# Anabolic Steroid Glucuronides

## Enzyme-Assisted Synthesis and Liquid Chromatographic–Mass Spectrometric Analysis

by

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## LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following four articles, which are referred to as **I–IV** in the text:

**I** Kuuranne, T., Aitio, O., Vahermo, M., Elovaara, E., and Kostiainen, R. Enzyme-assisted synthesis and structure characterization of glucuronide conjugates of methyltestosterone (17α-methylandrost-4-en-17β-ol-3-one) and nandrolone (estr-4-en-17β-ol-3-one) metabolites, Bioconjugate Chem. 13 (2002) 194–199.

**II** Kuuranne, T., Kurkela, M., Finel, M., Thevis, M., Schänzer, W., and Kostiainen, R. Structure–function relationships in the glucuronidation of anabolic androgenic steroids by recombinant human UDP-glucuronosyltransferases, Drug Metab. Dispos. (2002) submitted.

**III** Kuuranne, T., Vahermo, M., Leinonen, A., and Kostiainen, R. Electrospray and atmospheric pressure chemical ionization tandem mass spectrometric behavior of eight anabolic steroid glucuronides, J. Am. Soc. Mass Spectrom. 11 (2000) 722–730.

**IV** Kuuranne, T., Kotiaho, T., Pedersen-Bjergaard, S., Rasmussen, K.E., Leinonen, A., Westwood, S., and Kostiainen, R. Feasibility of a liquid-phase microextraction sample clean-up and LC-MS/MS screening method for selected anabolic steroid glucuronides in biological samples, J. Mass Spectrom. 38 (2003) 16–26.

Some unpublished data are included.

## ABBREVIATIONS

GENERAL ABBREVIATIONS

| | |
|---|---|
| 3HSD | 3α-hydroxysteroid dehydrogenase enzyme |
| 3-KSR | 3-ketosteroid reductase enzyme |
| 5α-R | 5α-reductase enzyme |
| 5β-R | 5β-reductase enzyme |
| 17HSD | 17β-hydroxysteroid dehydrogenase enzyme |
| 17-KSR | 17-ketosteroid reductase enzyme |
| AAS | anabolic–androgenic steroids |
| ADME | administration, distribution, metabolism, excretion |
| APCI | atmospheric pressure chemical ionization |
| API | atmospheric pressure ionization |
| cDNA | complementary deoxyribonucleic acid |
| EI | electron impact |
| ER | endoplasmic reticulum |
| ESI | electrospray ionization |
| FAB | fast atom bombardment |
| GC | gas chromatography |
| Glu | glucuronic acid |
| HPLC | high-performance liquid chromatography |
| IAC | immunoaffinity chromatography |
| i.d. | internal diameter |
| ISTD | internal standard |
| LC | liquid chromatography |
| LLE | liquid–liquid extraction |
| LPME | liquid-phase microextraction |
| m/z | mass-to-charge ratio |
| MRM | multiple reaction monitoring |
| MS | mass spectrometry |
| MS/MS | tandem mass spectrometry |
| NMR | nuclear magnetic resonance |
| PA | proton affinity |
| RP | reversed phase |
| $S_N2$ | bimolecular nucleophilic substitution |
| SPE | solid-phase extraction |
| SSI | sonic spray ionization |
| TIS | turbo ionspray |
| TSI | thermospray ionization |
| TMS | trimethylsilyl |
| UDPGA | uridine-5’-diphosphoglucuronic acid |

| | |
|---|---|
| UGT | uridine diphosphoglucuronosyltransferase |
| UV | ultraviolet |

STEROID GLUCURONIDE ABBREVIATIONS*

| | |
|---|---|
| 3-OHSTG | 3'-hydroxystanozolol glucuronide |
| 5α-1-MEG | 1-methyl-5α-androst-1-en-3-one-17β-*O*-glucuronide |
| 5α-AG | 5α-androstane-3α-ol-17β-*O*-glucuronide |
| 5α-DHTG | 5α-androstane-3-one-17β-*O*-glucuronide |
| 5α-DROSTG | 2α-methyl-5α-androstane-17-one-3α-*O*-glucuronide |
| 5α-MEG | 1-methylen-5α-androstane-17-one-3α-*O*-glucuronide |
| 5α-MESM1G | 1α-methyl-5α-androstane-17-one-3α-*O*-glucuronide |
| 5α-MESM2G | 1α-methyl-5α-androstane-17β-ol-3α-*O*-glucuronide |
| 5α-MTG | 17α-methyl-5α-androstane-17β-ol-3α-*O*-glucuronide |
| 5α-NG | 5α-estran-17-one-3α-*O*-glucuronide |
| 5β-BOLDG | 5β-androst-1-en-3-one-17β-*O*-glucuronide |
| 5β-EPIMG | 17β-methyl-5β-androst-1-ene-17α-ol-3α-*O*-glucuronide |
| 5β-LMTG | 17α-$CD_3$-5β-androstane-17β-ol-3α-*O*-glucuronide (internal standard) |
| 5β-MTG | 17α-methyl-5β-androstane-17β-ol-3α-*O*-glucuronide |
| 5β-NG | 5β-estran-17-one-3α-*O*-glucuronide |
| 7α-BOLAG | 7α,17α-dimethyl-5β-androstane-17β-ol-3α-*O*-glucuronide |
| 7β-CALUG | 7β,17α-dimethyl-5β-androstane-17β-ol-3α-*O*-glucuronide |
| AG | 5α-androstane-17-one-3α-*O*-glucuronide; androsterone glucuronide |
| ETCG | 5β-androstane-17-one-3α-*O*-glucuronide; etiocholanolone glucuronide |
| ETG | 4-androsten-3-one-17α-*O*-glucuronide; epitestosterone glucuronide |
| MTG | 17α-methyl-4-androsten-3-one-17β-*O*-glucuronide; methyltestosterone glucuronide |
| NG | estr-4-en-3-one-17β-*O*-glucuronide; nandrolone glucuronide |
| TG | 4-androsten-3-one-17β-*O*-glucuronide; testosterone glucuronide |

* The abbreviation for the corresponding aglycone is obtained by removing the G for glucuronide.

## ABSTRACT

Anabolic–androgenic steroids (AAS) are testosterone derivatives, widely misused by athletes because of their potential enhancing effect on physical performance. Within the human body, AAS are transformed by phase-I and phase-II metabolic reactions and most often they are excreted in urine as glucuronide-conjugates. Electron impact (EI) and GC–MS based methods have conventionally been applied in the analysis of AAS metabolites in urine. The methods rely on the detection of hydrolyzed and derivatized steroid aglycons and, for that, laborious sample preparation must be carried out. Soft ionization methods, such as electrospray (ESI), enable the connection of liquid chromatographic (LC) separation to mass spectrometric (MS) detection, and thereby, the direct analysis of non-volatile, bulky, and polar compounds, such as the AAS glucuronides. Glucuronide-conjugated AAS standards are required for LC–MS method development, but only a few are commercially available.

An enzyme-assisted synthesis was optimized to produce milligrams of glucuronide-conjugated metabolites of the most widely misused AAS. The uridine diphosphoglucuronosyl-transferase (UGT) enzymes that catalyze the glucuronidation reaction were obtained from the hepatic microsomal fraction of induced Wistar rats. To allow characterization of regio- and stereoselectivity and substrate specificity of UGTs, the glucuronidation reaction was additionally examined *in vitro* with recombinant human UGT isoenzymes, as well as with human liver microsomes. After the structural characterization of the synthesized substances, the conjugates were utilized in the development of a liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method designed for the determination of intact AAS glucuronides in enzyme-kinetic assays and in human urine.

Enzyme-assisted synthesis was successfully applicable to the production of stereochemically pure AAS glucuronides in amounts sufficient for LC–MS/MS method development. The glucuronide-conjugated AAS were recovered in milligram amounts (1.3–6.5 mg), with yields (13-78%) highest for steroid substrates with 4-ene-3-one structure.

The only recombinant human isoenzymes showing evidence of regioselectivity were UGT1A8, 1A9, and 2B15, which appeared preferentially to catalyze 17β-hydroxyl glucuronidation. Most recombinant human UGTs did not exhibit a clear preference for conjugation to either the 3α-hydroxyl or the 17β-hydroxyl group. Apparent stereoselectivity was detected in the formation of nandrolone metabolites 5α-NG and 5β-NG with most of the UGT isoenzymes, but the corresponding isomeric pair of methyltestosterone metabolites, 5α-MTG and 5β-MTG, did not show the similar behavior, however. The substrate specificities were closely similar among the groups of structurally analogous UGTs, although inter-individual differences were observed in their relative activities. In a comparison of rat and human liver preparations and recombinant UGT isoenzymes, the main difference was found in the conjugation of methyltestosterone, which was glucuronidated only with the human and rat liver microsomal UGTs, from which the induced rat liver UGTs were clearly more active.

The optimized LC–ESI-MS/MS method enabled the direct analysis of glucuronide conjugates. Two structure-specific product ions from both the analyte and the deuterium-labeled internal standard were monitored in positive ion ESI-MS/MS, and the structure-specific fragmentation that occurred allowed differentiation of most of the isobaric AAS glucuronides with identical product ion spectra. Chromatographic separation was achieved with an end-capped $C_{18}$ column and ammonium acetate buffered acetonitrile–water gradient. The optimized method was applied in metabolic *in vitro* studies of AAS glucuronides. The complex urine matrix samples required more effective sample purification, and for that, a liquid-phase microextraction (LPME) method was developed.

Enzyme-assisted synthesis with rat liver microsomal UGTs is a suitable approach to the small-scale synthesis of glucuronide-conjugated AAS. Relative to recombinant UGT isoenzymes, the activity of glucuronidation is significantly higher with the liver microsomal preparations, and these are recommended for future synthesis work. UGT isoenzymes are of great importance in the examination of the glucuronidation reaction and in future, selected UGT isoenzymes may offer an *in vitro* model to predict *in vivo* glucuronidation of xenobiotics in drug discovery development. The LPME–LC–ESI-MS/MS method is suitable for the direct detection of AAS glucuronides in biological samples, offering detection at 1–5 ng/ml level in simple sample matrixes and 2–20 ng/ml for most analytes in urine. Because of the interference of endogenous compounds in urine, future method development should be focused on enhancement of the specificity and, for that, the main task is the improvement of chromatographic separation.

# 1. INTRODUCTION

Anabolic–androgenic steroids (AAS) are widely misused compounds among athletes and, despite the ban since 1976, the doping use of these substances continues to be a problem for sports authorities. Anabolic effects of AAS include the enhancement of skeletal muscle strength and the balancing of catabolic conditions after stress; the androgenic effects, such as cardiovascular and hepatic disorders, are mainly considered as side effects. Synthetic AAS are testosterone derivatives that are designed to deliver the anabolic effects but not the androgenic effects of the endogenous analogue. Despite many attempts to synthesize compounds without the androgenic effects, both types of effect remain closely associated with the AAS activity.

Because of their non-polar character, AAS are extensively modified by phase-I and phase-II metabolic reactions in the human body prior to their excretion in urine. Phase-I reactions – oxidation, hydrolysis, and reduction – introduce new functional groups to the steroid structure, which increase the polarity of the parent compound. Often they also serve as sites for conjugation in subsequent phase-II reactions. The most common conjugation reaction for AAS in the human body is glucuronidation and the main site for the reaction is the liver. Glucuronidation is catalyzed by uridine diphosphoglucuronosyltransferases (UGTs), which are membrane-bound enzymes of the endoplasmic reticulum (ER). The UGT enzyme family has several members with a variety of substrate specificities, which makes them capable of conjugating substrates with diverse structures of both endogenous and exogenous origin. Through glucuronidation the parent compounds are generally transformed into less toxic metabolites. In addition to detoxification, coupling with glucuronic acid moiety increases the polarity of the steroid aglycone, leading to easier excretion of metabolites in urine.

In the detection of AAS misuse it is useful to focus on the long-term urinary excreted metabolites, most of which are glucuronide-conjugated compounds. At present, the analytical methods for glucuronide-conjugated AAS are based on gas chromatographic (GC) separation and mass spectrometric (MS) detection of hydrolyzed and derivatized steroid metabolites. Although the GC–MS methods are robust and sensitive, sample preparation is time-consuming and GC–MS sample throughput is relatively low. The development of faster and simpler methods based on the direct analysis of steroid conjugates is thus of great interest. Relatively new developments in analytical instrumentation, especially soft ionization methods such as electrospray (ESI) in mass spectrometry, have enabled the direct analysis of non-volatile biomolecules. A combination of liquid chromatographic (LC) separation with ESI and tandem mass spectrometric (MS/MS) detection provides information on the molecular weight and structure of compounds, offering an effective analytical approach to qualitative and quantitative analysis of AAS glucuronides.

Reference compounds are needed for the LC–MS/MS method development, but today only a few AAS glucuronides are commercially available. *In vivo* production of the reference substances runs into ethical problems, as well as practical problems associated with the

isolation of pure metabolites from urine. Chemical syntheses have been published for several glucuronide conjugates, but a common difficulty is the formation of a racemic mixture and unwanted by-products. An alternative approach to chemical *in vitro* glucuronide synthesis is to catalyze the reaction with UGTs obtained from animal tissues such as rat liver microsomes or with individual recombinant UGT isoenzymes. The main advantage of the enzyme-assisted synthesis over the chemical synthesis is that the stereospecificity of the enzymes allows synthesis of stereo-specifically pure conjugates.

Enzyme-assisted synthesis of glucuronide-conjugated AAS was attempted in this study. Rat liver microsomes were the source of the UGT enzymes, which were used to produce milligram amounts of AAS glucuronides for concomitant LC–MS/MS method development **(I)**. Catalytic activities of human liver microsomes and recombinant human UGT enzymes towards AAS metabolites were investigated in detail **(II)**. The analytical part of the study focused on the development of an LC–MS/MS method for direct analysis of steroid glucuronides in human urine. Traditional sample cleanup procedures of liquid–liquid extraction (LLE) and solid-phase extraction were compared with the more recently developed liquid-phase microextraction (LPME) **(IV)**. Optimization of the instrumental conditions was focused on chromatographic separation and structure-specific MS/MS fragmentation of glucuronide-conjugated steroid metabolites **(III, IV)**.

## 2. REVIEW OF THE LITERATURE

### 2.1 ANABOLIC–ANDROGENIC STEROIDS

Anabolic–androgenic steroids (AAS) are synthetic testosterone derivatives, which are designed to maintain the anabolic (beneficial effects) and to minimize the androgenic (side effects) activities of the endogenous prototype (Haupt and Rovere, 1984). More than 600 testosterone analogues have been synthesized, a particularly large number of them during the 1940s and 1950s. A single hormonal receptor apparently mediates both androgenic as well as anabolic actions of testosterone, and the complete separation of these two effects has not yet been achieved (Celotti and Negri-Cesi, 1992).

Medical use of AAS was initially intended for the treatment of hypogonadism and catabolic states (Kennedy, 1992; Lukas, 1993). There is no question about the capability of AAS to promote protein synthesis in skeletal muscles (Lamb, 1984), but owing to weaknesses in experimental studies (e.g. limited number of test subjects, dosing, non-uniform test environments and short experimental periods), it is not clear whether AAS are actually able to improve athletic performance (Wilson and Griffin, 1980; Haupt and Rovere, 1984; Celotti and Negri-Cesi, 1992). Although the increase in total body weight following androgen administration is indisputable, it is unclear whether this is due to true increase in lean body mass or merely to salt and water retention (Kennedy, 1992). Some other benefits claimed for AAS use are increased blood volume and hemoglobin concentration (Lamb, 1984), together with anticatabolic effects (Wilson and Griffin, 1980; Wu, 1997). The metabolism of the various AAS is different, leading to differing patterns of side effects, which can roughly be categorized as androgenic, dermatological, hematological and cardiovascular, hepatic, psychiatric and neurological, renal, and skeletal and muscular (Wilson and Griffin, 1980; Kennedy, 1992; Lukas, 1993; Rockhold, 1993; Huhtaniemi, 1994).

Despite the initial ban of the International Olympic Committee at the Olympic Games 1976 in Montreal, AAS still represent a major group of misused compounds in sports. The doping analytical methodology is faced with a wide variety of target compounds in diverse concentrations, as the non-medical administration of AAS is typically performed either in on/off cycles with one steroid (6–12 weeks or more per cycle), by continuous stacking of more than one steroid at a time, or in a long-term pyramid program with gradually increased dosing of several compounds (Rogol and Yesalis, 1992).

#### *2.1.1 AAS structure and nomenclature*

As depicted in Figure 1, the steroid structure consists of three six-member rings (A-C) and one five-member ring (D), thus forming a bulky and non-polar perhydrocyclopentano-phenanthrene steroid skeleton. Steroids with 4-ene-3-one structure are planar and rigid, as are steroids with 5α-oriented proton. In 5β-orientation the A/B ring juncture is bent, so that, for

example, the orientation of the axial substituents is switched to equatorial and vice versa (Kirk and Marples, 1995). Various structural modifications of testosterone have been designed to bypass the extensive first pass metabolism in the body and so enhance the potency, or to delay absorption from the injection site (Kennedy, 1992). These modifications have been undertaken by alkylation of the 17α-position (Liao, 1973), which allows oral administration of the compound, or by esterification of the 17β-position, leading to a compound that can be administered intramuscularly (Wilson and Griffin, 1980). Androgen activity can also be modified through ring additions or substitutions, such as addition of the pyrazol moiety in stanozolol or C-4 substitution of a chlorine atom in clostebol.

