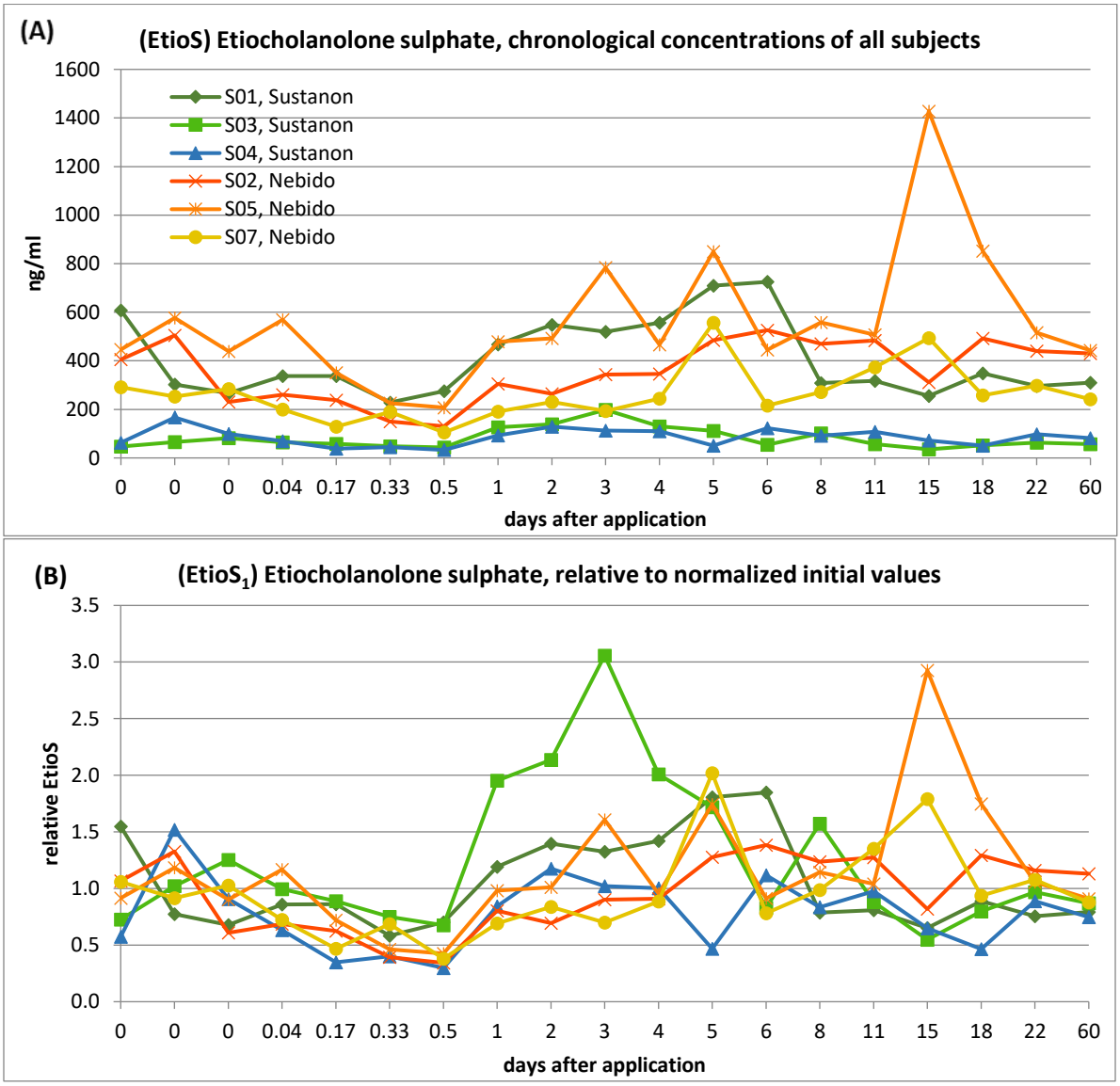

Figure 16: Excreted etiocholanolone sulphate concentrations (A) and relative values referring to normalized mean initial urinary EtioS before application (B) of all subjects over all 19 sample points.

3.2.3. Ratios of sulphates and glucuronides

The change of the ratio of testosterone sulphate and testosterone glucuronide in the urine of the 6 subjects as a function of time is shown in Figure 17. The diagram indicated with (A) shows the ratio calculated with the absolute concentrations, whereas the diagram indicated with (B) shows the relative change compared to the base ratios, which were normalized to the mean value of the 3 initial ratios before the application. The base ratios of the different subjects is quite different as its range goes from about 0.02 in subject S02 to 1.07 in subject S05. S05 is the only test person with a higher initial TS concentration than initial TG concentration. Right after the application the ratio drops dramatically in the samples of the Sustanon® subjects. That is, the base TS/TG ratio in S01 is about 0.55, then it decreases quickly and from 8 hours to 15 days after the application it is between 0.04 (7 %) and 0.18 (33 %). In S03 the ratio starts with 0.18. At 0.33 days (8 hours) after the application it is down at 13 % and at day 5 it is at only 2 % before it increases slowly and comes back to the initial value on day 60. S04 starts with a mean TS/TG of 0.56 and develops the same way, with a minimum of 0.01 (2 %) at day 8. In the Nebido® samples there is also a decrease in the TS/TG ratio, but it is slower as the slower individual response of TG and TS in these samples. In S02 the mean initial ratio is at 0.05 and on day 4 it is at 13 % of that. At day 8 there is the lowest TS/TG value with 0.003, which is 6 % of the starting ratio. In S05 and S07 the initial TS/TG ratio is 1.00 and 0.65 and needs also 4 days to decrease to less than 30 %. Thus, the TS/TG ratio of all subjects no matter what preparation they were given decreases to less than 10 % of the ratio before the application, so there is no outlier. The biggest difference is the time needed.

