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T.KUURANNE, A.LEINONEN, O.AITIO, K.KUOPPASALMI, R.KOSTIAINEN:  
Enzyme-assisted Synthesis and Mass Spectrometric Behaviour of Anabolic Steroid  
Glucuronides

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## Enzyme-assisted synthesis and mass spectrometric behavior of anabolic steroid glucuronides

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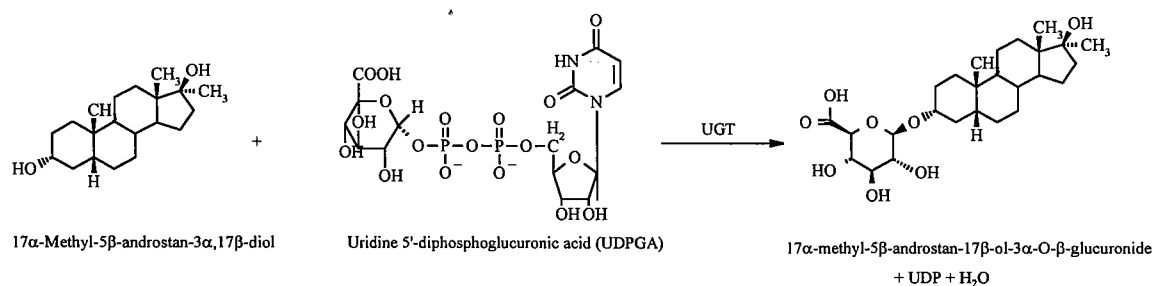
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### INTRODUCTION

Anabolic androgenic steroids (AAS) undergo rapid metabolic reaction in human body. First phase metabolic reactions of endoplasmic reticulum bound 5 $\alpha$ - and cytoplasmic 5 $\beta$ -reductase enzymes together with 3 $\alpha$ -hydroxysteroid-dehydrogenase enzyme convert the parent steroid structure and form suitable sites for the further second phase metabolic reactions, the conjugation [1]. Glucuronidation is the most common conjugation pathway in human body, as in all mammals. It is enzymatically controlled by uridindiphosphoglucuronosyl-transferases (UGT), which is the family of close related membrane-bound enzymes in the endoplasmic reticulum. From the steroid point of view the most interesting subfamily is UGT 2B and its members 2B4, 7, 10, 11, 15 and 17 have been reported to participate in the androgen metabolism in human body [2].

Mechanism of the reaction catalyzed by UGT is a bimolecular nucleophilic substitution (S<sub>N</sub>2). The attack of the acceptor group of the substrate, i.e. first phase metabolite of a certain steroid, to C<sub>1</sub> of the pyranose acid ring of UDPGA results in the formation of the glucuronide conjugate. Inversion of the configuration occurs and the resulting conjugate has  $\beta$ -configuration (Fig. 1).



**Fig. 1.** Glucuronidation reaction

The conjugate is generally less toxic, water-soluble and therefore more easily excreted in urine than the parent compound. Structures and nomenclature of the steroid glucuronides under examination in this study are presented in Table 1.