***Figure 1.*** *Examples of AAS structural modifications. A) Testosterone, B) testosterone undecanoate (17β-esterified), C) methyltestosterone (17α-alkylated), D) stanozolol (additional pyrazol ring), and E) clostebol (C-4 substitution).*

### *2.1.2 AAS metabolism in the human body*

Several metabolic reactions enhance the excretion of AAS by transforming them into less toxic, less active, and/or more polar form. In general, metabolic pathways may be divided into phase-I and phase-II reactions, and often the two classes of reactions will occur in parallel for a certain compound. The practical goal of the study of AAS metabolism in doping control is

to ensure that the monitoring is correctly targeted; for most analytes are transformed in some way and are very seldom excreted as the parent compounds (Gower *et al.*, 1995).

Phase-I reactions, often referred to as functionalization reactions, typically modify the parent compound via hydrolysis, oxidation, and/or reduction (Gibson and Skett, 1994; Rendic, 1997). The initial and also rate-limiting step in the metabolism of 4-ene-3-one structured steroids (e.g. nandrolone) is the non-reversible reduction of the C-4,5 double bond, which leads to an asymmetric center at C-5 (Figure 2). Two isomers will be formed, in a ratio depending on the relative catalyzing effects of 5α- and 5β-reductase enzymes (Schänzer, 1996). As soon as the double bond is reduced, the 3-keto group is transformed, predominantly by 3α-hydroxysteroid dehydrogenase (3HSD). In D-ring metabolism, 17β-hydroxysteroid dehydrogenase (17HSD) has a strong tendency to form 17-keto metabolites (Gower, 1995). Spontaneous 17-epimerization has also been reported for 17α-methyl-17β-hydroxy structured steroids, originating in decomposition of the corresponding 17β-sulphate conjugate in urine and resulting in an inversion of configuration (Bi and Massé, 1992; Schänzer *et al.*, 1992; Gower *et al.*, 1995; Schänzer, 1996). Although phase-I reactions already increase the polarity and the excretion of AAS, these modifications are most often preparative stages for reactions that expose reactive sites of the analyte structure for the following phase-II, i.e. conjugation reactions. For AAS the main phase-II reactions are glucuronidation and sulfation. However, for doping control purposes urinary AAS screening is typically performed in free and glucuronide fraction, sulfate-conjugated metabolites remaining undetected (Uralets and Gillette, 2000).

***Figure 2.*** *Phase-I metabolism of nandrolone (A). Modifications with 5α- and 5β-reductase (5α/β-R), 3α-hydroxysteroid dehydrogenase (3HSD), and 17β-hydroxysteroid dehydrogenase (17HSD) enzymes leading to the formation of 5α-estran-3α-ol-17-one (B) and 5β-estran-3α-ol-17-one (C).*

## 2.2 GLUCURONIDATION AND UGT ISOENZYMES

Conjugation with glucuronic acid is the major conjugation reaction in all mammals. Various functional groups have the potential of reacting with glucuronic acid to form *O*-, *S*-, *N*-, and *C*-glucuronides, which means that a wide variety of compounds are metabolized via the glucuronidation pathway (Mulder *et al.*, 1990). Glucuronidation is a bimolecular nucleophilic substitution ($S_N2$) reaction, which is catalyzed by uridine diphosphoglucuronosyltransferases (UGTs; Enzyme Classification E.C. 2.4.1.17) and uses uridine-5'-diphosphoglucuronic acid (UDPGA) as the co-substrate. The reaction leads to the attachment of the polar sugar moiety to the steroid structure with the immediate inversion of the configuration to yield a β-glycosidic bond (Figure 3). As a result, these metabolic reactions in most cases terminate the activity of xenobiotics and endobiotics. Some notable exceptions exist (Ritter, 2000); morphine-6-*O*-glucuronide (Paul *et al.*, 1989) and the D-ring glucuronide conjugates of 17β-hydroxy estrogens, testosterone, and dihydrotestosterone, for example, are more toxic than the original compounds (Vore and Slikker, 1985). The main site of glucuronidation is the liver, although extra-hepatic glucuronidation has been observed in kidney, intestines, lung, and prostate (Bélanger *et al.*, 1998; Hum *et al.*, 1999; Tukey and Strassburg, 2000).

17α-methyl-5β-androstane-3α,17β-diol + Uridine 5'-diphosphoglucuronic acid (UDPGA) → (UGT) 17α-methyl-5β-androstan-17β-ol-3α-O-β-glucuronide + UDP + $H_2O$

***Figure 3.*** *A UGT-catalyzed glucuronidation reaction between 17α-methyl-5β-androstane-3α,17β-diol (5β-MT) and uridine-5'-diphosphoglucuronic acid (UDPGA).*

UGTs are a family of enzymes bound in the membrane of the endoplasmic reticulum, which catalyze the glucuronidation of various endogenous and exogenous compounds, including steroids (Mackenzie *et al.*, 1997). At least 16 different UGTs, ranging from 526 to 533 amino acids in size, are encoded by the human genome. The highly homologous carboxyl terminal is suggested to contain the domain critical for catalysis and for binding of UDPGA, whereas the amino terminal is responsible for the substrate specificity (Mackenzie, 1990). The expressed UGT proteins have been categorized into two families (UGT1 and UGT2) on the basis of the protein sequence similarity (Figure 4), which is higher than 38% within a single family (Tukey and Strassburg, 2000;2001). According to the sequence homology, the enzyme families are further divided into subfamilies (Burchell *et al.*, 1991; Mackenzie *et al.*, 1997). The most important enzymes involved in steroid glucuronidation are members of subfamilies UGT1A and UGT2B (Hum *et al.*, 1999).

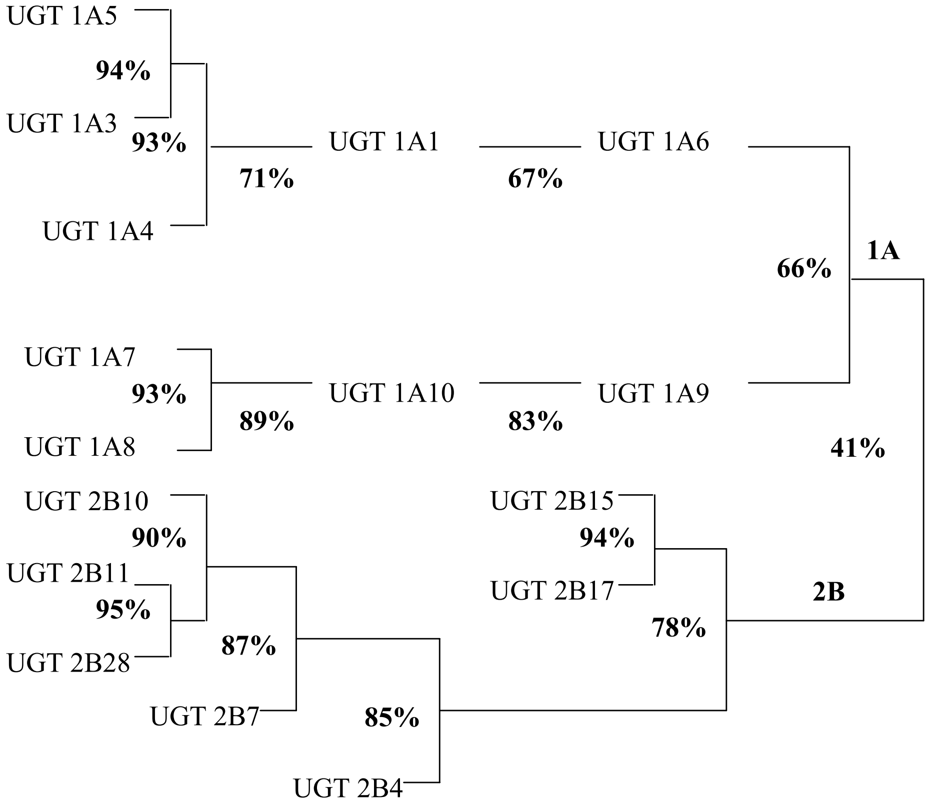



***Figure 4.*** *Sequence similarity of human UGT isoenzymes (according to Tukey and Strassburg, 2000; Lévesque et al., 2001).*

An earlier approach to the detection of human UGT specificity was the isolation and purification of the enzymes from hepatic microsomes by chromatofocusing (Irshaid and Tephly, 1986). With the development of cDNA cloning and expression techniques, the availability of recombinant isoenzymes has expanded the characterization of enzyme activities. Several human UGT proteins have been cloned and studied to characterize steroidal substrate specificity of the isoenzymes, the main focus of the research being on the glucuronidation of clinically important endogenous steroids, such as testosterone, 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol, and androsterone (Figure 5). The latter three compounds are testosterone metabolites, easily converted to each other in the liver, and their glucuronidation has an influence, therefore, on the level of these hydroxysteroids in the body (Jin *et al.*, 1997; Bélanger *et al.*, 1998). For example, the level of glucuronide-conjugated androgen metabolites in serum has been suggested to correlate with the total pool of androgens in men (Labrie *et al.*, 1997).

The data relevant to the activity and specificity of UGT enzymes toward androgens has come from several laboratories using different experimental conditions and analytical methods, which means that occasionally the data are inconsistent and do not easily support conclusions. A rough overview of the role of UGT isoenzymes in human androgen glucuronidation, with related references, is nevertheless presented in Table 1. According to earlier investigations,

human UGT1 proteins are actively involved in the glucuronidation of steroids, especially of the steroids having C18 structure (Hum *et al.*, 1999). In addition, conjugation capability toward C19 steroids has been reported for UGT1A3, UGT1A4, UGT1A8, and UGT1A10; however, their activities are higher for estrogens and catechol estrogens.

***Figure 5.*** *Interrelation of metabolism of A) testosterone, B) 5α-dihydrotestosterone, C) 5α-androstane-3α,17β-diol, D) androstenedione, E) 5α-androstanedione, and F) androsterone (according to Rittmaster et al., 1988). Abbreviations: 5α-R=5α-reductase, 17-KSR=17-ketosteroid reductase, 3-KSR=3-ketosteroid reductase.*

Members of the UGT2B subfamily are well known for their ability to glucuronidate hydroxysteroids, showing interesting evidence of the regio- and stereoselective conjugation of endogenous androgens and pregnanes (Jin *et al.*, 1997). Isoenzyme UGT2B4 exhibits reactivity toward 5α-reduced androgens, e.g. 5α-androstane-3α,17β-diol (5α-A) and androsterone (A), although at a significantly lower level than UGT2B7, 2B15, or 2B17 (Turgeon *et al.*, 2001). UGT2B7, probably the most widely examined isoenzyme with respect to androgen glucuronidation, has been found capable of conjugating several endogenous hydroxysteroids with 3-hydroxyl and/or 17-hydroxyl structure, and demonstrates more efficient glucuronidation of 5α-A than any other human UGT2B isoform (Turgeon *et al.*, 2001). So far the only member without any reported androgen substrate is UGT2B10. Isoenzyme UGT2B11, which is closely similar to UGT2B10, has also been inactive toward androsterone and testosterone (Jin *et al.*, 1993; Lévesque *et al.*, 2001), but it has been found active toward the 3α-hydroxyl structure of 5α-A (Jin *et al.*, 1997). UGT2B15 is suggested to prefer 17β-conjugation, as well as for 5α-androstane compounds over the corresponding 5β-

structures (Chen *et al.*, 1993; Green *et al.*, 1994). The last two isoenzymes of the UGT2B family, UGT2B17 and UGT2B28, have been found capable of glucuronidating several endogenous C19 steroids at the hydroxyl group at both 3α- and 17β-position (Beaulieu *et al.*, 1996; Lévesque *et al.*, 2001).

***Table 1.*** *UGT isoenzyme tissue distribution and examples of experiments carried out with androgens. See nomenclature in Table 3. n.d. = not detected, * = and several other hydroxysteroids.*

| Enzyme | Tissue distribution | Reported androgen substrates | References |
|---|---|---|---|
| UGT1A1 | Liver, bile ducts, stomach, colon | n.d. | King *et al.* , 1996<br>Strassburg *et al.* , 1998 |
| UGT1A3 | Liver, bile ducts, stomach, colon | n.d. (Green *et al.* , 1998)<br>A | Mojarrabi *et al.* , 1996;<br>Green *et al.* , 1998;<br>Gall *et al.* , 1999 |
| UGT1A4 | Liver, bile ducts, colon | 5α-A | Green *et al.* , 1996; |
| UGT1A5 | ND | n.d. | Tukey and Strassburg, 2000 |
| UGT1A6 | Liver, bile ducts, stomach, colon, brain | n.d. | Tukey and Strassburg, 2000 |
| UGT1A7 | Esophagus, stomach | n.d. | Strassburg *et al.* , 1998<br>Tukey and Strassburg, 2000 |
| UGT1A8 | Esophagus, ileum, jejunum, colon | n.d. (Strassburg *et al.* , 1998)<br>5α-DHT, ET, T | Strassburg *et al* ., 1998<br>Cheng *et al.* , 1999 |
| UGT1A9 | Liver, colon, kidney | n.d. | Tukey and Strassburg, 2000 |
| UGT1A10 | Esophagus, stomach, bile ducts, intestine, colon | 5α-DHT, A | Strassburg *et al.* , 1998<br>Cheng *et al.* , 1999<br>Tukey and Strassburg, 2000 |
| UGT2B4 | Liver, prostate, testis, mammary gland, lung, kidney | 5α-A, A | Hum *et al.* , 1999<br>Lévesque *et al* ., 2001<br>Turgeon *et al.* , 2001 |
| UGT2B7 | Liver, mammary gland, lung, kidney | 5α-A, 5α-DHT, A, ET, T, * | Coffman *et al.* , 1998<br>Gall *et al.* , 1999<br>Lévesque *et al.* , 2001<br>Turgeon *et al.* , 2001 |
| UGT2B10 | Liver, prostate, testis, mammary gland, lung, kidney | n.d. | Jin *et al.* , 1993<br>Hum *et al.* , 1999<br>Lévesque *et al.* , 2001 |
| UGT2B11 | Liver, prostate, mammary gland, lung, kidney | n.d.<br>5α-A (Jin et al., 1997) | Jin *et al.* , 1993;1997<br>Hum *et al.* , 1999<br>Lévesque *et al.* , 2001 |
| UGT2B15 | Liver, prostate, testis, mammary gland, lung, kidney | 5α-A, 5α-DHT, T | Green *et al.* , 1994<br>Hum *et al.* , 1999<br>Lévesque *et al.* , 2001<br>Turgeon *et al.* , 2001 |
| UGT2B17 | Liver, prostate, testis, mammary gland, lung, kidney | 5α-A, 5α-DHT, A, ETCH, T | Beaulieu *et al.* , 1996<br>Hum *et al.* , 1999<br>Lévesque *et al.* , 2001 |
| UGT2B28 | Liver, mammary gland | 3α-diol, A, T | Lévesque *et al.* , 2001 |

The prevalence of genetic polymorphism has been demonstrated for UGT1A1, 1A6, 1A7, 2B4, 2B7 and 2B15, although the functional significance has been undisputedly shown only for UGT1A1 with conjugation of bilirubin (Miners *et al.*, 2002). With androgens, studies have been carried out with two allelic forms of UGT2B7 (H268 and Y268) showing glucuronidation for androsterone (3α-hydroxyl) but not for testosterone (17β-hydroxyl), even though they both were readily conjugated by human liver microsomes within the same study (Gall *et al.*, 1999). The result is in good agreement with that of another study (Coffman *et al.*, 1998), which also reported the conjugation of androsterone and non-conjugation of testosterone with both UGT2B7 isomers. Interestingly, however here they both glucuronidated the 17α-hydroxyl group of epitestosterone. The altered catalytic activity of mutants has been proposed to be of toxicological significance in general glucuronidation (Miners *et al.*, 2002), and the polymorphism has been suggested to offer a plausible explanation for some of the observed ethnic differences in steroid hormone profiles and drug metabolism (Lampe *et al.*, 2000). These suggestions are still without confirmation, however.