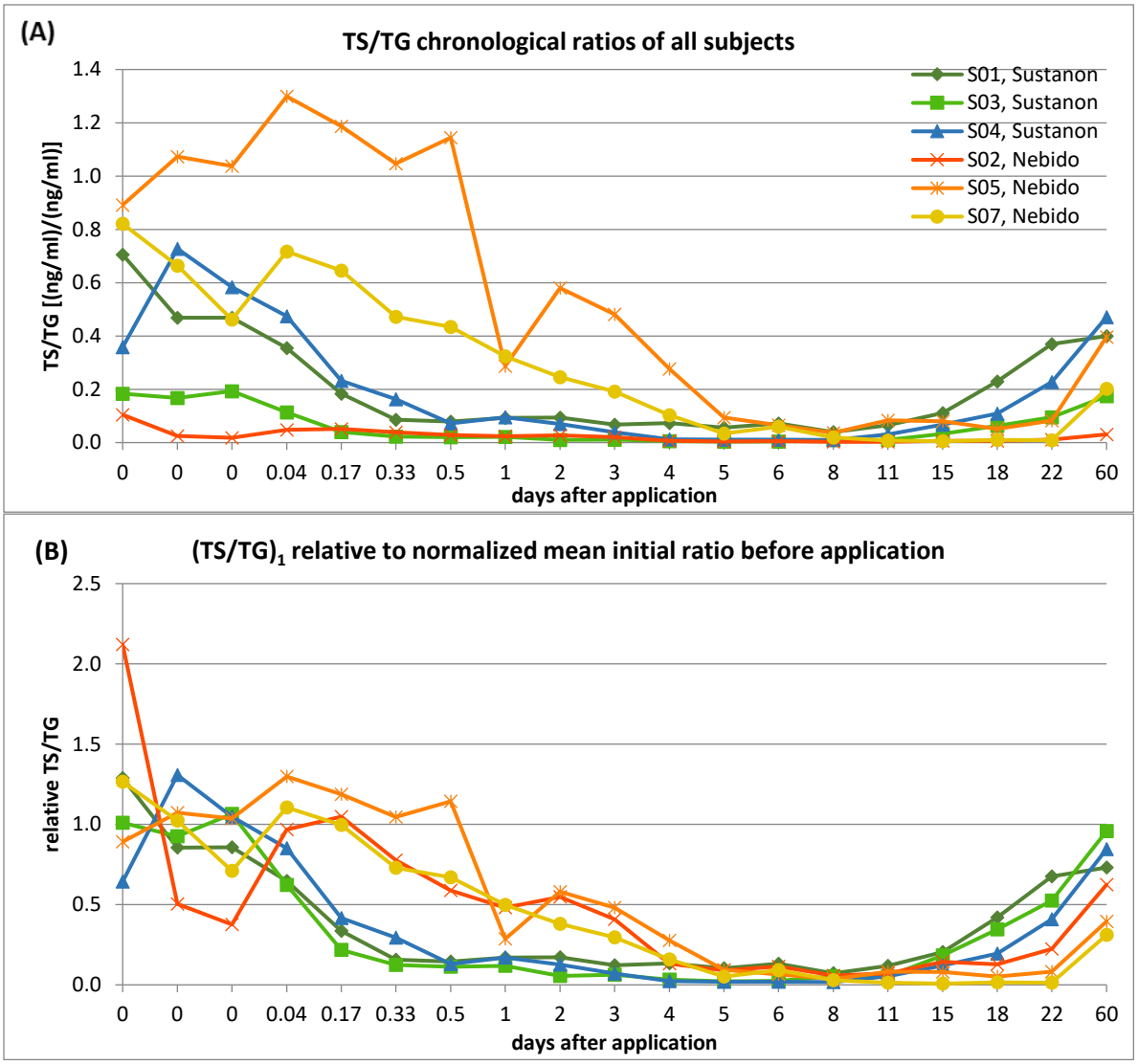

Figure 17: Ratios of testosterone sulphate to glucuronide (TS/TG) (A) and relative values referring to normalized mean initial urinary TS/TG before application (B) of all subjects over all 19 sample points.

Epitestosterone sulphate to epitestosterone glucuronide ES/EG is shown in Figure 18. In contrast to the TS/TG ratio, the ES/EG ratio is slightly increasing after several days. The Sustanon® subjects S01, S03 and S04 are starting with ES/EG mean values of 0.85, 0.89 and 1.00 and are reaching their maximum at days 4 and 5 after the application. In S01 it is just 1.4 times higher than the initial ratio, in S03 and S04 it is more than 3 times the original value. After 15 days ES/EG is down at the initial conditions. The Nebido® subjects S02, S05 and S07 have starting ratios of 0.19, 0.34 and 0.99. In S02 this value increases slowly until day 15 to 1.1 which is about 6 times higher and stays there for a few days until it drops again rapidly. S05 reaches a ratio about 3 times higher than the initial ratio between 11 and 22 days and in S07 it is also 3 times the initial ratio at day 6 after the application. Here the change in ES/EG ratio is also comparable between nearly all subjects.