## **EXPERIMENTAL**

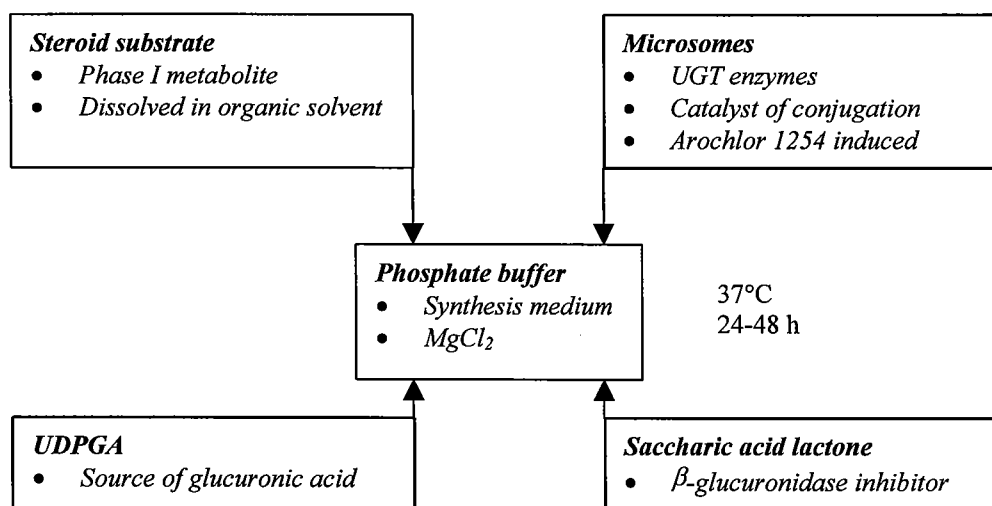
### *Synthesis*

*In vitro* performed synthesis imitates the glucuronidation conditions in the body. Synthesis is carried out in phosphate buffer, the pH of which is adjusted to physiological 7.4. This buffer also contains magnesium chloride, which is needed to ensure the enzyme function. The substrate is a first phase metabolite of certain steroid, metabolites of nandrolone and 17 $\alpha$ -methyltestosterone in this study, which were either purchased from Steraloids (Newport, USA) or synthesized in our laboratory. The substrate was dissolved in organic solvent, usually in ethanol or methanol. The total portion of organic solvent must not exceed 10% of the total volume of synthesis mixture to avoid the precipitation of the proteins and termination of enzyme activity. As the metabolites are quite sparingly soluble in organic solvents, the solubility of the starting material seems to be the limiting step of the synthesis.

UGT-enzymes were obtained from microsomal fraction of rat liver tissue. This homogenate was a pool of nine female Wistar rats (Helsinki University Breeding Center, Finland) whose liver enzymes were induced with a strong inductor, Arochlor 1254, which is a mixture of polychlorinated biphenyls (Foxboro Company, North Haven, CT, USA). The preparation of microsomes is described more detailed elsewhere [3]. The protein concentration of the homogenate was determined spectrophotometrically [4] with commercial protein standards.

The tissue homogenate includes other enzymes in addition to UGTs, some of which may be crucial for the glucuronidation. Because of the bond-breaking feature of  $\beta$ -glucuronidase, *D*-saccharic acid 1,4-lactone (Sigma, St. Louis, USA), a  $\beta$ -glucuronidase inhibitor, was added to the reaction mixture.

The glucuronide moiety of the conjugate is obtained from the commercially available co-substrate, uridin-5'-diphosphoglucuronate (UDPGA, Boehringer Mannheim GmbH, Germany). As an expensive reagent, it often limits the scale of the steroid glucuronide production. Reaction mixture is prepared in ice bath because of the easy degradation of tissue homogenate but the reaction itself is carried out in water bath of 37°C for 24 to 48 hours with continuous stirring with magnet (Fig. 2). Precipitating the proteins with dichloromethane interrupts the synthesis. In this step not only the proteins are precipitated but the free substrate is also extracted to the organic layer. After centrifugation the water phase including the glucuronide conjugates is further cleaned up with C<sub>18</sub> solid phase extraction. Final elution of the glucuronides is made with methanol, which is then evaporated to dryness under nitrogen stream. The dry residue is further suspended to water and lyophilised. The yield of the synthesis depends strongly on the steroid aglycone, being approximately 10-30%. In these experiments the best substrate has been 5β-MT and the worst 5α-MT.



**Fig. 2.** Composition of the reaction mixture.

### *NMR experimental*

NMR studies were carried out with Varian Unity 500 (Palo Alto, CA, USA) instrument using a nanoprobe. The samples were dissolved in deuterated acetone prior DQFCOSY (Double Quantum Filtered CORrelation SpectroscopY), HMQC (Heteronuclear Multiple Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Coherence) spectra were recorded.