## 2.3 MASS SPECTROMETRY IN THE DETECTION OF ANABOLIC STEROIDS

Spectroscopic methods for the trace analysis (i.e. nano and picomolar concentrations) of steroids in biological fluids have been available since the end of the 1960s, gas chromatography with packed columns then being the separation method with highest resolving power (Jaakonmäki *et al.*, 1967; Horning *et al.*, 1968). In the early 1970s, immunoassays were introduced for steroid measurements (Barnard *et al.*, 1995). With the later development of labels, detection systems and automation, steroid immunoassay methods have become of great importance in routine clinical chemistry. Although thin-layer chromatography (TLC) is not applied in human doping control, both TLC and radioautographic techniques are successfully used in the detection of radiolabeled steroid glucuronides in kinetic studies on AAS (Green *et al.*, 1994; Gall *et al.*, 1999). Immunoassays have also been applied as screening methods in doping control of AAS (Catlin *et al.*, 1987). Because of the rapid improvement in sensitivity and specificity, as well as in data processing systems, mass spectrometric (MS) detection of AAS metabolites has nevertheless replaced almost all other methods in doping control laboratories, for both screening and confirmatory analysis (Gower *et al.*, 1995).

Analysis of ions as a function of their mass-to-charge ratio (m/z) gives MS its unique power in identification, the specificity of the technique often being compared to human fingerprints (McLafferty and Lory, 1981), especially when tandem mass spectrometric methods are applied (McLafferty, 1981). In MS measurements the compounds pass through two or three stages, namely 1) chromatographic separation in the case of a mixture of analytes, 2) ionization, and 3) analysis of the produced ions according to their m/z values. There are several options for each stage, and the combination of options chosen will depend on the analytes, as well as on the requirements for the analysis (e.g. high resolution for accurate mass measurements). The significance of chromatographic separation is diminishing with the

recent arrival of the high-throughput applications, and the provision of specificity is being transferred to the MS analysis. Most often, however, gas chromatography (GC) or liquid chromatography (LC) is still applied for the separation of analytes.

### *2.3.1 Gas chromatography–mass spectrometry (GC–MS)*

The analysis of AAS metabolites in urine has conventionally been carried out by electron impact (EI) and GC–MS based methods for the total fraction of the steroids, i.e. both free and conjugated fractions (Figure 6). The first step in the procedure is solid-phase extraction (SPE), which may be carried out, for example, with $C_{18}$ cartridges (Massé *et al.*, 1989) or XAD-2 resin (Schänzer and Donike, 1993). This provides the preliminary purification of the urine sample by removing salts and polar impurities. An additional purification step, liquid–liquid extraction (LLE), is often carried out with diethyl ether (Ayotte *et al.*, 1996), pentane, or *tert.*-butyl methyl ether (Schänzer *et al.*, 1996). Specific antibody–antigen binding properties of immunoaffinity chromatography (IAC) have also been exploited for the isolation of AAS in urine (van Ginkel, 1991), especially in confirmatory analysis (Schänzer *et al.*, 1996).

The analysis of a conjugate fraction is indirect, since the glucuronide conjugates (G) and sulfate-conjugated (S) steroids are hydrolyzed enzymatically (G,S) or chemically (S), or via methanolysis (G,S) before further stages of the procedure (Sample and Baezinger, 1989; Massé *et al.*, 1989; Tang and Crone, 1989). Non-volatile compounds such as AAS metabolites are not amenable to GC separation as such, and the hydrolyzed analytes are most often modified to trimethylsilyl (TMS) derivatives (Chambaz and Horning, 1969;Donike and Zimmermann, 1980;Donike *et al.*, 1984). Recently, TMS derivatization and GC–MS analysis has been applied for the characterization of chemically synthesized intact AAS glucuronides to be used as pure reference compounds (Thevis *et al.*, 2001a;b) and for the characterization of endogenous androgen glucuronides in human urine (Choi *et al.*, 2000).

In general terms, the GC–MS methods in AAS analysis are sensitive and robust. However, the multi-staged procedure is tedious; especially the enzymatic hydrolysis step (Figure 6). Moreover, some problems may arise in the hydrolysis step, as the competitive or non-competitive inhibition of the enzyme may lead to incomplete hydrolysis in urine matrix (Bowers and Sanaullah, 1996), and, in certain cases, contaminants in the enzyme preparation may lead to the conversion of steroid structures (Messeri *et al.*, 1984). These potential problems of GC–MS make the development of alternative methods, such as direct measurement of AAS conjugates by LC–MS, highly attractive.

*2.3.2 Liquid chromatography–mass spectrometry (LC–MS)*

The lack of chromophores and fluorophores in AAS glucuronide structures prevents the use of UV and fluorescence detectors, the standard detectors in LC. In this respect, the development of instrumentation providing interfacing of LC to MS, has opened up broad new possibilities for the direct analysis of thermolabile, non-volatile, bulky, and polar compounds, such as the AAS glucuronides. By means of LC–MS also the simultaneous detection of the total steroid fraction (i.e. free, sulfate- and glucuronide-conjugated AAS) becomes possible. Negative ion desorption chemical ionization, by applying ethanolic solution to a Pt wire, was applied for underivatized steroid glucuronides as long ago as 1981 (Bruins, 1981). Immediately after the introduction of this technique, moving belt (Alcock *et al.*, 1982) and fast atom bombardment (Cole *et al.*, 1987; Gaskell, 1988; Tomer and Gross, 1988) were presented for the ionization of steroid glucuronides.

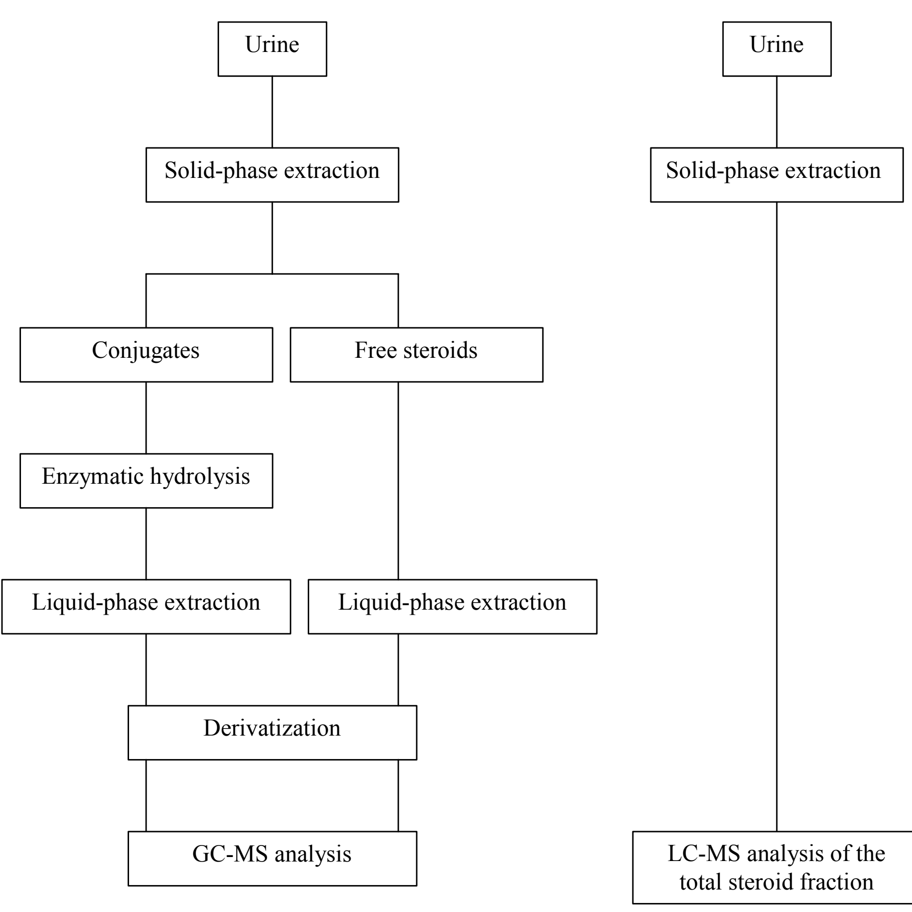



***Figure 6.*** *Comparison of principles of gas chromatographic–mass spectrometric (GC–MS) and liquid chromatographic–mass spectrometric (LC–MS) analysis.*

The introduction of atmospheric pressure ionization (API) techniques enabled the effective breakthrough of LC–MS methods. Atmospheric pressure chemical ionization (APCI; Mück and Henion, 1990; Sjöberg and Markides, 1998; Joos and Van Ryckeghem, 1999; Draisci *et al.*, 2001), atmospheric pressure photoionization (APPI; Robb *et al.*, 2000), electrospray (ESI; Bowers and Sanaullah, 1996; Sanaullah and Bowers, 1996; Bean and Henion, 1997; Draisci *et*

*al.*, 1997; Williams *et al.*, 1999; Borts and Bowers, 2000; Que *et al.*, 2000; Nielen *et al.*, 2001; Leinonen *et al.*, 2002; Van Poucke and Van Peteghem, 2002), sonic spray (SSI; Jia *et al.*, 2001), and thermospray ionization (TSI; Watson *et al.*, 1986; Liberato *et al.*, 1987) have been applied in the detection of free as well as glucuronide- and sulfate-conjugated AAS in pharmaceutical preparations and biological matrixes.

Electrospray ionization (ESI) has been the method of choice for AAS glucuronides. In ESI the ions are transferred from the charged initial droplets to gas phase, either directly by evaporation from the small droplets near the Rayleigh limit (Iribarne *et al.*, 1976) or through consecutive steps of coulombic fission, which eventually lead to the formation of droplets containing only one ion (Schmelzeisen-Redeker *et al.*, 1989) (Figure 7). ESI is characterized as a soft ionization process where there is little if any addition of internal energy to the ions (Kebarle and Tang 1993). Analytes are typically observed as protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecules, in positive or negative ion ESI, respectively, which has been demonstrated for AAS glucuronides (Bowers and Sanaullah, 1996; Bean and Henion, 1997; Borts and Bowers, 2000). In addition to AAS analysis, ESI-based LC–MS methods have become widespread in forensic science and biochemical and pharmaceutical analysis (Henion *et al.*, 1993; Maurer, 1998; Niessen, 1999; Bogusz, 2000; Griffiths *et al.*, 2001).

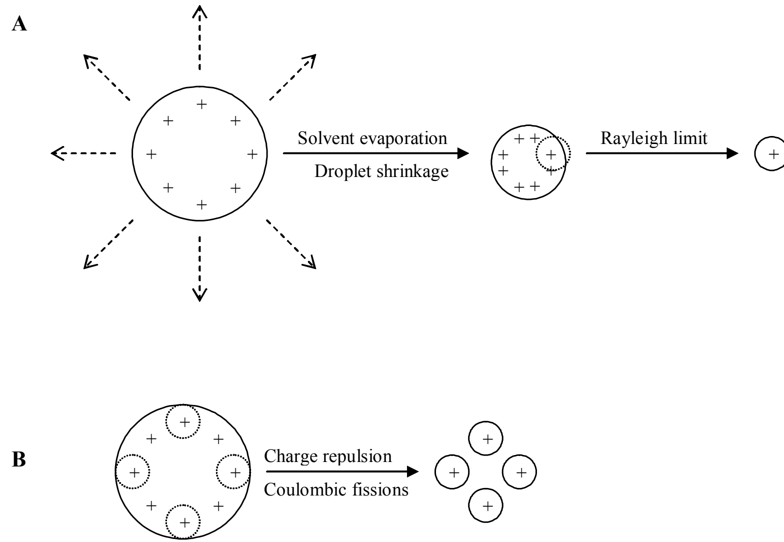



***Figure 7.*** *Schematic picture of the formation of gas phase ions in electrospray ionization (ESI) according to A) ion evaporation theory and B) charge residue theory.*

Conjugated reference material is needed for the analysis of intact steroid glucuronides by LC–MS, but for exogenous AAS in particular only a few conjugates are commercially available. Several chemical syntheses have been described for steroid glucuronides (Conrow and Bernstein, 1971; Chung *et al.*, 1992; Hadd, 1994; Sanaullah and Bowers, 1996; Stachulski and Jenkins, 1998; Thevis *et al.*, 2001a;b). These classical syntheses produce AAS glucuronides in milligram amounts, but the potential formation of the corresponding α-anomers and other side-products is a problem, so that further purification is required for the isolation of the desired isomer (Conrow and Bernstein, 1971).

An alternative to the classical chemical syntheses is the enzymatically driven pathway, using tissue preparations or recombinant isoenzymes as the source of the catalyzing UGT enzymes for glucuronidation. Given the high specificity of UGTs, the strength of the enzyme-assisted synthesis lies in the formation of a stereochemically pure product (Mackenzie *et al.*, 1992). Enzymatically driven syntheses have been demonstrated, for example, for the production of glucuronide-conjugated androsterone, androstanediol, dihydrotestosterone (Rittmaster *et al.*, 1989), epitestosterone (Falany and Tephly, 1983), and testosterone (Rao *et al.*, 1976; Numazawa *et al.*, 1977).

## 3. AIMS OF THE STUDY

- The main goal of the study was to optimize an enzyme-assisted synthesis procedure, and to produce milligrams of glucuronide-conjugated metabolites of the most widely misused anabolic androgenic steroids (AAS). The syntheses were carried out with hepatic UGT enzymes obtained from the microsomal fraction of induced Wistar rats. In addition to this larger-scale production of the conjugates, the AAS glucuronidation reaction was examined *in vitro* with a set of 11 recombinant human UGT isoenzymes, as well as with human liver microsomes, in order to characterize the potential regio- and stereoselectivity and substrate specificity of UGTs.

- In the analytical part of the study, the structures of the synthesized substances were characterized, and the conjugates were utilized in the development of a liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method. LC separation and MS detection steps were optimized for the determination of intact AAS glucuronides in enzyme-kinetic assays. When the LC–MS/MS method was implemented in the analysis of AAS glucuronides in human urine, impurities in the samples required the adaptation of a relatively new sample purification and concentration method, liquid-phase microextraction (LPME).

# 4. MATERIALS AND METHODS

Only the major experimental features are described in this section. More detailed descriptions can be found in the original publications **I–IV**.

## 4.1 REAGENTS

The detailed list of reagents, solvents, and gases of the study is presented in Table 2. For MS work all the glassware was flushed with 5% nitric acid and rinsed with ion-exchanged water (Millipore, Milli-Q Plus, France).

***Table 2.*** *Solid reagents, solvents, and gases of the study, with their quality and source.*

| **Reagent** | **Quality** | **Producer** | **Country** |
|---|---|---|---|
| Ammonium acetate | Pro analysi | Merck | Germany |
| Disodium hydrogen phosphate dihydrate | Pro analysi | Merck | Germany |
| Magnesiumchlorid-6-hydrat | Pro analysi | Riedel-de Haën | Germany |
| Potassium chloride | Pro analysi | Riedel-de Haën | Germany |
| Potassium dihydrogen phosphate | Pro analysi | Merck | Germany |
| Saccharic acid 1,4-lactone | Desiccate | Sigma | MO, USA |
| Uridine-5’-diphospho-glucuronic acid | Disodium salt | Sigma | MO, USA |
| **Solvent** | **Quality** | **Producer** | **Country** |
| Acetic acid | HPLC grade | Rathburn | Scotland |
| Acetone-$d_6$ | 99.50 % | Aldrich | WI, USA |
| Acetonitrile | HPLC grade | Rathburn | Scotland |
| Dichloromethane | HPLC grade | Baker | The Netherlands |
| Diethyl ether | Analytical grade | Riedel-de Haën | Germany |
| Ethyl acetate | Pro analysi | Merck | Germany |
| Formic acid | Analytical grade | Riedel-de Haën | Germany |
| Methanol | HPLC grade | Baker | The Netherlands |
| Methanol-$d_4$ | 99.50 % | Acros Organics | Belgium |
| *n* -Octanol | Extrapure | Merck | Germany |
| 2-Octanone | Purum | Fluka | Germany |
| Pentylacetate | Pro analysi | Fluka | Germany |
| Perchloric acid | Pro analysi | Merck | Germany |
| Water | Milli-Q Plus | Millipore | France |
| **Gas** | **Filter system** | **Producer** | **Country** |
| Air | CD-2 | Atlas Copco | Belgium |
| Nitrogen | 75-72 nitrogen generator | Whatman | MA, USA |

## 4.2 STEROIDS, STEROID GLUCURONIDES AND URINE SAMPLES

The AAS glucuronides in the study consisted mainly of phase-I modified steroid metabolites, but some parent compounds were also included. The structures, nomenclature, and sources of the AAS glucuronides and the sources of steroid aglycones used as precursors in enzyme-assisted syntheses are presented in Table 3. Preliminary LC–MS/MS work was done with pure reference compounds, which were spiked in a solvent system consisting of 15 mM ammonium acetate in water–acetonitrile (50–50, V/V). For the positive ion mode ionization the pH was adjusted to 4.2 with formic acid. Further LC–MS/MS method development was performed with spiked urine samples; for these, drug-free male and female pools of spot urine samples were obtained as a generous gift from United Laboratories Ltd. (Helsinki, Finland).

For the synthesis of the internal standard 5β-LMTG (17α-$CD_3$-labeled structural analogue of 5β-MTG), the deuterium-labeled steroid aglycone was synthesized by chemical method (Shinohara *et al.*, 1984) and then conjugated via enzyme-assisted reaction identically with 5β-MTG. The resulting $d_3$-labeled glucuronide conjugate was applied as internal standard in all LC–MS/MS experiments.