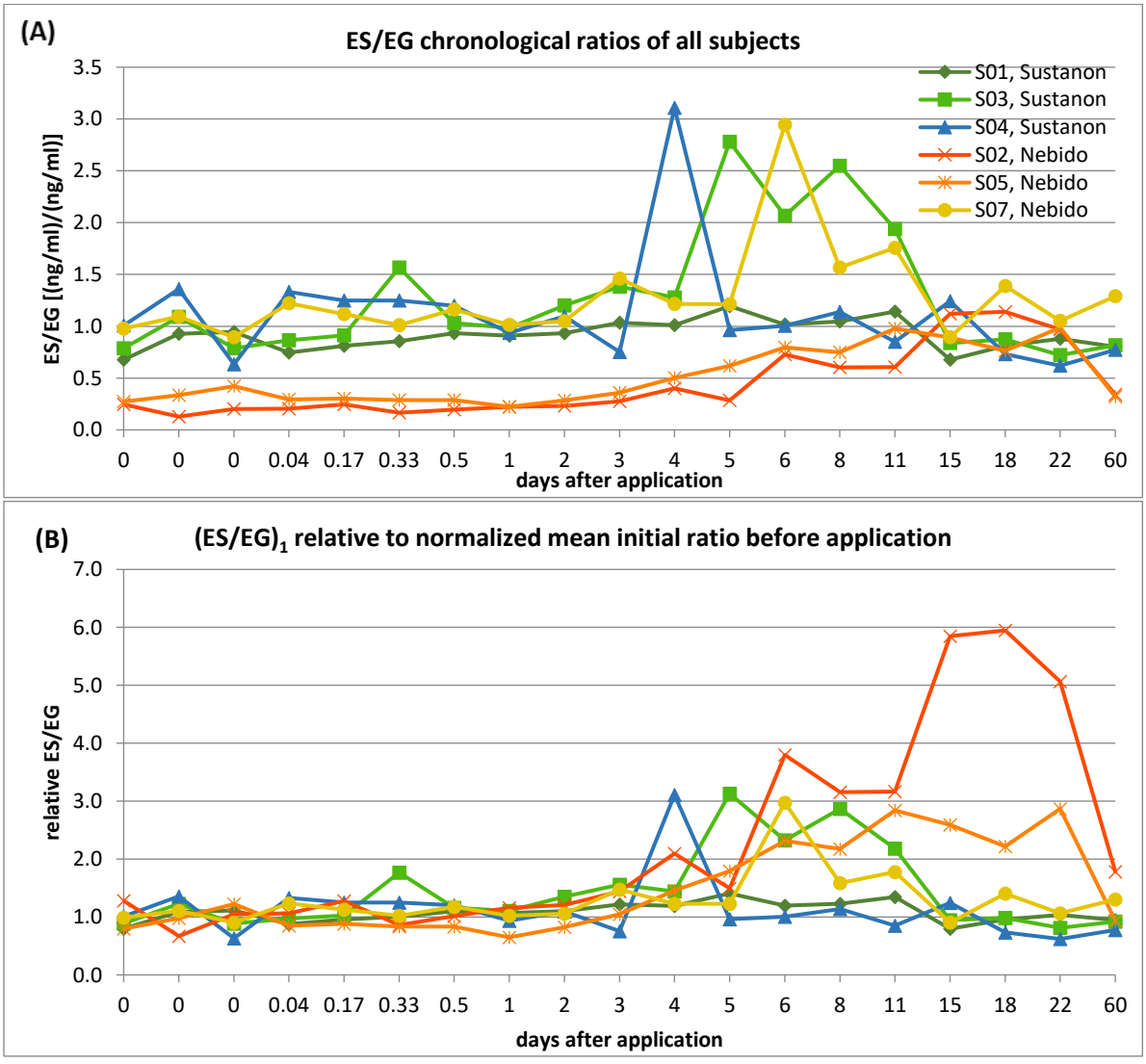

Figure 18: Ratios of epitestosterone sulphate to glucuronide (ES/EG) (A) and relative values referring to normalized mean initial urinary ES/EG before application (B) of all subjects over all 19 sample points.

The changes in AS/AG and EtioS/EtioG ratios is not quite consistent as they seem to go up and down randomly and never exceeding a value much more than 2 times higher or less than a third than the mean initial ratio of point 0. This applies to all subjects (Figure 19 and Figure 20).

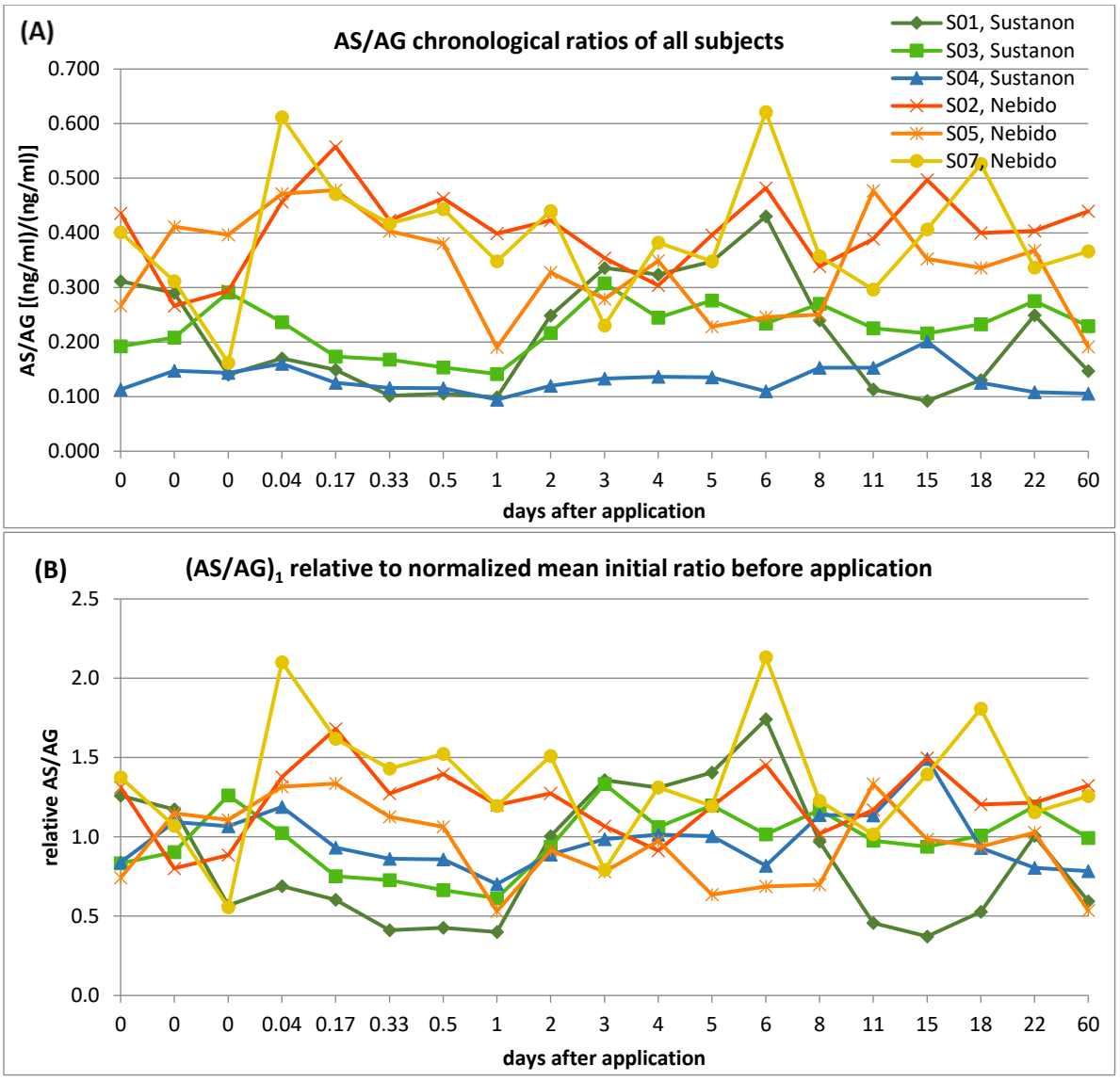

Figure 19: Ratios of androsterone sulphate to glucuronide (AS/AG) (A) and relative values referring to normalized mean initial urinary AS/AG before application (B) of all subjects over all 19 sample points.