## 4.3 METHODS

### *4.3.1 Enzyme-assisted synthesis of steroid glucuronides*

Liver microsomes were prepared from Aroclor 1254 induced (a single dose of 500 mg/4.5 ml olive oil/body weight) male Wistar rats (n=5) at the Department of Industrial Hygiene and Toxicology (Finnish Institute of Occupational Health, Helsinki, Finland) according to a previously described procedure (Luukkanen *et al.*, 1997). The treatment of the animals was approved by the local ethical committee for animal studies. Specific UGT activity of the preparation was not measured, but a commercial BCA protein assay kit (Pierce, IL, USA) was used for the determination of protein concentration, which was used to standardize the amount of microsomal enzymes in the syntheses (**I**).

The incubation matrix was 50 mM phosphate buffer (pH 7.4) with 5 mM $MgCl_2$. Because of the risk of bond-breaking enzymes in the tissue preparate, constant concentration (5 mM) of saccharic acid 1,4-lactone, β-glucuronidase inhibitor was added to the reaction mixture. Optimal concentrations of the steroid substrate (aglycone), uridine-5'-diphospho-glucuronic acid (UDPGA) and microsomal protein were determined in the small-scale incubations (100 μl) within the corresponding ranges of 1–1000 μM, 0.5–10 mM, and 0.1–1.75 mg/ml. The steroid substrate was dissolved in methanol, the amount of which was 10% of the total incubation volume. The reaction was initiated with UDPGA without pre-incubation and carried out in water bath of 37°C for 12–15 hours with continuous magnetic stirring. The reaction was terminated by transferring the incubation mixture to an ice bath, and enzymatic

***Table 3.*** *Structures, nomenclature and sources of steroid glucuronides and sources of the steroid aglycones used as starting material in enzyme-assisted syntheses. * 17α-$CD_3$-labeled analogue of 5β-MTG (internal standard); UH University of Helsinki; DSHS Deutsche Sporthochschule, Cologne, Germany; NARL National Analytical Reference Laboratory; Pymble, Australia; Steraloids, Wilton, NH; where two or more sources are indicated, the sources after the slash (/) are for the steroid aglycones.*

| Abbreviation | Compound | Precursor | Structure | Source |
|---|---|---|---|---|
| 3-OHSTG | 3'-hydroxystanozolol glucuronide | stanozolol | | NARL |
| 5α-1-MEG | 1-methyl-5α-androst-1-en-3-one-17β-*O*-glucuronide | methenolone | | UH / DSHS |
| 5α-AG | 5α-androstane-3α-ol-17β-*O*-glucuronide | testosterone | | UH / DSHS |
| 5α-DHTG | 5α-androstane-3-one-17β-*O*-glucuronide | testosterone | | NARL |
| 5α-DROSTG | 2α-methyl-5α-androstane-17-one-3α-*O*-glucuronide | drostanolone | | NARL |
| 5α-MEG | 1-methylen-5α-androstan-17-one-3α-*O*-glucuronide | methenolone | | UH / DSHS<br>NARL |
| 5α-MESM1G | 1α-methyl-5α-androstane-17-one-3α-*O*-glucuronide | mesterolone | | NARL |
| 5α-MESM2G | 1α-methyl-5α-androstane-17β-ol-3α-*O*-glucuronide | mesterolone | | NARL |
| 5α-MTG | 17α-methyl-5α-androstane-17β-ol-3α-*O*-glucuronide | mestanolone<br>methyltestosterone<br>oxymetholone | | UH / DSHS; Steraloids<br>NARL |
| 5α-NG | 5α-estran-17-one-3α-*O*-glucuronide | nandrolone | | UH / DSHS; Steraloids<br>NARL |
| 5β-BOLDG | 5β-androst-1-en-3-one-17β-*O*-glucuronide | boldenone | | NARL |

***Table 3.*** *See the previous page for the title of the table.*

| Abbreviation | Compound | Precursor | Structure | Source |
|---|---|---|---|---|
| 5β-EPIMG | 17β-methyl-5β-androst-1-ene-17α-ol-3α-*O*-glucuronide | metandienone | | UH /DSHS |
| 5β-MTG<br>5β-LMTG* | 17α-methyl-5β-androstane-17β-ol-3α-*O*-glucuronide | metandienone<br>methandriol<br>methyltestosterone | | UH / UH; Steraloids |
| 5β-NG | 5β-estran-17-one-3α-*O*-glucuronide | nandrolone | | UH / Steraloids<br>NARL |
| 7α-BOLAG | 7α,17α-dimethyl-5β-androstane-17β-ol-3α-*O*-glucuronide | bolasterone | | NARL |
| 7β-CALUG | 7β,17α-dimethyl-5β-androstane-17β-ol-3α-*O*-glucuronide | calusterone | | NARL |
| AG | 5α-androstane-17-one-3α-*O*-glucuronide | androsterone | | SIGMA / NARL |
| ETCG | 5β-androstane-17-one-3α-*O*-glucuronide | etiocholanolone | | NARL |
| ETG | 4-androsten-3-one-17α-*O*-glucuronide | epitestosterone | | NARL |
| MTG | 17α-methyl-4-androsten-3-one-17β-*O*-glucuronide | methyltestosterone | | UH / DSHS |
| NG | estr-4-en-3-one-17β-*O*-glucuronide | nandrolone | | UH / DSHS; Diosynth |
| TG | 4-androsten-3-one-17β-*O*-glucuronide | testosterone | | UH / Makor Chemicals<br>NARL |

protein was precipitated with dichloromethane, which also extracted the excess of starting material. The aqueous phase was removed and further purified in solid-phase extraction (SPE) with $C_{18}$ cartridge (Figure 8). The AAS glucuronide fraction of SPE was evaporated to dryness in 60°C with nitrogen. The organic layer containing the excess of AAS aglycone was filtered (HV 0.45 µm, Millipore, USA), evaporated to dryness in a rotavapor system, and finally applied in the prospective AAS glucuronide synthesis.

Mass spectrometric structure characterization of AAS glucuronides was performed with LC–ESI-MS in positive ion mode and LC–ESI-MS/MS in positive and negative ion modes, according to the methods described in section *4.3.3*. In NMR structure characterization, DQFCOSY, HMQC, and HMBC experiments were carried out with a Varian Unity 500 instrument (**I**) with the AAS glucuronides dissolved in either acetone-$d_6$ or methanol-$d_4$.

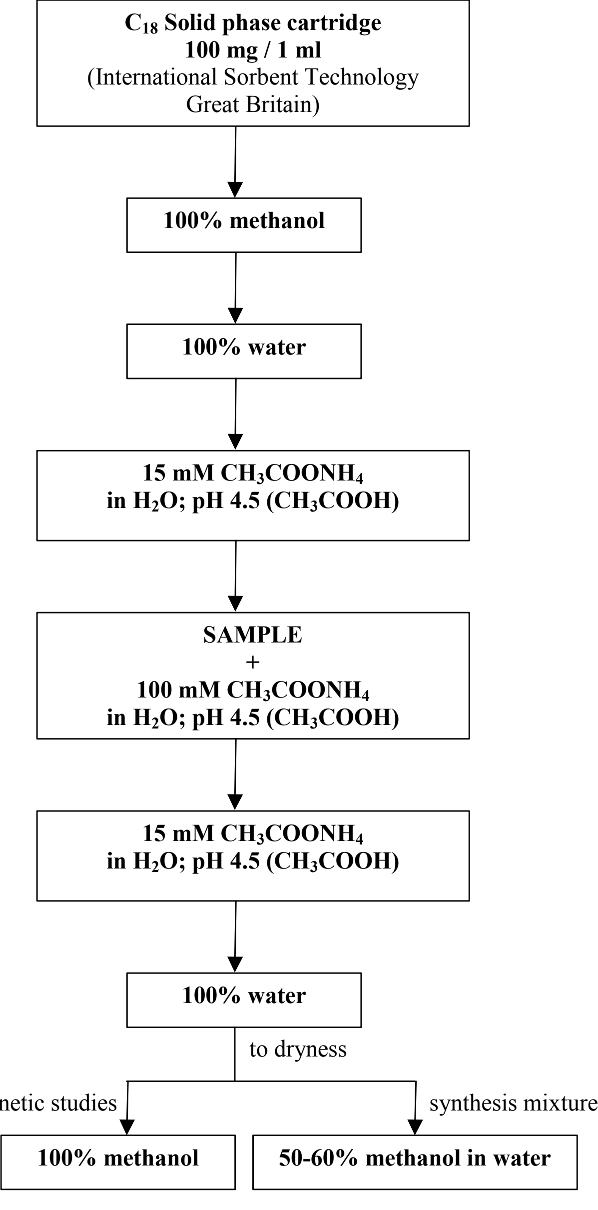



***Figure 8.*** *Flowchart of solid-phase extraction procedures for AAS glucuronide bulk synthesis and kinetic studies.*

*4.3.2 UGT isoenzyme studies*

Enzyme kinetic assays were incubated in a total volume of 100 μl, the matrix consisting of 50 mM phosphate buffer (pH 7.4, 5 mM $MgCl_2$), 5 mM saccharic acid lactone, 5 mM UDPGA, and 0.5 mg/ml enzymatic protein (50 μg per assay). The same conditions were used in the studies with human liver (Gentest, Woburn, MA) and rat liver microsomal homogenates, both of which were pools of five individuals. The rat liver microsomal pool was the same as used for the bulk syntheses and it is described in section *4.3.1*. Human UGTs of the studies were cloned and expressed in baculovirus-infected insect cells and prepared according to the method described by Kurkela *et al.*, 2003. The activity of isoenzymes was confirmed with specific substrates; ethinylestradiol (UGT1A1), scopoletin (1A3, 2B15), 4-aminobiphenyl (1A4), α-naphtole (1A6), entacapone (1A7, 1A8, 1A9, 1A10), estriol (2B4), and morphine (2B7).

Preliminary screening for glucuronidation was performed with a substrate concentration of 50 μM. The glucuronidation reaction was initiated with UDPGA addition, carried out for two hours in a dry bath of 37°C, and finally terminated by precipitating the enzymatic protein with 10 μl of 4 M perchloric acid. After centrifugation, the internal standard (5β-LMTG) was added to the supernatant, which was purified by SPE (Figure 8). The final eluate was evaporated to dryness and dissolved in LC–ESI-MS/MS buffer. For those isoenzymes that showed activity in the screening, the apparent Michaëlis-Menten constant, $K_m$, was determined using substrate concentration levels of 1, 2.5, 5, 10, 25, 50, 100, 250, 500, and 1000 μM. The peak areas of one specific ion of the analyte and one of the internal standard were plotted, $v = A_{(ANAL)}/A_{(ISTD)}$, to obtain the variable (v) for calculations of $K_m$. Calculations were done with Leonora Michaëlis-Menten software (Cornish-Bowden, version 1.0) with the least absolutes method. The structure-specific ions of the AAS glucuronides are presented in section *5.3.2*.

*4.3.3 LC–ESI-MS/MS analysis of steroid glucuronides*

Initial characterization of the mass spectrometric (MS) and tandem mass spectrometric (MS/MS) behavior of the AAS glucuronides was done with direct infusion of the reference compounds to the ESI ion source (**III**). The samples were dissolved in an eluent consisting of 7.5 mM ammonium acetate in water–methanol (50–50, V/V). In negative ion mode the eluent was neutral, whereas in positive ion mode the pH was adjusted to 4.2 with formic acid.

The optimized LC method, which was applied both in enzyme kinetic studies and in the detection of AAS glucuronides in urine, utilized acetonitrile–water gradient and 15 mM ammonium acetate buffer. The pH of the eluent was adjusted to 4.2 with formic acid. An endcapped $C_{18}$ column (Purospher RP-18e, 125 mm x 3.0 mm i.d., particle size 5 μm, Merck, Germany) was used for the chromatographic separation and a guard column of the same phase

material was also employed. The solvent flow rate of 0.5 ml/min was post-column split (Acurate, LC-Packings, The Netherlands) in the ratio of 1:10 before it was introduced to the turbo ionspray (TIS) ion source (**IV**).

MS and MS/MS analyses were performed both in negative and in positive ion mode. The measurements were carried out mainly with the triple quadrupole MS, but the additional data of fragmentation were obtained from the experiments with the ion trap MS. Instrument details are given in Table 4. Although ESI ionization was used in the final application, the suitability of APCI was examined for eight AAS glucuronides (**III**). The fragmentation of the AAS glucuronides was studied in positive and negative ion mode MS/MS to identify structure-specific AAS glucuronide fragments suitable for multiple reaction monitoring (MRM) in the LC–ESI-MS/MS method. Collision offset voltages were ramped within the range of 5–60 V, in the steps of 5 V.

In the development of the LC–ESI-MS/MS method, a relatively new sample purification and concentration method, liquid-phase microextraction (LPME), was developed for urinary AAS glucuronides. This was finally compared with the modified LLE (Thevis *et al.*, 2001a) and standard SPE procedures (Borts and Bowers, 2000). LPME optimization and the details of the LLE and SPE procedures are described in paper **IV**.

***Table 4.*** *Description of the liquid chromatography–mass spectrometry (LC–MS) instrumentation applied in the study. Atmospheric pressure chemical ionization (APCI); atmospheric pressure photoionization (APPI); electrospray ionization (ESI); turbo ionspray (TIS).*

| **LIQUID CHROMATOGRAPHS** | | | | | |
|---|---|---|---|---|---|
| Abbreviation | Type | Producer | Country | Autosampler | Pump |
| LC1 | Series 1100 | Agilent | Germany | G1367A | G1312A binary |
| LC2 | Series 1100 | Agilent | Germany | G1313A | G1312A binary |
| LC3 | LC 200 micro | Perkin Elmer | USA | Series 200 | Series 200 micro |
| **MASS SPECTROMETERS** | | | | | |
| Abbreviation | Type | Producer | Country | Analyzer type | Ion sources |
| MS1 | API 300 | PE Sciex | Canada | triple quadrupole | ESI, APCI |
| MS2 | API 3000 | Applied Biosystems | Canada | triple quadrupole | ESI, TIS, APPI |
| MS3 | Esquire | Bruker | Germany | LC ion trap | ESI |

## 5. RESULTS AND DISCUSSION

The main results obtained in this work are shortly described in this section. More details can be found in the original publications **I–IV**.

### 5.1 ENZYME-ASSISTED SYNTHESIS OF AAS GLUCURONIDES

Optimization of AAS glucuronide synthesis was carried out in small-scale studies, with a total incubation volume of 100 µl. Glucuronidation activity was followed as a function of steroid substrate concentration, UDPGA concentration, protein amount, and incubation time (Figure 9). Rat liver microsomes were found to be capable of conjugating all the tested steroid aglycones, and only slight differences were detected in the optimal conditions for the various substrates (Table 5). Substrate inhibition was not observed for any of the steroids within the concentration range of 1–1000 µM, but a plateau of glucuronidation was reached at 500–1000 µM, except for 5α-NG, for which it was 250 µM. The first point of the plateau was selected as the substrate concentration for the further synthesis. The most probable reason for the limited formation of AAS glucuronide was the sparing solubility of the non-polar steroid substrate in phosphate buffer. The amount of organic modifier, which was methanol, was limited to approximately 10% because of potential precipitation of the enzymatic protein.

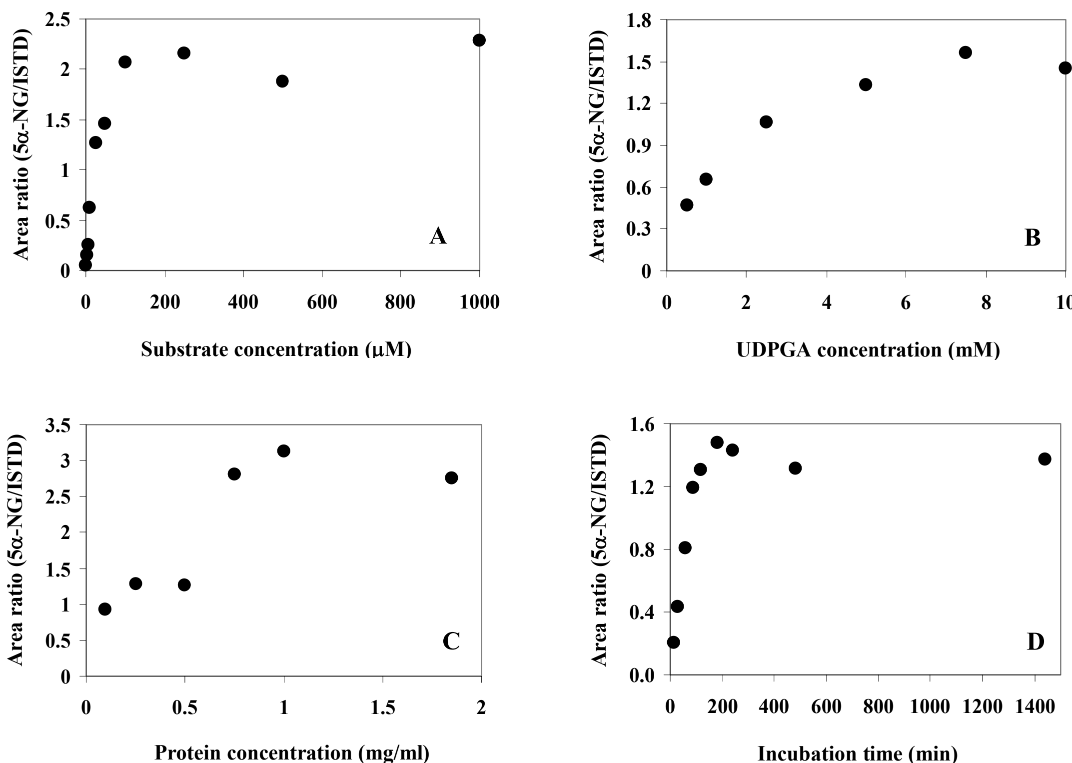


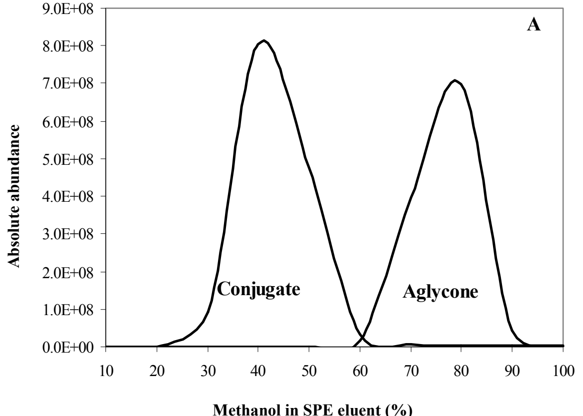

***Figure 9.*** *Optimization of conditions of AAS glucuronide synthesis for 5α-NG. Formation of the conjugate as function of A) substrate concentration, B) UDPGA concentration, C) microsomal protein concentration, and D) incubation time. Constant conditions: 50 µM substrate,5 mM UDPGA, 0.5 mg/ml protein, and 2 hrs incubation time.*

Optimal concentration of the co-substrate, UDPGA, was 2.5–5 mM, except for methyltestosterone, which required 7.5 mM UDPGA for maximal production of MTG. The formation of the glucuronide conjugates was linearly proportional to the amount of protein and became optimal when the amount of protein was 0.5–1 mg/ml. The effect of incubation time was examined up to 24 hours. Glucuronidation was linear within the first six hours, but because there was no evidence of breakdown of the AAS glucuronide with longer reaction times, the incubations were carried out overnight (12–15 hours).

***Table 5.*** *Parameters selected for the optimized synthesis of AAS glucuronides. Positions of deuterium label indicated with an asterisk. See Table 3 for nomenclature.*

| Abbreviation | Structure | Substrate (μM) | UDPGA (mM) | Protein (mg/ml) |
|---|---|---|---|---|
| 5α-1-MEG | | 500 | 5 | 1 |
| 5α-AG<br>$d_5$-5α-AG | | 500 | 2.5 | 1 |
| 5α-MEG | | 500 | 2.5 | 0.75 |
| 5α-MTG | | 1000 | 2.5 | 0.5 |
| 5α-NG<br>$d_4$-5α-NG | | 250 | 5 | 1 |
| 5β-EPIMG | | 500 | 5 | 1 |
| 5β-MTG<br>5β-LMTG | | 1000 | 2.5 | 0.5 |
| 5β-NG | | 500 | 5 | 0.75 |
| MTG | | 1000 | 7.5 | 0.5 |
| NG<br>$d_3$-NG | | 500 | 5 | 1 |
| $d_3$-TG | | 500 | 5 | 1 |

The excess starting material (steroid substrate) was removed in liquid–liquid extraction (LLE) with dichloromethane, which also simultaneously precipitated the enzymatic protein and stopped the conjugation reaction. Although LLE enabled the collection and recycling of the precious starting material, some residues were detected in the aqueous glucuronide fraction. An additional solid-phase extraction (SPE) step was therefore attached to the procedure. Retention of the steroid substrate and the glucuronide conjugate was examined as a function of methanol percentage (0-100%) in the SPE eluent in intervals of 10% (Figure 10). The difference between the polarities of the two compounds was sufficient for the complete isolation of the AAS glucuronide when methanol–water (60–40, V/V) was applied in the final elution.



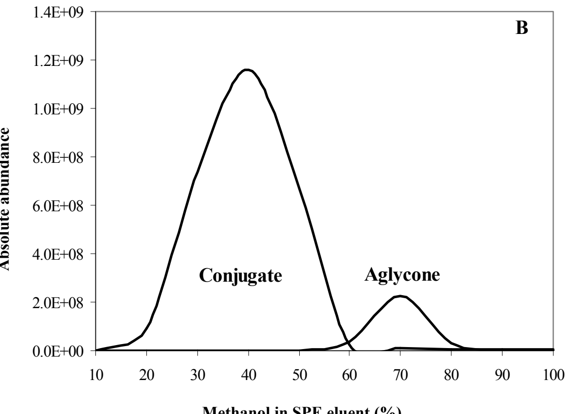




***Figure 10.*** *Elution of AAS glucuronide and non-reacted steroid aglycone from the reaction mixture of 5α-1-MEG (A) and NG (B) as function of methanol concentration in the eluent of solid-phase extraction.*

Fourteen AAS glucuronides, including four deuterium-labeled analogues, were synthesized in milligram amounts (1.3-6.5 mg) with yields of 13-78% (Table 6). A significant difference was observed between the yields of NG, $d_3$-NG, and $d_3$-TG, which are 4-ene-3-one structured substrates, and the yields of other substrates, the former showing superior yields of 77-78% and the latter yields of 13-28%.

***Table 6.*** *List of synthesized steroid glucuronides and their yields.*

| Compound | Yield (mg) | Yield (%) | Compound | Yield (mg) | Yield (%) |
|---|---|---|---|---|---|
| 5α-1-MEG | 1.7 | 24 | 5β-EPIMG | 1.4 | 25 |
| 5α-AG | 1.9 | 16 | 5β-MTG | 2.6 | 26 |
| $d_5$-5α-AG | 2.1 | 26 | 5β-NG | 1.1 | 13 |
| 5α-MEG | 2.5 | 28 | MTG | 1.2 | 14 |
| 5α-MTG | 2.1 | 22 | NG | 3.5 | 77 |
| 5α-NG | 1.3 | 16 | $d_3$-NG | 4.4 | 78 |
| $d_4$-5α-NG | 1.7 | 18 | $d_3$-TG | 6.5 | 77 |

Although methyltestosterone also has 4-ene-3-one structure, the yield of MTG was exceptionally low, 14%. The only possible conjugation site of methyltestosterone is the sterically hindered 17β-hydroxyl group, which has earlier been proposed to inhibit the catalysis of the bulky UGT enzymes. According to Schänzer, 1996, there is evidence of 17β-glucuronidation for metandienone, fluoxymesterone, and 4-chloro-1,2-dehydro-17α-methyltestosterone, though detailed results have not been published. On the basis of the formation of MTG, but in low yield, it is proposed here that the 17α-methyl-17β-hydroxyl structure, while not favorable for glucuronidation, nevertheless, allows some conjugation in the absence of a more favorable hydroxyl group. The formation of 5β-EPIMG supports the suggestion, as conjugation occurs with the 3α-hydroxyl group rather than with the epimeric 17β-methyl-17α-hydroxyl structure. The syntheses with the rat liver microsomal preparation were not significantly affected by the orientation of the 5α/β-proton, because the yields of isomeric pairs 5α-NG and 5β-NG, as well as of 5α-MTG and 5β-MTG were closely identical.

Characterization of the AAS glucuronides was carried out by mass spectrometric (MS; see section *5.3.2*) and nuclear magnetic resonance (NMR) analyses, which confirmed the structures of the synthesized conjugates (**I**). The substrates with two hydroxyl groups, and thus two potential sites for conjugation, were of special interest in the NMR studies. Substrates having both a tertiary 17-hydroxyl group (either 17α- or 17β-oriented) and a 3α-hydroxyl group, that is, aglycones 5β-EPIM, 5α-MT, and 5β-MT, formed 3-*O*-glucuronides, whereas 5α-A, with a secondary 17β-hydroxyl group and a 3α-hydroxyl group, formed 17-*O*-glucuronide.

Systematic stability testing of the AAS glucuronides was not carried out, but methylation of the glucuronic acid group was observed after a two-week period in acidic methanolic solution. A follow-up of our synthesis products showed that the storage either as dry substance or in neutral aprotic environment, such as in acetonitrile, is sufficient to prevent the degradation of the AAS glucuronides at least during six months.

The absolute amounts of AAS glucuronides produced in the enzyme-assisted synthesis were relatively small and the procedure was carried out completely manually. For LC–MS/MS method development purposes the amount of reference material was clearly sufficient, enabling thousands of injections. The procedure would hardly be suitable, however, for scaling up or commercializing of the enzymatic synthesis. Although a larger volume of reaction mixture would provide more of the synthesis product, the higher consumption of UDPGA would increase the cost of the synthesis dramatically. One approach to enhancing the production would be to screen for microsomal UGT enzymes in other rat races or animal species. The use of liver preparations for bulk synthesis would require sacrifice of many animals, and run into severe ethical problems. Probably, however, the most critical point in the method development is to increase the solubility of the steroid aglycone in the reaction mixture without damaging the UGT enzymes. It would be sensible to explore the use of additives such as phospholipids, bovine serum albumin, and cyclodextrins rather than

applying a high concentration of organic solvent, although the addition of new components to the reaction would demand a careful re-evaluation of the purification procedure. A higher level of automation could be achieved by applying preparative or semi-preparative HPLC fractioning to the purification of the AAS glucuronides.

## 5.2 SUBSTRATE SPECIFICITY OF RECOMBINANT UGT ISOENZYMES

The activity of 11 human recombinant UGTs toward 11 AAS substrates was examined to determine the possible of regio- or stereoselectivity of the UGTs (Table 7). The structures of the AAS substrates in the study were closely related, and the only potential sites of conjugation were the 3α-, 17α-, and/or 17β-hydroxyl group(s) in various combinations. LC–ESI-MS/MS method was used in the detection of the AAS glucuronides that formed. Comparison of the results of these studies with the results obtained with human and rat liver microsomal preparations was made to determine whether the UGT isoenzymes could be used as an *in vitro* model of AAS glucuronidation and in regio- and stereospecific production of AAS glucuronides (**II**).

The activity of isoenzymes was confirmed with known substrates, as described in section *4.3.2*. Methanol, ethanol, and dimethyl sulfoxide (DMSO) were all tested at the levels of 5% and 10% in reaction mixture to ensure the maximal solubility of the non-polar steroid substrate with minimal interference with the enzyme function (Figure 11). From these solvent environments especially ethanol was found to have a crucial effect on the protein, and 10% DMSO was selected for the following screening of the glucuronidation. Similar sensitivity to organic solvent was not detected for liver microsomal preparations.

Among the UGTs, the only isoenzymes without detectable conjugation activity for any of the tested steroid substrates were UGT1A6 and UGT1A7. The behavior of UGT1A10 was exceptional, and it was as an enzyme of its own class, possessing both high substrate selectivity and high activity toward the test compounds. The other the enzymes could be divided into two main groups: group A enzymes (UGT1A1, 1A8, 1A9, and 2B15) with weak but selective glucuronidation activity, and group B enzymes (UGT1A3, 1A4, 2B4, and 2B7) with high activity toward most substrates. With respect to regioselectivity, most UGTs did not show a clear preference in glucuronidation for the 3α- or the 17β-hydroxyl group, as all of the tested enzymes that were active in the formation of 3α-*O*-glucuronides also catalyzed the production of 17β-*O*-glucuronides. Exceptional isoenzymes were UGT1A8, 1A9, and 2B15, which appeared to preferentially catalyze 17β-*O*-glucuronidation. This is in good accordance with the results obtained for UGT2B15 (Chen *et al.*, 1993; Green *et al.*, 1994). Among the AAS glucuronides, 5α-AG was of particular interest because regioselective conjugation has been reported to occur in either the 17β-hydroxyl group (Rittmaster *et al.*, 1988; Beaulieu *et al.*, 1996) or the 3α-hydroxyl group (Jin et *al.*, 1997).

***Table 7.*** *Substrate specificity of UGT isoenzymes toward steroid aglycones. + = Detected formation of glucuronide conjugate (increase of symbol amount highlighting the difference of two decades of peak area); - = No evidence of steroid glucuronide formation; * = Evidence of substrate inhibition; n.d. = not determined.*

| UGT | 5α-N | 5β-N | 5α-ME | MT | 5α-MT | 5β-MT | 5β-EPIM | T | N | 5α-1-ME | 5α-A |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1A1 | + | + | - | - | - | - | - | + | + | - | ++ |
| $K_m$ | | 375 μM | | | | | | 55 μM | 16 μM | | 38 μM |
| 1A3 | + | ++ | ++ | - | + | + | + | ++ | ++ | + | ++ |
| $K_m$ | n.d. | 136 μM | 37 μM | | 12 μM | 12 μM | n.d. | 131 μM | 44 μM | n.d. | 33 μM |
| 1A4 | + | ++ | ++ | - | ++ | ++ | - | + | ++ | ++ | ++ |
| $K_m$ | 4 μM | 55 μM | 36 μM | | 7 μM | 19 μM | | 44 μM | 32 μM | 26 μM | 28 μM |
| 1A6 | - | - | - | - | - | - | - | - | - | - | - |
| $K_m$ | | | | | | | | | | | |
| 1A7 | - | - | - | - | - | - | - | - | - | - | - |
| $K_m$ | | | | | | | | | | | |
| 1A8 | - | - | - | - | - | - | - | + | ++ | + | ++ |
| $K_m$ | | | | | | | | 72 μM | 72 μM | 70 μM | 9 μM |
| 1A9 | - | - | - | - | - | - | - | + | + | + | ++ |
| $K_m$ | | | | | | | | 61 μM | 81 μM | n.d. | 21 μM |
| 1A10 | - | + | - | - | - | - | + | ++ | ++ | ++ | ++ |
| $K_m$ | | 45 μM | | | | | n.d. | 60 μM | 47 μM | 46 μM | 14 μM |
| 2B4 | + | ++ | ++ | - | + | + | ++ | + | + | + | ++ |
| $K_m$ | n.d. | 17 μM | 21 μM | | n.d. | n.d. | n.d. | 6 μM | 31 μM | n.d. | 5 μM |
| 2B7 | ++ | ++ | ++ | - | + | ++ | ++ | + | + | + | ++ |
| $K_m$ | 21 μM | n.d. | n.d. | | 19 μM | * | * | * | * | * | * |
| 2B15 | - | - | - | - | - | - | - | + | + | + | ++ |
| $K_m$ | | | | | | | | n.d. | n.d. | n.d. | * |
| Human | ++ | +++ | +++ | + | ++ | ++ | +++ | ++ | ++ | ++ | +++ |
| Rat liver | ++ | +++ | +++ | +++ | ++ | +++ | +++ | +++ | +++ | ++ | +++ |

In this study, 5α-AG was the only AAS glucuronide to form abundantly with every active isoenzyme, but unfortunately, in the small incubation volumes of the study, the amount of the resulting conjugate was too small for NMR determination of the conjugation site. The behavior of methyltestosterone was also unusual, as the formation of MTG was not detected with any of the UGT isoenzymes. Until now, the liver has been considered as the main site of steroid glucuronidation (Hum *et al.*, 1999) with members of the subfamily UGT2B mainly in charge of the conjugation. The formation of AAS glucuronides by members of the subfamily UGT1A, especially by gastrointestinal isoenzymes UGT1A8 and UGT1A10, was thus a highly interesting finding.