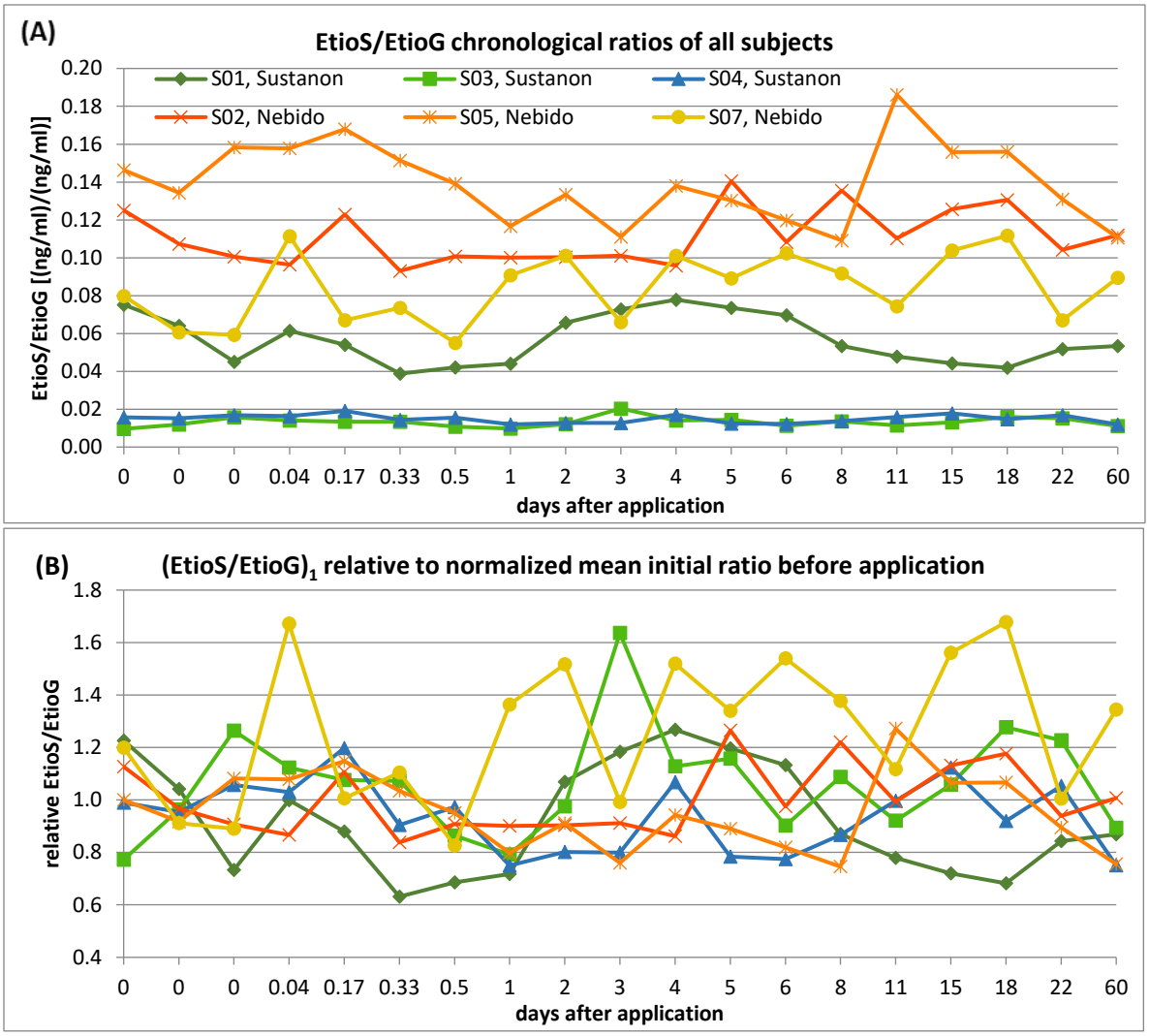

Figure 20: Ratios of etiocholanolone sulphate to glucuronide (EtioS/EtioG) (A) and relative values referring to normalized mean initial urinary EtioS/EtioG before application (B) of all subjects over all 19 sample points.

3.2.4. Ratio of ratios (ES/EG)/(TS/TG) versus T/E

The diagrams indicated with (A) in the following figures show the ratio of ratios of ES to EG and TS to TG, that is (ES/EG)/(TS/TG), of each subject. They also show the corresponding T/E on the secondary axis because the values are often more than 10 times lower than the (ES/EG)/(TS/TG). The diagrams indicated with (B) visualise the relative change after all values were relativized to the mean point 0 value, which was set to 1.

S01 (Figure 21) starts with a mean initial T/E of 1.7 and a (ES/EG)/(TS/TG) of 1.7, so these are quite similar. Half a day after the application of the Sustanon® preparation there is a first T/E peak with the highest value of this subject of 14.4, which is 8.5 times the initial value. The limit of a T/E of 4 was exceeded already 8 hours after the application. It then decreases until day 4 and has a second peak at day 5 before it goes back slowly to less than 4 at day 15 and on to nearly the original level at day 60. In case of the (ES/EG)/(TS/TG) there are several peaks, one higher than the other, and the first one is also at 12 hours with a value of 11.8. The next ones are at day 3 and 5 and the highest at day 8 with 26.5, which is 16 times the initial value. At day 60 after the application the starting level is nearly reached. One can conclude that there is a fast response in S01 to the preparation concerning the T/E as well as the ratio of ratios consisting of glucuronide and sulphate conjugates originating from the phase II metabolism, but this response is comparatively higher.

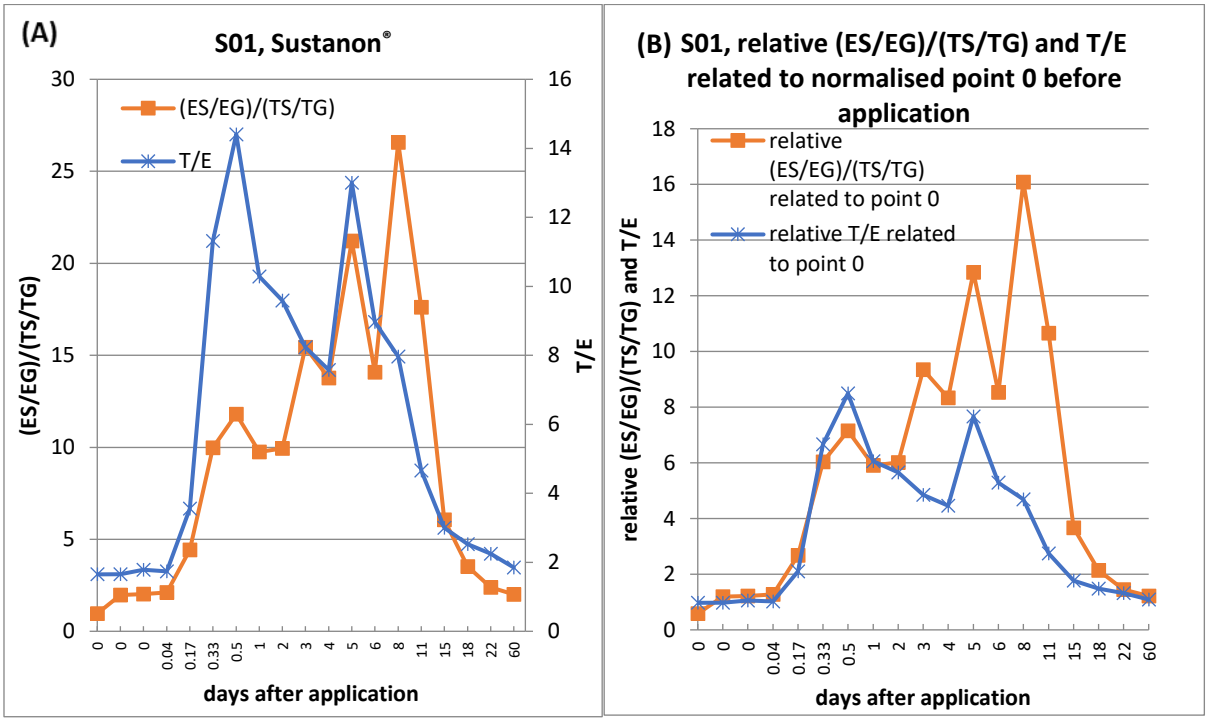

Figure 21: Ratio of ratios (ES/EG)/(TS/TG) in comparison with the T/E values in urine of subject S01 over all sample points after Sustanon® application with independent values presented on two different y-axes (A) and relative change referring to normalized mean initial (ES/EG)/(TS/TG) and T/E (B).