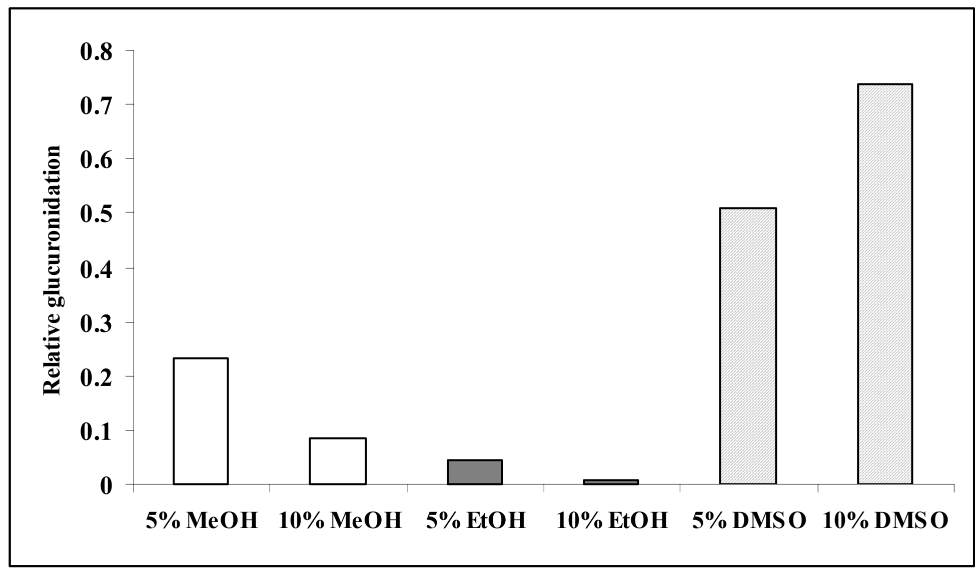



***Figure 11.*** *Effect of organic solvent on the formation of 5α-AG with UGT2B15. Amount of the formed glucuronide expressed as area ratio to internal standard 5β-LMTG. Abbreviations: MeOH=methanol, EtOH=ethanol, and DMSO=dimethyl sulfoxide.*

Stereoselective glucuronidation by UGT2B7 of an endogenous 5α/β-diasteromeric pair, androsterone and etiocholanolone, has been reported by Jin *et al.* (1997). The orientation of the proton has a dramatic effect on the steroid ring structure, as the A/B-*cis* junction of 5β-steroids changes the spatial ring geometry to a severely bent form and the 3α-bond to equatorial orientation: 5α-steroids, in turn, exhibit the planar A/B ring system with axial 3α-bond (Kirk and Marples, 1995). In the present study, stereoselectivity was detected in the formation of the conjugated nandrolone metabolites 5α-NG and 5β-NG (Table 7), as the formation of 5β-NG was favored over 5α-NG with most of the UGT isoenzymes (UGT1A1, 1A3, 1A4, 1A10, and 2B4). In contrast to 5α-NG and 5β-NG, no stereoselectivity was detected in the formation of 5α-MTG and 5β-MTG. Also, differing from earlier results, UGT2B7 did not show clear selectivity toward the orientation of C-5 hydrogen.

The observed differences in substrate specificity between the structurally highly homologous isoforms (Figure 4) were actually due more to the activities of the enzymes than the substrate

specificity. Thus, isoenzymes UGT1A8, 1A9, and 1A10 in most cases were active toward the same substrates, namely those that were planar and possessed the 17β-hydroxyl group for conjugation (T, N, 5α-1-ME, and 5α-A). Glucuronidation of 5β-N and 5β-EPIM only with 1A10 were exceptions to this rule. Considering the isoenzyme homology, UGT1A7 was exceptional in that it was not active toward any of the steroid substrates (Table 7). Substrate selectivity of two other closely similar isoenzymes, UGT1A3 and 1A4, was virtually identical: they were active toward the same substrates and toward most substrates of the study; the conjugation of 5β-EPIM solely with UGT1A3 was the only observed deviation. Nevertheless, differences were observed between the activities of UGT1A3 and 1A4 in the formation of 5α-MTG, 5β-MTG, TG, and 5α-1-MEG.

To characterize the affinity of the different UGTs for the steroid substrates, reaction kinetics was examined by determining the apparent $K_m$, i.e. the Michaëlis-Menten constant. The UGT2B subfamily members are often referred to as steroid metabolizing enzymes. In this study, the observed $K_m$ values fully support that characterization, as in most cases the $K_m$ values for a given substrate were lower for UGT2B4 and 2B7 than for members of the UGT1A subfamily (Table 7). An exceptionally high $K_m$ value was obtained in the formation of 5β-NG with UGT1A1 (375 μM), indicating low affinity of the isoenzyme toward the substrate. Similarly to UGT1A1, $K_m$ was also high for 5β-N formed with UGT1A3. Despite the close similarity of the amino acid sequences, significant differences were detected between the affinities of UGT1A3 and 1A4 in the formation of 5β-NG (136 vs. 55 μM) and TG (131 vs. 44 μM). For the other substrates that showed glucuronidation with UGT1A3 and 1A4, the apparent $K_m$ values were closely similar (Table 7). Apparent $K_m$ values obtained with homologous enzymes UGT1A8, 1A9, and 1A10 for substrates T, N, 5α-1-ME, and 5α-A were also closely similar to each other. Although UGT2B7 is reported to have only one binding site for xenobiotics and endobiotics, its catalytic capacity was high in inhibition studies for *in vivo* drug–drug interactions with opioid substrates (Rios and Tephly, 2002). In view of this finding, it was unexpected to observe substrate inhibition, which interfered heavily the determination of $K_m$ with UGT2B7 for most of the present steroid substrates (Table 7). These results, however, were obtained in the methanolic environment, and therefore, should be confirmed with DMSO to exclude the solvent effects on isoenzymes.

The most significant difference in steroid glucuronidation with human recombinant UGTs, human liver preparation, and rat liver preparation was observed for methyltestosterone, which was not conjugated with any isoenzyme, only scarcely with human liver microsomal UGTs, but to high extent with induced rat liver microsomal UGTs. The possible correlation between AAS glucuronidation with isoenzymes and with human liver microsomes was studied by determining the relative activities of the enzyme preparations toward different aglycones. To minimize the effect of the various expression levels of the enzymes, the activities obtained for a particular preparation were normalized to the formation of 5α-AG.

***Table 8.*** *Glucuronidation of the steroid substrates relative to the glucuronidation of 5α-androstane-3α,17β-diol (5α-A). Comparison of the human liver preparate with selected UGT isoenzymes.*

| Structure | Substrate | Formation of steroid glucuronides relative to 5α-AG | | | | | |
|---|---|---|---|---|---|---|---|
| | | Human liver microsomes | UGT1A3 | UGT1A4 | UGT2B4 | UGT2B7 | UGT1A10 |
| | 5β-N | 1.423 | 0.565 | 0.040 | 1.232 | 6.809 | 0.022 |
| | 5α-A | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| | 5β-EPIM | 0.748 | 0.030 | - | 5.907 | 2.018 | 0.024 |
| | 5α-ME | 0.539 | 2.998 | 0.439 | 2.702 | 0.699 | - |
| | T | 0.073 | 0.236 | 0.013 | 0.003 | 0.029 | 0.085 |
| | 5β-MT | 0.068 | 0.079 | 0.138 | 0.097 | 0.177 | - |
| | N | 0.062 | 0.196 | 0.029 | 0.007 | 0.004 | 0.796 |
| | 5α-N | 0.047 | 0.097 | 0.014 | 0.033 | 0.248 | - |
| | 5α-1-ME | 0.039 | 0.031 | 0.170 | 0.020 | 0.034 | 0.896 |
| | 5α-MT | 0.035 | 0.079 | 0.060 | 0.095 | 0.068 | - |
| | MT | 0.0002 | - | - | - | - | - |

According to the results of experiments measuring the relative aglycone preference, glucuronidation patterns of the hepatic isoenzymes UGT1A3, 1A4, 2B4 and 2B7 generally resembled the glucuronidation pattern of human liver microsomes (Table 8). Comparison of the different glucuronidation assays suggested that, with the set amount of membrane protein used (mg protein/incubation), the activity of the recombinant isoenzymes was significantly lower than that of the liver preparations. One possible reason for this is the difference between the expression levels of individual UGTs in liver preparations, but also the organic solvent in the reaction mixture may interfere differently with the activity of the different recombinant enzymes.

## 5.3 LC–ESI-MS/MS ANALYSIS OF AAS GLUCURONIDES

Radioactivity detection based on the use of $^{14}$C-labeled UDPGA was investigated at an early stage of the development of an enzyme kinetic assay for AAS glucuronides. Because of the low yield of the glucuronidation reaction and small amount of the $^{14}$C-labeled AAS glucuronide, however, the poor sensitivity and high price of the labeled co-substrate became restrictive in application of the method. Most AAS glucuronides in the study did not possess either chromophores or fluorophores, which also excluded the use of UV or fluorescence detectors. Instead, mass spectrometric detection in combination with API techniques was investigated as a suitable approach in the method development. Because of the ionization mechanism, only volatile buffers and additives are suitable with the LC–ESI-MS/MS system. For the analyst this often means that modifications must be made to traditional high-performance liquid chromatographic (HPLC) methods, which typically rely on phosphate or citrate buffer.

### *5.3.1 Liquid chromatography*

Although MS was used as the detector, the method development was focused on chromatographic separation of the glucuronides, because of the particular analytes with the same precursor ion and similar product ion spectra. Moreover, separation was considered advantageous to maintain the dynamic range and to minimize the risk of ion suppression in electrospray ionization (ESI). Acetonitrile rather than methanol was chosen as the organic modifier because of its slightly higher resolving power. The difference between acetonitrile and methanol was most evident in the separation of the nandrolone metabolites 5α-NG and 5β-NG, which completely co-eluted when methanol gradients were applied. Switching to acetonitrile did not solve the problem completely, but it provided partial separation with the resolution $R_s$=0.5. Moderately high ammonium acetate concentration, 15 mM, was required to achieve acceptable peak widths and shapes, and the eluent pH was adjusted to 4.2 according to an earlier method introduced for the LC–ESI-MS/MS analysis of TG and ETG (Bowers and Sanaullah, 1996).

An endcapped $C_{18}$ column with linear acetonitrile gradient was applied in the final LC method (**II, IV**). Within the total analysis time of 15 minutes, AAS glucuronides eluted in 3–8.5 min, and most of the isobaric analytes with similar MS/MS behavior were clearly separated (**IV**). Orientation of the relatively bulky and highly polar glucuronide moiety had a significant effect on separation of the isomers: TG (17β-*O*-glucuronidation) and ETG (17α-*O*-glucuronidation) was the most clearly separated pair, with resolution $R_s$=13.0. The pair of regioisomers 5α-MESM1G/5α-DROSTG was also clearly separated ($R_s$=3.3). Orientation of a methyl group (7α/7β) was sufficient to produce chromatographic separation in $C_{18}$ column, where 7α-BOLAG and 7β-CALUG were clearly distinguishable ($R_s$=1.9). In some cases even 5α/β orientation of a proton led to separation, for example, AG from ETCG ($R_s$=1.2); and, as

described above, 5α-NG was to some extent separated from 5β-NG ($R_s$=0.5). The group consisting of three isomers, 5α-MTG, 5β-MTG, and 5α-MESM2G, was slightly more problematic, as the diastereomers 5α-MTG and 5β-MTG completely co-eluted. However, their regioisomer 5α-MESM2G was separated with $R_s$=0.76 from the first two compounds.

A column with narrower internal diameter (Luna $C_{18}$, 150 mm x 1.0 mm i.d., particle size 5 μm, Phenomenex, CA, USA) was tested in the development stage with the solvent flow rate decreased to 100 μl/min. Despite the slightly improved resolution, better separation was not achieved for 5α-MTG/5β-MTG/5α-MESM2G. Additionally, the analysis time expanded to 30 min, mainly due to the equilibration step after the gradient, and irreproducibility was still observed in the retention times. This result suggests the use of the micro-LC pumps that are specially designed for the handling of gradients with low solvent flow rates.

### *5.3.2 Mass spectrometry (MS) and tandem mass spectrometry (MS/MS)*

Electrospray ionization (ESI) was suitable for the ionization of every glucuronide-conjugated analyte of the study, both in positive and negative ion mode (**III, IV**). In positive ion ESI the ratio of the protonated molecule $[M+H]^+$ to the corresponding ammonium adduct $[M+NH_4]^+$ was found to depend strongly on the structure, i.e. on the proton affinity of the compound, the high proton affinity (PA) of conjugated double bond systems favoring the formation of protonated form. This was nicely observed with ETG, MTG, NG and TG with 4-ene-3-one, as well as in the spectra of 5α-1-MEG and 5β-BOLDG with 1-ene-3-one structure (Table 3). For the analytes with saturated A-ring, and thus with lower PA (Harrison, 1982), the $[M+H]^+$ was often totally absent from the spectra, or its relative abundance was below 10%. In comparison with the other AAS glucuronides, the ionization efficiency of 3-OHSTG was exceptionally high. It is present in ionized form at the eluent pH of 4.2 and, furthermore, the basic pyrazol ring most probably allows the proton transfer reaction in the gas phase. As a result, strong and stabile $[M+H]^+$ was the base peak of positive ion ESI of 3-OHSTG, without $[M+NH_4]^+$.

In negative ion ESI an intensive deprotonated molecule $[M-H]^-$ was observed as the base peak of all AAS glucuronides (Table 9). Because the steroid aglycones were not ionized in negative ion ESI, it is likely that the deprotonation occurs in the carboxylic acid moiety of the glucuronic acid part of the conjugate.

In positive ion MS/MS the precursor ion was either $[M+H]^+$ or $[M+NH_4]^+$, according to results in MS studies. The main fragments were produced by losses of one or two water molecules from the precursor ion $[M+H-nH_2O]^+$ and by subsequent neutral cleavage of the glucuronide moiety $[M+H-Glu-nH_2O]^+$. The positive charge stayed predominantly on the aglycone side in the dissociation process, and only weak fragment ions were derived from the glucuronide moiety; e.g. $[Glu+H]^+$=177, $[Glu+H-H_2O]^+$=159, $[Glu+H-2H_2O]^+$=141. The origin of these fragments was investigated in $MS^n$ measurements with the ion trap instrument.

Since neither the steroid glucuronide nor the cleaved aglycone produced the ions m/z 177, 159, or 141 in consecutive isolation and fragmentation steps, these ions were most probably fragments of the glucuronide moiety (Figure 12).

***Table 9.*** *Positive and negative ion ESI MS of AAS glucuronides; m/z (rel. abundance), n.d. = not detected. See Table 3 for structures and nomenclature.*

| | POS ESI | | | NEG ESI |
|---|---|---|---|---|
| **Compound** | $[M+H]^+$ | $[M+NH_4]^+$ | $[M+Na]^+$ | $[M-H]^-$ |
| 3-OHSTG | 521 (100) | n.d. | 543 (76) | 519 (100) |
| 5α-1-MEG | 479 (89) | 496 (100) | 501 (11) | 477 (100) |
| 5α-AG | n.d. | 486 (100) | 491 (6) | 467 (100) |
| 5α-DHTG | 467 (9) | 484 (39) | 489 (100) | 465 (100) |
| 5α-DROSTG | 481 (3) | 498 (41) | 503 (100) | 479 (100) |
| 5α-MEG | 479 (2) | 496 (57) | 501 (100) | 477 (100) |
| 5α-MESM1G | 481 (2) | 498 (46) | 503 (100) | 479 (100) |
| 5α-MESM2G | 483 (3) | 500 (48) | 505 (100) | 481 (100) |
| 5α-MTG | n.d. | 500 (100) | 505 (36) | 481 (100) |
| 5α-NG | n.d. | 470 (100) | n.d. | 451 (100) |
| 5β-BOLDG | 465 (76) | 482 (24) | 487 (100) | 463 (100) |
| 5β-EPIMG | n.d. | 498 (100) | n.d. | 479 (100) |
| 5β-MTG | n.d. | 500 (100) | 505 (38) | 481 (100) |
| 5β-NG | n.d. | 470 (100) | 475 (43) | 451 (100) |
| 7α-BOLAG | 497 (2) | 514 (49) | 519 (100) | 495 (100) |
| 7β-CALUG | 497 (3) | 514 (45) | 519 (100) | 495 (100) |
| AG | 467 (3) | 484 (100) | 489 (15) | 465 (100) |
| ETCG | n.d. | 484 (100) | 489 (83) | 465 (100) |
| ETG | 465 (100) | 482 (77) | 487 (42) | 463 (100) |
| MTG | 479 (94) | 496 (100) | 501 (15) | 477 (100) |
| NG | 451 (100) | n.d. | 473 (50) | 449 (100) |
| TG | 465 (100) | 482 (22) | 487 (48) | 463 (100) |
| **Deuterated compound** | $[M+H]^+$ | $[M+NH_4]^+$ | $[M+Na]^+$ | $[M-H]^-$ |
| 5β-LMTG | n.d. | 503 (100) | 508 (76) | 484 (100) |
| $d_4$-AG | n.d. | 488 (100) | 493 (61) | 469 (100) |
| $d_3$-5α-DHTG | 470 (8) | 487 (36) | 492 (100) | 468 (100) |
| $d_3$-ETG | 468 (74) | 485 (100) | 490 (98) | 466 (100) |
| $d_3$-TG | 468 (100) | 485 (41) | 490 (66) | 466 (100) |

Collision offset voltages were optimized to produce structure-specific product ions of AAS glucuronides for the multiple reaction monitoring (MRM) in LC–ESI-MS/MS. These were ions that originated from the steroid aglycone, i.e. $[M+H-Glu-nH_2O]^+$. Selected collision offset voltages were 20–35 V, with slightly higher voltages for 17-*O*-conjugated than for 3-*O*-conjugated AAS glucuronides (Table 10). The fragmentation behavior of 3-OHSTG was unique: the only product ion at the optimal collision offset voltage (25 V) was the steroid aglycone $[M+H-Glu]^+$=345. Most probably this was because of the presence of the pyrazole ring, which is capable of resonance stabilization of the positive charge. Further increment of collision energy led only to the formation of fragments in low abundance in the low m/z

region. Thus, monitoring of a single target ion was possible with 3-OHSTG. Among the studied AAS glucuronides there were several groups of isobaric analytes with similar product ion spectra: TG/ETG, 5α-NG/5β-NG, 5α-MTG/5β-MTG/5α-MESM2G, 5α-MESM1G/5α-DROSTG, 7α-BOLAG/7β-CALUG, and AG/ETCG (Table 3). In positive ion MS/MS it was possible to distinguish between TG and ETG, where the isomerism occurs specifically at the conjugation site 17β/α-OH. The molecular strain is clearly greater in ETG than in TG because the precursor ion $[M+H]^+$ of ETG was significantly less stable than that of TG. With the nandrolone metabolites 5α-NG and 5β-NG, differences were seen in the stability of $[M+NH_4]^+$, which was higher for 5β-NG than for 5α-NG. Furthermore, at the optimal collision offset voltage the ratio of the relative abundances of product ions $[M+H\text{-}Glu]^+/[M+H\text{-}Glu\text{-}H_2O]^+$ was significantly higher for 5β-NG than for 5α-NG, which allowed the two diastereomers to be distinguished. Mesterolone metabolite 5α-MESM2G could be distinguished from the methyltestosterone metabolites 5α-MTG and 5β-MTG, because the relative abundance ratio of ions $[M+H\text{-}Glu\text{-}H_2O]^+/[M+H\text{-}Glu\text{-}2H_2O]^+$ was higher for 5α-MESM2G than for 5α-MTG and 5β-MTG. Compounds 5α-MTG/5β-MTG, 5α-MESM1G/5α-DROSTG, 7α-BOLAG/7β-CALUG, and AG/ETCH yielded identical spectra at every collision offset voltage and thus were indistinguishable without chromatographic separation.

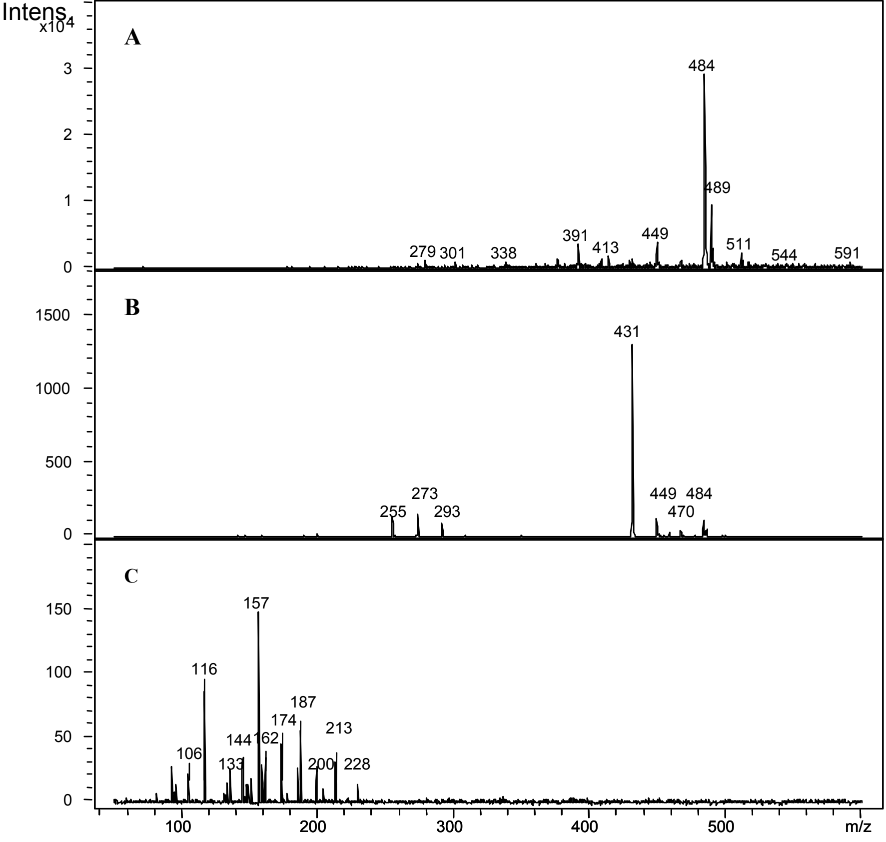



***Figure 12.*** *An example of $MS^n$ studies with androsterone glucuronide (AG). A) Positive ion MS spectrum, B) MS/MS spectrum of $[M+NH_4]^+$=484, and C) MS/MS/MS spectrum of $[M+H\text{-}Glu\text{-}2H_2O]^+$=255. Suggested glucuronic acid fragments $[Glu+H]^+$=177, $[Glu+H\text{-}H_2O]^+$=159, $[Glu+H\text{-}2H_2O]^+$=141 were not detected.*

***Table 10.*** *Positive ion mode ESI-MS/MS; m/z (rel. abundance), n.d. = not detected. See Table 3 for structures and nomenclature. Collision offset voltage noted for each analyte. The table continues in the next page.*

| Ions | Steroid glucuronide | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3-OHSTG 25 V | 5α-1-MEG 30 V | 5α-AG 20 V | 5α-DHTG 25 V | 5α-DROSTG 20 V | 5α-MEG 20 V | 5α-MESM1G 20 V | 5α-MESM2G 20 V | 5α-MTG 20 V | 5α-NG 20 V | 5β-BOLDG 20 V | 5β-EPIMG 20 V | 5β-MTG 20 V |
| **Precursor** | | | | | | | | | | | | | |
| $[M+NH_4]^+$ | | 496 (1) | 486 (4) | 484 (1) | 498 (7) | 496 (7) | 498 (3) | 500 (4) | 500 (13) | 470 (11) | | 498 (5) | 500 (9) |
| $[M+H]^+$ | 521 (100) | | | | | | | | | | 465 (11) | | |
| **Product ions** | | | | | | | | | | | | | |
| $[M+NH_4-NH_3]^+$ | | 479 (100) | n.d. | 467 (2) | 481 (1) | 479 (1) | 481 (1) | 483 (1) | n.d. | 453 (6) | | 481 (4) | n.d. |
| $[M+NH_4-H_2O]^+$ | | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 482 (4) | n.d. | | | 482 (4) |
| $[M+H-H_2O]^+$ | n.d. | n.d. | 451 (7) | 449 (1) | 463 (14) | 461 (6) | 463 (21) | 465 (7) | 465 (15) | 435 (28) | 447 (5) | n.d. | 465 (13) |
| $[M+H-2H_2O]^+$ | n.d. | n.d. | 433 (1) | n.d. | 445 (8) | 443 (2) | 445 (7) | 447 (2) | 447 (5) | 417 (74) | 431 (3) | n.d. | 447 (7) |
| $[M+H-Glu]^+$ | 345 (18) | 303 (18) | n.d. | 291 (20) | 305 (9) | 303 (8) | 305 (5) | n.d. | n.d. | 277 (60) | 289 (100) | 305 (3) | n.d. |
| $[M+H-Glu-H_2O]^+$ | n.d. | 285 (11) | 275 (34) | 273 (100) | 287 (100) | 285 (100) | 287 (100) | 289 (100) | 289 (44) | 259 (100) | 271 (21) | 287 (4) | 289 (58) |
| $[M+H-Glu-2H_2O]^+$ | n.d. | 267 (4) | 257 (100) | 255 (48) | 269 (28) | 267 (10) | 269 (39) | 271 (48) | 271 (100) | 241 (14) | 253 (5) | 269 (100) | 271 (100) |
| $[Glu+H]^+$ | n.d. | 177 (1) | 177 (13) | 177 (8) | 177 (12) | n.d. | 177 (15) | 177 (20) | 177 (19) | 177 (22) | n.d. | 177 (2) | 177 (6) |
| $[Glu+H-H_2O]^+$ | n.d. | 159 (4) | 159 (7) | 159 (24) | 159 (4) | 159 (1) | 159 (5) | 159 (5) | 159 (7) | 159 (16) | 159 (4) | 159 (1) | 159 (3) |
| $[Glu+H-2H_2O]^+$ | n.d. | 141 (5) | 141 (6) | 141 (71) | 141 (4) | 141 (1) | 141 (7) | 141 (5) | 141 (5) | 141 (16) | 141 (3) | n.d. | 141 (4) |

***Table10.*** *See the previous page for the title of the table.*

| Ions | Steroid glucuronide | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5β-NG 20 V | 7α-BOLAG 20 V | 7β-CALUG 20 V | AG 25 V | ETCG 25 V | ETG 25 V | MTG 30 V | NG 30 V | TG 25 V | 5β-LMTG 20 V | $d_4$-AG 25 V | $d_3$-5α-DHTG 25 V | $d_3$-ETG 25 V | $d_3$-TG 25 V |
| **Precursor** | | | | | | | | | | | | | | |
| $[M+NH_4]^+$ | 470 (100) | 514 (4) | 514 (2) | 484 (1) | 484 (1) | | 496 (2) | | | 503 (3) | 488 (1) | 487 (1) | | |
| $[M+H]^+$ | | | | | | 465 (0) | | 451 (100) | 465 (100) | | | | 468 (1) | 468 (100) |
| **Product ions** | | | | | | | | | | | | | | |
| $[M+NH_4-NH_3]^+$ | 453 (23) | n.d. | n.d. | 467 (1) | 467 (1) | | 479 (100) | | | n.d. | 471 (1) | 470 (3) | | |
| $[M+NH_4-H_2O]^+$ | n.d. | 496 (1) | 496 (1) | n.d. | n.d. | | n.d. | | | 485 (1) | n.d. | n.d. | | |
| $[M+H-H_2O]^+$ | 435 (21) | 479 (4) | 479 (2) | 449 (5) | 449 (2) | n.d. | 461 (2) | n.d. | n.d. | 468 (4) | 453 (3) | 452 (1) | n.d. | n.d. |
| $[M+H-2H_2O]^+$ | 417 (92) | 461 (1) | 461 (4) | 431 (14) | 431 (21) | n.d. | n.d. | n.d. | n.d. | 450 (3) | 435 (22) | n.d. | n.d. | n.d. |
| $[M+H-Glu]^+$ | 277 (67) | n.d. | n.d. | 291 (20) | 291 (48) | 289 (99) | 303 (32) | 275 (71) | 289 (55) | n.d. | 295 (23) | 294 (21) | 292 (56) | 292 (45) |
| $[M+H-Glu-H_2O]^+$ | 259 (42) | 303 (33) | 303 (66) | 273 (100) | 273 (100) | 271 (100) | 285 (71) | 257 (24) | 271 (14) | 292 (48) | 277 (100) | 276 (100) | 274 (100) | 274 (13) |
| $[M+H-Glu-2H_2O]^+$ | 241 (13) | 285 (100) | 285 (100) | 255 (63) | 255 (61) | 253 (29) | 267 (34) | 239 (14) | 253 (10) | 274 (100) | 259 (47) | 258 (36) | 256 (18) | 256 (8) |
| $[Glu+H]^+$ | 177 (4) | 177 (1) | 177 (4) | 177 (20) | 177 (4) | 177 (5) | 177 (9) | n.d. | 177 (1) | 177 (3) | 177 (16) | 177 (14) | n.d. | 177 (5) |
| $[Glu+H-H_2O]^+$ | 159 (4) | 159 (1) | 159 (2) | 159 (37) | 159 (12) | 159 (16) | 159 (15) | 159 (12) | 159 (9) | 159 (5) | 159 (26) | 159 (21) | 159 (12) | 159 (8) |
| $[Glu+H-2H_2O]^+$ | 141 (5) | 141 (1) | 141 (3) | 141 (40) | 141 (13) | 141 (6) | 141 (4) | 141 (6) | 141 (6) | 141 (4) | 141 (38) | 141 (72) | 141 (7) | 141 (5) |

*__Table 11.__ Negative ion mode ESI-MS/MS; m/z (rel. abundance), n.d. = not detected. See Table 3 for structures and nomenclature. Collision offset voltage 40 V. The table continues in the next page.*

| | Steroid glucuronide | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Ions** | 3-OHSTG | 5α-1-MEG | 5α-AG | 5α-DHTG | 5α-DROSTG | 5α-MEG | 5α-MESM1G | 5α-MESM2G | 5α-MTG | 5α-NG | 5β-BOLDG | 5β-EPIMG | 5β-MTG |
| **Precursor** | | | | | | | | | | | | | |
| $[M-H]^-$ | 519 (1) | 477 (14) | 467 (26) | 465 (22) | 479 (62) | 477 (56) | 479 (100) | 481 (81) | 481 (89) | 451 (52) | 463 (16) | 479 (100) | 481 (100) |
| **Product ions** | | | | | | | | | | | | | |
| $[M-H-H_2O]^-$ | n.d. | 459 (1) | 449 (1) | 447 (2) | 461 (2) | 459 (7) | 461 (4) | 463 (4) | 463 (6) | 433 (5) | 445 (1) | 461 (8) | 463 (6) |
| $[M-H-Glu]^-$ | 343 (41) | 301 (26) | 291 (6) | 289 (3) | n.d. | 301 (5) | 303 (1) | 305 (1) | 305 (1) | n.d. | 287 (4) | 303 (9) | n.d. |
| $[M-H-Glu-2H]^-$ | n.d. | 299 (22) | 289 (11) | 287 (2) | 301 (4) | n.d. | 301 (3) | 303 (1) | 303 (1) | 273 (12) | 285 (5) | 301 (1) | 303 (4) |
| $[Glu-H]^-$ | 175 (16) | 175 (2) | 175 (2) | 175 (2) | 175 (4) | 175 (4) | 175 (3) | 175 (6) | 175 (28) | 175 (2) | 175 (1) | 175 (6) | 175 (15) |
| $[Glu-H-H_2O]^-$ | 157 (2) | 157 (5) | 157 (6) | 157 (5) | 157 (6) | 157 (11) | 157 (15) | 157 (15) | 157 (36) | 157 (11) | 157 (3) | 157 (9) | 157 (18) |
| $[Glu-H-H_2O-CO]^-$ | 129 (5) | 129 (6) | 129 (6) | 129 (8) | 129 (7) | 129 (5) | 129 (8) | 129 (10) | 129 (16) | n.d. | 129 (4) | 129 (9) | 129 (9) |
| m/z 113 | (100) | (55) | (47) | (51) | (100) | (100) | (87) | (88) | (90) | (75) | (100) | (57) | (59) |
| m/z 99 | (11) | (9) | (5) | (7) | (5) | (5) | (2) | (5) | (4) | (6) | (6) | (9) | (4) |
| m/z 97 | n.d. | (4) | (4) | (4) | (1) | n.d. | (1) | (2) | (1) | (1) | (5) | (4) | (1) |
| m/z 95 | (7) | (15) | (18) | (15) | (9) | (8) | (13) | (10) | (10) | (12) | (9) | (8) | (6) |
| m/z 85 | (19) | (100) | (100) | (100) | (95) | (55) | (96) | (100) | (100) | (100) | (89) | (61) | (41) |
| m/z 75 | (6) | (70) | (60) | (88) | (71) | (85) | (100) | (98) | (89) | (82) | (82) | (40) | (49) |
| m/z 59 | (6) | (7) | (6) | (5) | (11) | (18) | (3) | (7) | (12) | (6) | (6) | (4) | (3) |