S03 (Figure 22) has a base level T/E ratio of 2.6 and this is the highest of all subjects. The initial (ES/EG)/(TS/TG) is 5. The T/E ratio has exceeded 4 already 4 hours after the application and after 12 hours it is already at 32. After a short drop to 20 it increases till it reaches 47, its top value 6 days after the application, from where it decreases and comes to the base level at day 15 after the experiment. (ES/EG)/(TS/TG) increases as well and after 4 hours it is already 4 times higher than the initial value. The maximal value is 709, which is 143 times the initial value, 5 days after the application. At the last sampling point the base level is reached again. Here the difference in relative change is enormous, since the highest T/E is only 18 times the initial value. Also, at day 18 after the application the (ES/EG)/(TS/TG) is still about 3 times the base level, whereas the T/E is already down there 3 days earlier.

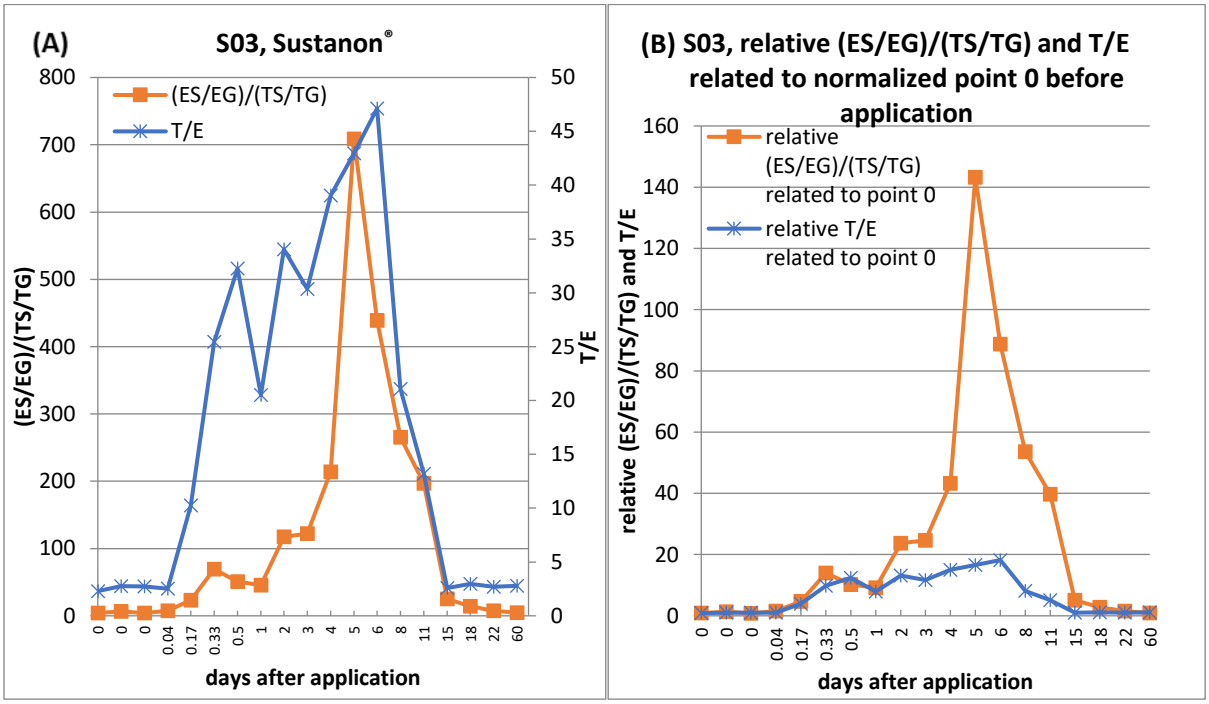

Figure 22: Ratio of ratios (ES/EG)/(TS/TG) in comparison with the T/E values in urine of subject S03 over all sample points after Sustanon® application with independent values presented on two different y-axes (A) and relative change referring to normalized mean initial (ES/EG)/(TS/TG) and T/E (B).

In S04 (Figure 23) the mean initial T/E is 1.8 and (ES/EG)/(TS/TG) is 1.9. 4 hours after the application the T/E is 4.5 and the ratio is 5.4. A first peak with a T/E of 19 is visible at the 12 hours point and a second with 21 at day 4 after the application, which is 12 times the initial T/E. After this the T/E decreases until it is less than 4 at day 15 and back at the initial value at the last sampling point. (ES/EG)/(TS/TG) increases too and has one very high value of 248 – 129 times the initial value – at day 4 after the application. There is a second smaller peak with a ratio value of 117 at day 8, before it decreases to reach the starting value also 60 days after the application. As in S03 the relative change of (ES/EG)/(TS/TG) is much higher than of T/E especially a few days after the application of the Sustanon® preparation.

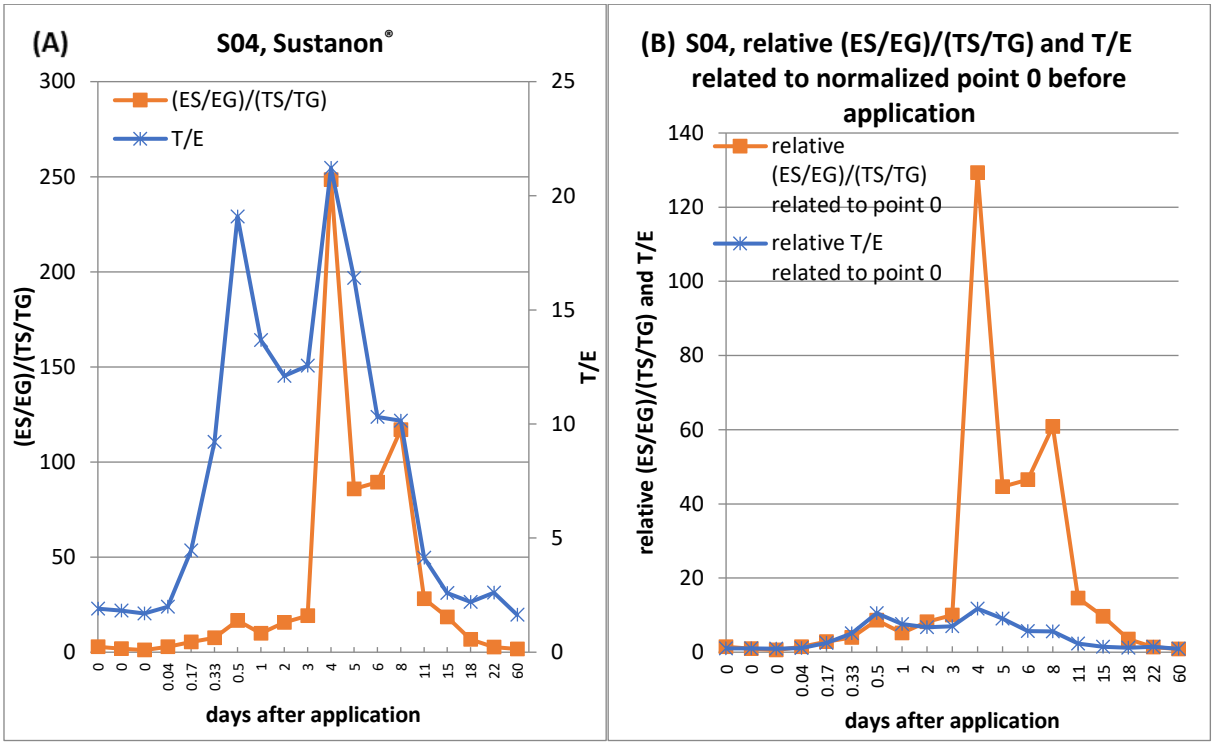

Figure 23: Ratio of ratios (ES/EG)/(TS/TG) in comparison with the T/E values in urine of subject S04 over all sample points after Sustanon® application with independent values presented on two different y-axes (A) and relative change referring to normalized mean initial (ES/EG)/(TS/TG) and T/E (B).

Nebido® subject S02 (Figure 24) has a relatively low initial T/E ratio of 0.8. The mean initial (ES/EG)/(TS/TG) is 6.1. After the application of the slow-acting preparation the T/E increases slowly, is over 4 not till day 4 and its maximum value is 32 at day 6. It then decreases, but 60 days after the application it is still 3 times higher than before. The (ES/EG)/(TS/TG) increases as well and its top value is 211, which is 35 times the initial value, 8 days after the application. After a short drop there is a second lower peak with a ratio of 181 at day 18, before it finally decreases to 11 at the last sampling point. Here the highest values of T/E and (ES/EG)/(TS/TG) are nearly on the same day and maybe they are, but there is no sampling point at 7 days after the application. In contrast to the Sustanon® subjects there is no big difference in relative change of these two ratios as the change over time is very similar.

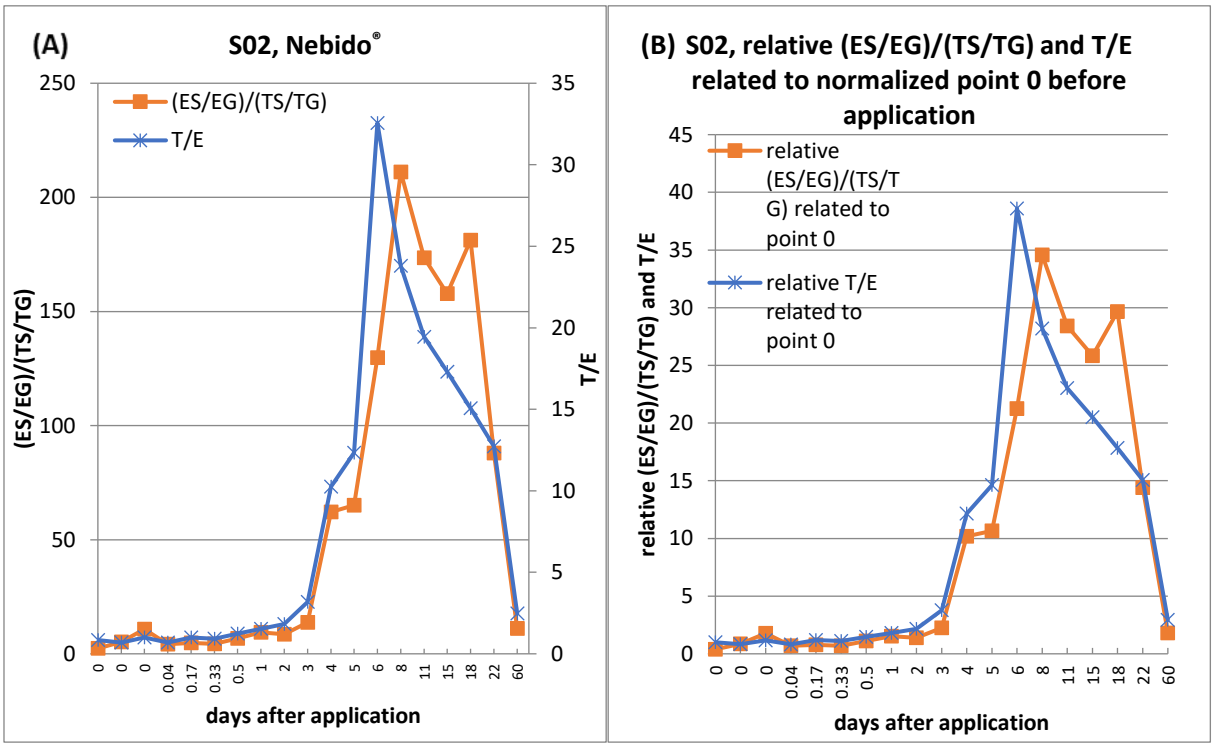

Figure 24: Ratio of ratios (ES/EG)/(TS/TG) in comparison with the T/E values in urine of subject S02 over all sample points after Nebido® application with independent values presented on two different y-axes (A) and relative change referring to normalized mean initial (ES/EG)/(TS/TG) and T/E (B).

S05 (Figure 25) starts with the lowest T/E of all, with 0.07, and the initial (ES/EG)/(TS/TG) is with 0.34 also the lowest. After 8 days the T/E increases until its maximum value of 3.66. At the last sampling point it is still twice the initial ratio. Also, after 8 days (ES/EG)/(TS/TG) is at its top with about 20 and as in S02 after a short drop rises to a second peak with a ratio of about 15 at day 18 after the application. At the last sampling point it has decreased to 0.8. The change of the two ratios over time is like in S02 very similar also concerning the relative change.