***Table11.*** *See the previous page for the title of the table.*

| Ions | Steroid glucuronide | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5β-NG | 7α-BOLAG | 7β-CALUG | AG | ETCG | ETG | MTG | NG | TG | 5β-LMTG | $d_4$-AG | $d_3$-5α-DHTG | $d_3$-ETG | $d_3$-TG |
| **Precursor** | | | | | | | | | | | | | | |
| $[M-H]^-$ | 451 (57) | 495 (100) | 495 (100) | 465 (59) | 465 (35) | 463 (11) | 477 (15) | 449 (11) | 463 (18) | 484 (95) | 469 (33) | 468 (23) | 466 (14) | 466 (10) |
| **Product ions** | | | | | | | | | | | | | | |
| $[M-H-H_2O]^-$ | 433 (3) | 477 (3) | 477 (4) | 447 (4) | 447 (2) | 445 (1) | 459 (2) | 431 (1) | 445 (1) | 466 (7) | 451 (1) | 450 (1) | 448 (1) | n.d. |
| $[M-H-Glu]^-$ | n.d. | 319 (1) | 319 (1) | 289 (6) | 289 (1) | 287 (6) | 301 (15) | 273 (32) | 287 (22) | n.d. | 293 (2) | 292 (2) | 290 (6) | 290 (6) |
| $[M-H-Glu-2H]^-$ | 273 (3) | 317 (1) | 317 (1) | 287 (8) | 287 (1) | 285 (6) | n.d. | 271 (23) | 285 (15) | 306 (14) | 291 (2) | 290 (1) | n.d. | n.d. |
| $[M-H-Glu-2D]^-$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 286 (1) | 286 (3) |
| $[Glu-H]^-$ | n.d. | 175 (4) | 175 (5) | 175 (13) | 175 (4) | 175 (4) | 175 (2) | 175 (4) | 175 (9) | 175 (28) | 175 (6) | 175 (4) | 175 (5) | 175 (3) |
| $[Glu-H-H_2O]^-$ | 157 (9) | 157 (3) | 157 (9) | 157 (25) | 157 (12) | 157 (9) | 157 (4) | 157 (9) | 157 (14) | 157 (21) | 157 (9) | 157 (6) | 157 (8) | 157 (6) |
| $[Glu-H-H_2O-CO]^-$ | 129 (6) | 129 (6) | 129 (5) | 129 (16) | 129 (8) | 129 (7) | 129 (5) | 129 (6) | 129 (10) | 129 (10) | 129 (10) | 129 (8) | 129 (6) | 129 (5) |
| m/z 113 | (55) | (11) | (58) | (100) | (82) | (100) | (48) | (75) | (92) | (81) | (86) | (72) | (89) | (82) |
| m/z 99 | (6) | (2) | (5) | (7) | (6) | (9) | (9) | (8) | (8) | (17) | (8) | (7) | (10) | (3) |
| m/z 97 | n.d. | (1) | (1) | (2) | n.d. | (1) | n.d. | (4) | (5) | n.d. | (1) | n.d. | n.d. | n.d. |
| m/z 95 | (7) | (4) | (8) | (12) | (11) | (12) | (12) | (11) | (11) | (21) | (14) | (14) | (14) | (11) |
| m/z 85 | (100) | (27) | (54) | (97) | (100) | (97) | (100) | (100) | (100) | (86) | (100) | (100) | (100) | (100) |
| m/z 75 | (82) | (31) | (51) | (95) | (86) | (90) | (63) | (77) | (80) | (100) | (95) | (78) | (95) | (72) |
| m/z 59 | (5) | (2) | (3) | (7) | (6) | (9) | (6) | (6) | (6) | (12) | (6) | (7) | (7) | (5) |

The precursor ion $[M-H]^-$ was more stabile in negative ion ESI than the precursor ion in positive ion ESI, because the carboxylic acid group of the glucuronide moiety delocalizes the negative charge. In the comparison of AAS glucuronides, it also became apparent that $[M-H]^-$ ions of 3-*O*-glucuronides are more stabile than those of 17-*O*-glucuronides (Table 11). The lesser stability can be explained by the additional strain caused by the methyl substitution at C-18. As in positive ion MS/MS, the purpose of collision offset voltage optimization was to find suitable conditions for the formation of structure-specific product ions. In negative ion mode these ions were those produced after cleavage of the glucuronide moiety $[M-H-Glu]^-$, after the abstraction of two additional hydrogens $[M-H-Glu-2H]^-$, and after the loss of water $[M-H-Glu-nH_2O]^-$. Collision offsets below 25 V were not sufficient to fragment the stabile $[M-H]^-$ ion, but as soon as the optimized 40 V was reached, the structure fragmented intensively. The most abundant fragments originated from the glucuronide moiety, unfortunately, and were identical with those of every other AAS glucuronide (Table 11). The characteristic ions were very weak in intensity, and thus, negative ion MS/MS was not suitable for the monitoring of trace levels of AAS glucuronides. Despite the inadequate structure-specific information on the steroid aglycone, negative ion MS/MS may offer a complementary method to positive ion MS/MS and could be employed as a tool, for instance in ADME studies, to indicate the presence of glucuronide conjugated drug metabolites.

Both positive and negative ion modes were also investigated with use of atmospheric pressure chemical ionization (APCI, **III**). The APCI process is thermally more energetic than ESI, however, which enhances the in-source fragmentation and hence decreases the sensitivity. The MS/MS fragmentation by APCI did not offer any information additional to ESI and ESI is thus the ionization method of choice for the further LC–MS/MS method development. Similarly to APCI, the excess of thermal energy also caused fragmentation in atmospheric pressure photoionization (APPI). In preliminary studies where toluene was used as the dopant solvent, ionization of TG was observed with both polarities of APPI, but the ionization was not as efficient as with ESI. APPI has been successfully applied to the analysis of non-conjugated AAS in urine (Robb *et al.*, 1999; Leinonen *et al.*, 2002), as well as to the identification of glucuronide-conjugated drug metabolites such as apomorphine, dobutamine, and entacapone in biological samples (Keski-Hynnilä *et al.*, 2002). In both two cases, however, the analytes were ionized most efficiently with ESI. It would be interesting to examine the feasibility of APPI for the ionization of AAS glucuronides where normal-phase chromatography was applied to the separation of the compounds, because the solvent systems might provide ionization of the analytes without the need for additional dopant solvent.

*5.3.3 Application of LC–ESI-MS/MS to the analysis of AAS glucuronides*

In enzyme-kinetic studies, with the relatively simple phosphate buffer matrix, precipitation of the proteins and solid-phase extraction (SPE) were sufficient sample preparation steps for the removal of the interfering salts and enabled the analysis of AAS glucuronides at low concentrations (1–5 ng/ml). Problems arose as soon as the analytes were spiked in urine, which contains a wide variety of metabolic end products, including endogenous steroid glucuronides (**IV**). An aspect worth noting, especially in quantitative analysis, is the limited dynamic range of ESI. This is relevant in any of the stages of the ionization process, i.e. in the formation of charged droplets, in the transfer of ions from liquid to gas phase, and in the introduction of sample ions to the mass analyzer (Kostiainen and Bruins, 1994). Each ion competes with the others in the ESI process that transfers ions from solution to gas phase, and the transfer is dependent on both the concentration and the relative ionization efficiency of the species (Kebarle, 2000). Thus, the analyte itself in high concentration, interfering background electrolytes, or nonvolatile solutes (e.g. impurities remaining after incomplete sample purification) may suppress the signal if these occupy the whole droplet surface or form solid precipitates within the droplet (King *et al.*, 2000). When this happens, the analytical result will be invalid and often the instrument will be contaminated as well.

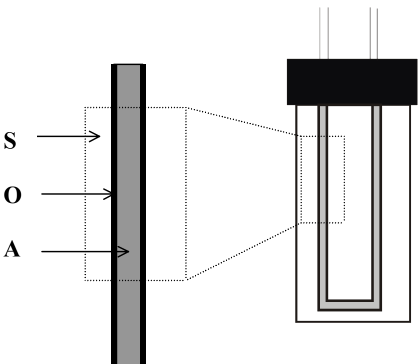



***Figure 13.*** *Three-phase solvent system of the liquid-phase microextraction (LPME) device: sample (S), organic layer (O), and acceptor phase (A).*

With the traditional liquid–liquid extraction (LLE) and SPE methods, the sample purification was insufficient and non-specific, producing high background interference in MRM ion traces. Accordingly, after optimization and comparison, a relatively new clean-up technique, liquid-phase microextraction (LPME) with hollow polypropylene membrane and three-phase solvent system (Figure 13) proved to be the most selective and provided the cleanest extracts. Detection limits of 2–20 ng/ml were achieved for most of the 12 AAS glucuronides (**IV**). LPME was initially introduced for the analysis of basic drugs (Pedersen-Bjergaard and Rasmussen 1999; Rasmussen *et al.* 2000; Ho *et al.* 2002) and anti-inflammatory analgesics (Pedersen-Bjergaard and Rasmussen 2000), but here it showed itself suitable for the extraction of acidic compounds as well. One explanation for the better specificity obtained

with LPME than with LLE or SPE is that the process involves two successive steps in which the solvent pH is adjusted. The deionized AAS glucuronides are first dissolved in an organic layer and then transferred further to an acceptor phase in ionic form. Although the specificity of the method was enhanced with LPME, the recoveries from urine were below 20% for all tested AAS glucuronides.

It was of interest to know whether the analytes remain in the sample solution or whether they are trapped into the organic layer and, in test of this, the distribution of the analytes among sample solution (S), organic solvents (O), and acceptor phase (A) was examined with LLE experiments with spiked samples and two phases at a time (S/O, O/A) (Figure 14). In the O/A experiment, with any of the organic solvents (*n*-octanol, octanone, or pentylacetate), the analytes were clearly transferred to the acceptor phase side. From this it may be concluded that trapping to the organic layer is not the explanation for the relatively low yields in LPME. In the S/O experiment, *n*-octanol extracted the AAS glucuronides most efficiently, showing that the initial step, the dissolution of the analyte from the sample solution to the organic phase, is the most critical step in LPME. The significance of the selection of organic solvent was supported by the experimental results for the complete LPME process, in which the only acceptable system relied on the use of *n*-octanol as the organic phase (**IV**).

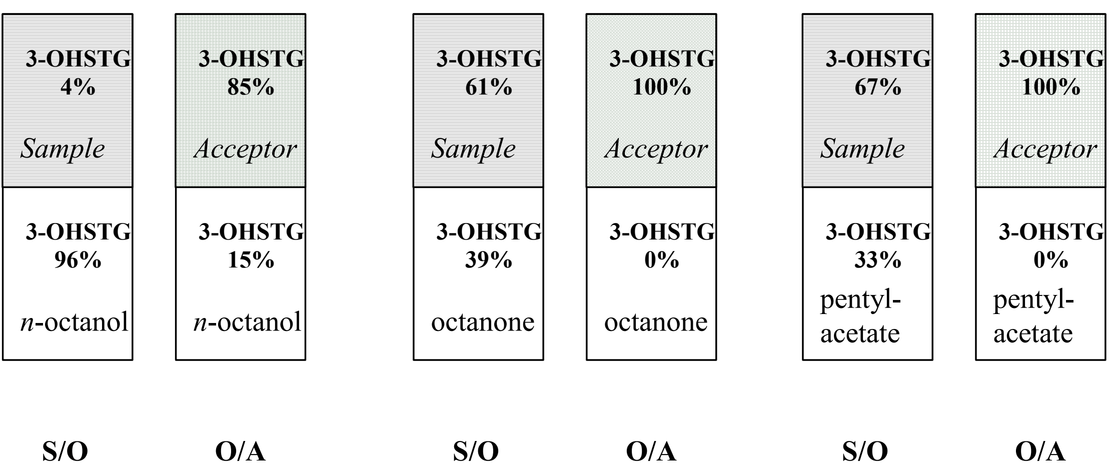



***Figure 14.*** *An example of the LPME distribution studies with 3-OHSTG. Sample (S), organic layer (O), and acceptor phase (A).*

Despite the results achieved with LPME, the LC–ESI-MS/MS method for urine samples is still in need of development, as the background of endogenous compounds interferes with the detection of some of the AAS glucuronides. Although a pool of several female and male urine samples was used in the method development, differences between the matrix effects of individual samples can be expected (Matuszewski et *al.*, 1998). With respect to unsolved problems of specificity, sample preparation and chromatographic separation are the critical issues in the future method development.

## 6. SUMMARY AND CONCLUSIONS

Glucuronide-conjugated anabolic androgenic steroids (AAS) in milligram amount were produced by *in vitro* enzyme-assisted synthesis. Induced rat liver microsomes were used as the source of the conjugating uridine diphosphoglucuronosyltransferase enzymes (UGTs). Enzyme-assisted synthesis provided stereochemically pure AAS glucuronide conjugates, which makes this method superior to chemical synthesis, where racemic mixtures of α/β-anomers of the product and undesired by-products are often encountered. The yields of the syntheses were mostly 13–28%, though for substrates with conjugated double bond system 4-ene-3-one, they were at the significantly higher level of 77–78%. Because the composition of the reaction mixture was relatively simple, and the differences in the optimal conditions for the various substrates were relatively minor, the addition of a new AAS substrate to the test compound set should be straightforward.

Enzyme-assisted synthesis offers a practical pathway for the rapid production of small amounts of AAS glucuronides, such as needed in the build-up of an analytical method. Liver microsomal preparations should be used in the syntheses, rather than recombinant UGT isoenzymes, owing to their higher activity and tolerance against solvents and pH effects of the reaction mixture. UGT isoenzymes are nevertheless of unique importance in detailed examination of the glucuronidation reaction, for instance, in the screening of the metabolic pathways of drugs and drug candidates. For the synthesis of AAS glucuronides in greater than low milligram amounts, chemical synthesis is still the method of choice.

Regio- and stereoselectivity of glucuronidation was examined with 11 human recombinant UGTs toward 11 steroid substrates. The selected UGTs included members of the UGT1A and 2B subfamilies, and the substrates were either parent AAS or their phase-I modified metabolites with 3α-, 17α-, and/or 17β-hydroxyl groups as the potential sites for *O*-glucuronidation. Most UGTs did not exhibit clear preference for conjugation to the 3α-hydroxyl or the 17β-hydroxyl group; the isoenzymes showing evidence of regioselectivity were UGT1A8, 1A9, and 2B15, which appeared to preferentially catalyze 17β-hydroxyl glucuronidation. Formation of nandrolone metabolite 5β-NG was favored over the corresponding 5α-isomer, 5α-NG, with most of the UGT isoenzymes (UGT1A1, 1A3, 1A4, 1A10, and 2B4), but the similar stereoselectivity was not detected in the formation of 5α-MTG and 5β-MTG.

The highly homologous isoforms UGT1A8, 1A9, and 1A10 were similarly active toward the planar steroid substrates with 17β-hydroxyl group, whereas the fourth member of that enzyme group, UGT1A7, did not exhibit activity toward any substrate. The substrate specificities of the two structurally analogous isoenzymes UGT1A3 and 1A4 were also closely similar. Inter-individual differences were observed in the relative activities among both groups of enzymes, however. The main difference between the three types of enzyme preparations studied; recombinant UGTs, human liver UGTs, and rat liver UGTs, was detected in the conjugation

of methyltestosterone, which was glucuronidated only with the human and rat liver microsomal UGTs, the activity of the induced rat liver UGTs being significantly higher than those of the human liver preparation. Glucuronidation patterns of the hepatic isoenzymes UGT1A3, 1A4, 2B4, and 2B7 generally resembled those of human liver microsomes, which indicates that in future, an appropriate selection of UGT isoenzymes might be used as *in vitro* model to predict the glucuronidation reactions of a particular xenobiotic in the human body.

ESI was indicated as the method of choice for ionization of the AAS glucuronides, because the excess thermal energy in APCI and APPI decreased the sensitivity. Although both polarities were applicable in ESI, positive ion mode MS/MS was selected on the basis of the structure-specific fragmentation. Because of the relatively low proton affinities of AAS glucuronides, the precursor ion of the MRM pair was most often the ammonium adduct of the molecule $[M+NH_4]^+$, whereas the product ions originated from the loss of glucuronide moiety $[M+H-Glu]^+$ with additional loss of water $[M+H-Glu-nH_2O]^+$. From the differences between fragment ion ratios, it was also possible to distinguish between some isobaric AAS glucuronides with similar product ion spectra.

The optimized LC–ESI-MS/MS method enabled direct analysis of the glucuronide conjugates, in which an end-capped $C_{18}$ column and ammonium acetate buffered acetonitrile–water gradient were employed for chromatographic separation. Two structure-specific product ions, for both the analyte and the deuterium-labeled internal standard, were monitored in positive ion ESI-MS/MS. Combined with LLE, LPME, or SPE sample preparation, LC–ESI-MS/MS was successfully applied in the detection of AAS glucuronides in moderately simple matrixes such as those of *in vitro* metabolic studies. Nevertheless, the high background signal of the matrix interfered with the analysis of AAS glucuronides in urine, especially in the determination of the methyltestosterone metabolites 5α-MTG and 5β-MTG. The main goal in further method development will be the improvement of specificity, which most likely will be achieved through further development of the chromatographic separation. In this study, both the sample preparation and the chromatographic separation were based mainly on $C_{18}$ reversed-phase systems, i.e. non-polar interactions between the analyte and the sorbent or LC column. The switch to normal-phase chromatography would likely be difficult for ESI, as the solvents do not adequately support ion formation (Voyksner, 1997). Testing of normal-phase or ion-exchange based procedures for sample preparation could be a fruitful direction in which to proceed.

The real problem, however, is the abundance of endogenous steroid glucuronides, which are difficult to remove from samples because of their closely similar structures. In this respect, other avenues to explore for improved sample preparation are immunoaffinity chromatography (IAC), based, for example, on the specific 17α-methyl-17β-hydroxy-structure of synthetic AAS aglycones, and the use of multi-analyte antibodies (Crooks *et al.*, 1997). The alternative approach, modification of the chromatographic separation, would involve the testing of normal-phase LC-columns and solvent systems. With this combination the suitability of APPI for the ionization method should be examined. The use of more

specific techniques, such as immunoaffinity and chiral separation, could enhance the specificity of the method. The number of viable alternatives appears fewer in the MS detection. The fragmentation of the different AAS glucuronides is fairly similar, and the selection of structure-specific target ions is limited. Experiments with different scan modes (neutral loss and precursor ion scan) did not offer any additional data to the product ion scan of MRM either. The detection limits were nevertheless low when pure standards were measured in pure solvents, so the main focus of the method development should be on sample preparation and chromatographic separation.

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