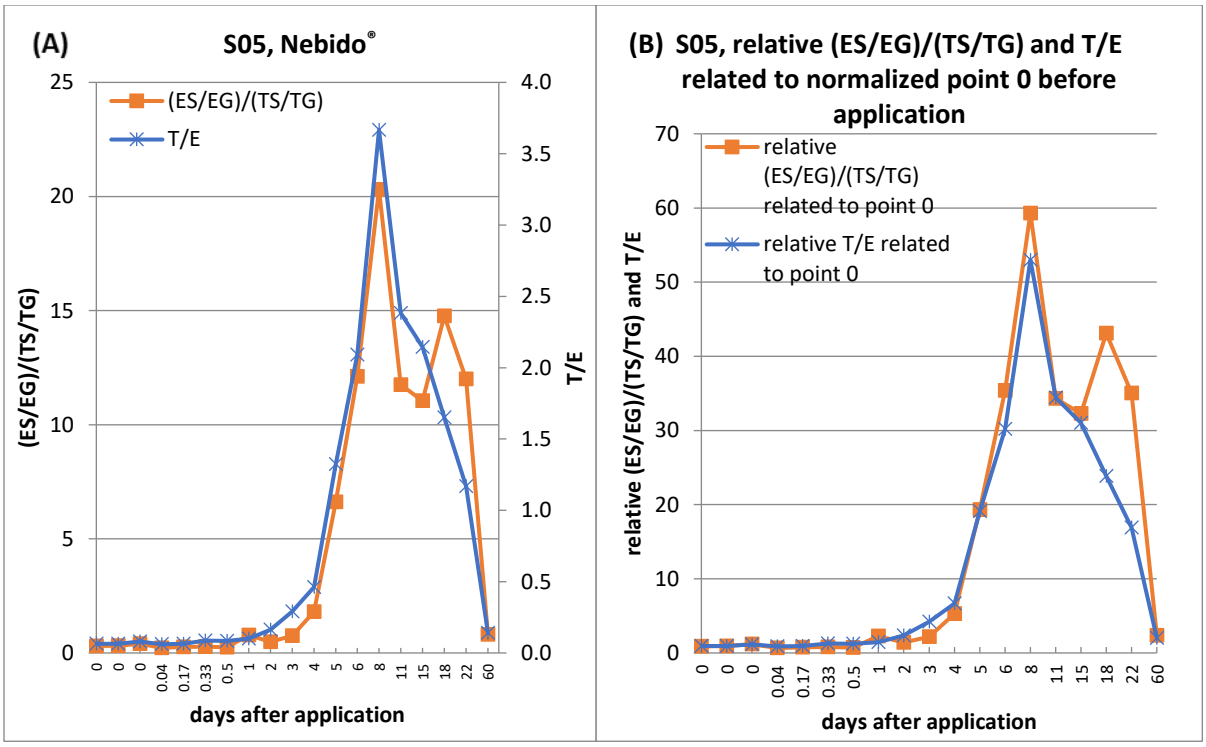

Figure 25: Ratio of ratios (ES/EG)/(TS/TG) in comparison with the T/E values in urine of subject S05 over all sample points after Nebido® application with independent values presented on two different y-axes (A) and relative change referring to normalized mean initial (ES/EG)/(TS/TG) and T/E (B).

The last Nebido® subject S07 (Figure 26) has a mean initial T/E of 1.28 and a (ES/EG)/(TS/TG) of 1.59. The highest T/E ratio is reached 11 days after the application with a value of about 18 and 60 days after the application it is still more than 4. The (ES/EG)/(TS/TG) increases with nearly the same speed at first, but after 4 days the slope is steeper and as it reaches its maximum value of 214 it is 134 times higher than before the application. At the last sampling point it is still 4 times the initial value. Here the difference in relative change between the T/E and the (ES/EG)/(TS/TG) is very high after a few days.

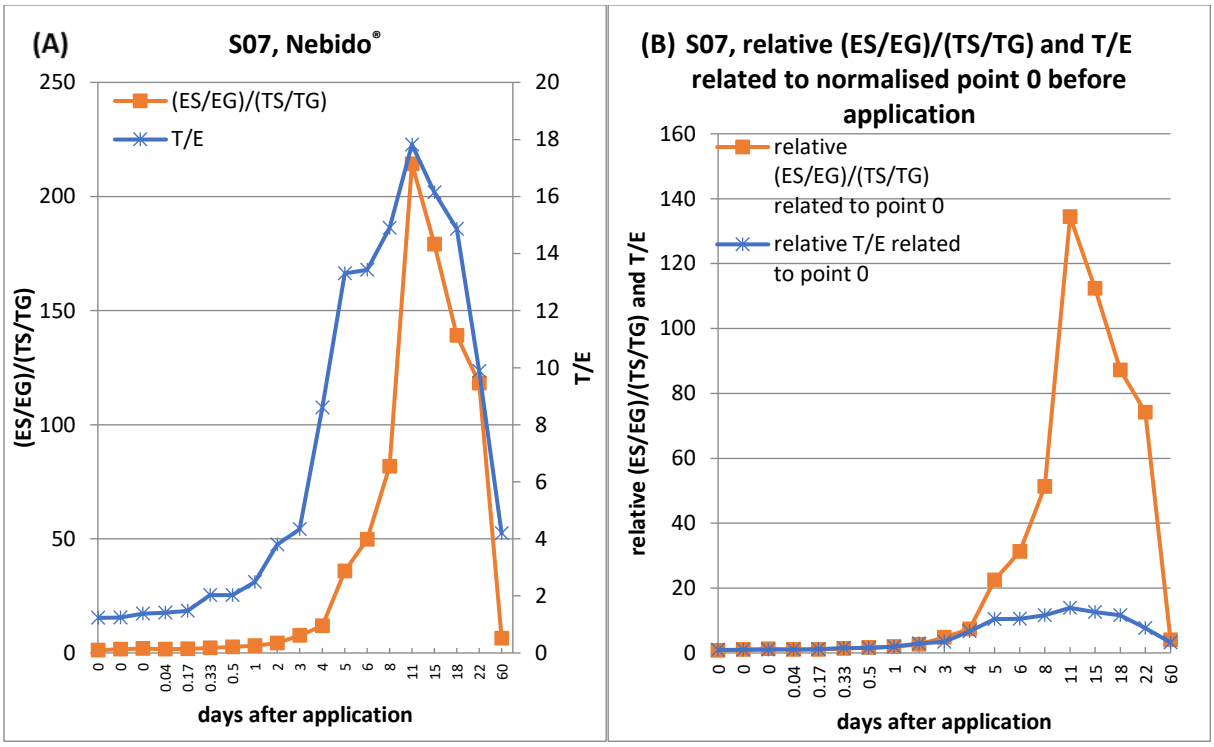

Figure 26: Ratio of ratios (ES/EG)/(TS/TG) in comparison with the T/E values in urine of subject S07 over all sample points after Nebido® application with independent values presented on two different y-axes (A) and relative change referring to normalized mean initial (ES/EG)/(TS/TG) and T/E (B).

4. Discussion and conclusion

In this project the influence of 2 testosterone ester preparations Sustanon® and Nebido® on the phase II metabolism concerning glucuro- and sulpho-conjugated main metabolites of testosterone in human urine was investigated. The analytical challenge was the separation of the intact conjugates by liquid chromatography in contrast to current methods consisting of gas chromatography mass spectrometry (GC-MS) after hydrolytic separation of the glucuronides. The detection uses high resolution mass spectrometry. The created method was validated according to WADA criteria of the International Standard for Laboratories 9.0[1] (ISL) and applied to study samples.

For the study, 6 subjects had provided 19 urine samples each up to 60 days after intramuscular injection of the preparations. The samples were stored at -20°C. The analytes were extracted via solid phase extraction (SPE) and measured via ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS). They were quantified individually, ratios were calculated and trends of each subject over the 60 days of sampling were evaluated. The results showed clear trends according the change of the urinary concentration of some analytes. That is, the concentration of testosterone glucuronide increased in samples of all subjects after application of their respective preparation, while testosterone sulphate decreased as well as epitestosterone glucuronide and sulphate. The rise in testosterone glucuronide concentration matched the expectation that it is a direct phase II metabolite of the administered testosterone esters. In contrast, the concentration of testosterone sulphate did not rise, but even dropped in every test person. A possible explanation of this phenomenon might be an altered metabolic – perhaps enzymatic – activity, caused by the sudden excess of testosterone in the muscle. That is, the suppression of the formation of sulphate metabolites in the testes as a negative feedback is clearly visible and there is no indication of the formation of TS outside the testes too.

Regarding the ratios of the sulphates to the corresponding glucuronides, a slight increase was visible for the ES/EG ratio, but a huge decrease for the TS/TG ratio due to highly increasing TG and decreasing TS concentration. The ratio of ratios (ES/EG)/(TS/TG) was calculated and depicted to compare the T/E, which was measured with the current GC-MS method, with information about the steroidal profile obtained with this LC-MS method supplemented by steroid-sulphates. For 4 of 6 subjects a highly significant increase could be demonstrated by maximum (ES/EG)/(TS/TG) values up to 11 times higher than the top T/E ratio related to their normalized initial values before the administration. Interestingly, there was no such effect for the two subjects with initial T/E values below 1, and their (ES/EG)/(TS/TG) versus T/E curves run nearly synchronously. Nevertheless, alterations happened faster in samples of subjects who received the Sustanon® injection and slower in

Nebido® subjects. This also matches the expectations since testosterone esters with lower chain length are hydrolysed faster[16]. For androsterone and etiocholanolone glucuronides and sulphates no distinct alterations in concentration could be seen, nor in the ratios of glucuronides to sulphates. A reason might be that those molecules are generated rather late in testosterone metabolism and when a sudden intramuscular boost of testosterone is mainly converted to testosterone glucuronide, their formation may not be affected as much. Also, the natural concentration of androsterone and etiocholanolone conjugates is very high compared with testosterone and epitestosterone. Small alterations could thus be less noticeable. The detection of phase II metabolites and their ratios is complementary to the already existing monitoring of the T/E ratio in the athlete´s biological passport. Complementary information provides a more stable basis to discriminate a steroid passport doping case from a natural or pathological condition.

The method developed within the frame of this master thesis demonstrated the ability to quantify steroid glucuronides and sulphates in human urine via UHPLC-HRMS after SPE. The method allows to monitor changes of urinary phase II metabolite concentrations and their respective ratios. Further investigation on base ratios of reference populations as well as the inclusion of these metabolites in the already existing steroid passport system of elite athletes must be performed to proceed in the detection of testosterone doping.

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Abstract

Intra-muscular injection of testosterone esters is a widely practised way of performance enhancement in sports. In doping control, the steroidal profile of athletes is monitored via the analysis of urine samples to detect abnormalities caused by administration of anabolic androgenic steroids (AAS). The aim of this project was to develop a complementary method that uses liquid chromatography and high resolution mass spectrometry to gain information about the glucuronide- and sulphate-conjugated phase II main metabolites of testosterone and their ratio changes after administration as testosterone esters. The method was validated according to the WADA criteria of the International Standard for Laboratories 9.0[1] (ISL) and applied on human samples. In a prior clinical study, 6 subjects had received a single injection of either Sustanon® – a mixture of 30 mg testosterone propionate, 60 mg testosterone phenylpropionate, 60 mg testosterone isocaproate and 100 mg testosterone decanoate – or Nebido® – 1000 mg of testosterone undecanoate. Urine samples were taken up to 60 days after the application. The samples were prepared via solid phase extraction. The results show the change of the concentrations and the ratios of glucuronides to sulphates over the sampling period. They were also compared with the T/E ratio, which was determined as usual via gas chromatography mass spectrometry after hydrolytic separation of glucuronic acid. Due to the simultaneous detection of the steroid sulphates and glucuronides, a partially high amplification effect of the alterations in post-administration samples could be seen. Though, the amplification seems to be dependent on the natural T/E ratio of the subject.

Zusammenfassung

Die intramuskuläre Injektion von Testosteronestern ist eine weit praktizierte Form der Leistungssteigerung im Sport. Bei der Dopingkontrolle wird das Steroidprofil der Athleten mittels Analyse von Harnproben beobachtet, um Unregelmäßigkeiten, die durch die Einnahme anaboler androgener Steroide entstehen können, zu detektieren. Ziel dieses Projekts war die Entwicklung einer Methode, in der Flüssigchromatographie und hochauflösende Massenspektrometrie dazu genutzt werden Informationen über die glucuronid- und sulfatkonjugierten Phase-II-Hauptmetaboliten von Testosteron und ihre verhältnismäßige Veränderung nach der Verabreichung in Form von Testosteronestern zu erhalten. Die Methode wurde nach den WADA Kriterien des Internationalen Standards für Laboratorien 9.0[1] (ISL) validiert und an menschlichen Proben angewandt. In einer früheren Studie erhielten 6 Probanden eine einzelne Injektion von entweder Sustanon® – eine Mischung von 30 mg Testosteronpropionat, 60 mg Testosteronphenylpropionat, 60 mg Testosteronisocaproat und 100 mg Testosterondecanoat – oder Nebido® – 1000 mg Testosteronundecanoat. Harnproben wurden bis 60 Tage nach der Verabreichung gezogen. Die Proben wurden mittels Festphasenextraktion zur Analyse vorbereitet. Die Ergebnisse zeigen die Änderung der Konzentrationen und der Verhältnisse von Glucuroniden und Sulfaten zueinander über die Probenahmezeit. Sie wurden auch mit dem T/E Verhältnis verglichen, der gewöhnlich via Gaschromatographie-Massenspektrometrie nach hydrolytischer Abtrennung der Glucuronsäure festgestellt wurde. Aufgrund der simultanen Detektion von Sulfat- und Glucuronidmetaboliten endogener Steroide konnte ein teilweise hoher Verstärkungseffekt der Veränderungen in Post-Injektionsproben festgestellt werden. Jedoch scheint die Verstärkung vom natürlichen T/E Verhältnis der Testpersonen abhängig zu sein.