### *Mass spectrometry*

The instrument used in MS and MS/MS studies was Perkin-Elmer Sciex API 300 triple quadrupole (Sciex, Concord, Canada) with pneumatic assisted ionspray, so called electrospray (ESI) as ion source. Atmospheric pressure chemical ionization (APCI) was also tested, but because of the higher solvent flow rate, the sample consumption and background noise were higher than in ESI, and thus ESI was a method of choice for ionization in these studies. MS and MS/MS studies were carried out in both negative and positive ion modes, capillary voltage being 5 kV. Orifice voltage was low, 10 V, in order to avoid the fragmentation already in the ion source. Samples were dissolved in the mixture of methanol and 7.5 mM ammonium acetate in water, which was used as the eluent from the syringe pump also. According to earlier results [5] pH was adjusted with formic acid (suprapure, Merck, Germany) to 4.3 in positive ion mode to enhance the ionization, and in negative ion mode the pH was 6.8.

## **RESULTS AND DISCUSSION**

### *Identification of the glucuronides*

The amount of the steroid glucuronides is small, but the use of a NMR-nanoprobe enabled the measurements. As a reference, NMR spectra were first obtained from AG, which is a commercially available compound having  $\beta$ -orientation of the glucuronide moiety and  $3\alpha$ -O-orientation of the conjugation at the aglycone side. Protons of the  $\alpha$ -carbon in the glucuronide moiety exhibit the coupling constant of 8 Hz, instead of 4 Hz, which reveals  $\beta$ -configuration of the glycosidic bond. Combining the data of all the spectra it was possible to conclude that conjugation is in 3-O-position in both nandrolone and methyltestosterone metabolites.  $17\beta$ -OH group seems to be sterically hindered from enzymatic conjugation, since the experimental synthesis with parent methyltestosterone did not yield any sign of conjugated compound.

### *Mass spectrometric studies*

For mass spectrometric studies, four steroid glucuronides more were taken in to account in addition to those now synthesised. Testosterone (TG), epitestosterone (ETG) and nandrolone (NG) are conjugated into 17-*O*-position whereas androsterone (AG) is 3-*O*-conjugated like similarly to 5 $\alpha$ -MTG, 5 $\beta$ -MTG, 5 $\alpha$ -NG and 5 $\beta$ -NG.

In negative ion mode MS all steroid glucuronides behaved alike. A stable and strong deprotonated molecule  $[M-H]^-$  with no fragmentation was seen in all the spectra (Table 2) and was chosen as a precursor ion for MS/MS studies. Relatively high offset voltage was needed to break down the precursor ion and only a few and weak structure specific product ions of the steroid aglycone were formed. Ions of the steroid aglycone and aglycone minus two hydrogen could be detected but their intensity was weak. Optimal offset voltage was 40 V and rising the energy of the collision led only to the formation of non-specific fragments originating from the glucuronide moiety of the molecule. As a result, all the steroid glucuronides gave almost similar MS/MS spectra in negative ion mode. Deprotonated molecules of the 3-*O*-glucuronides studied are more stable than  $[M-H]^-$  of 17-*O*-glucuronides, obviously due to methyl group at C-18, which may cause strain to 17-*O*-glucuronide structures. Fragment ions  $[M-H-Glu]^-$  and  $[M-H-Glu-2H]^-$  ions of TG and NG are more stable than corresponding ions of other steroid glucuronides, due to stabilizing effect of 4-ene-3-one structure. In the case of ETG, 17 $\alpha$ -orientation of the glucuronide may cause more molecular strain than 17 $\beta$ -orientation leading to less intense ions  $m/z$  287 and 285, i.e. ions accounting for the steroid aglycone (Table 3).

In positive ion mode the 4-ene-3-one structured 17-*O*-glucuronides yielded mainly the protonated molecule  $[M+H]^+$ , while 3-*O*-glucuronides without a conjugated double bond system were detected mainly as ammonium adducts  $[M+NH_4]^+$ . Both types of glucuronides exhibited also sodium adducts  $[M+Na]^+$  (Table 2). The orifice voltage was adjusted to 10 V, but still some breakdown occurred in the ion source. According to MS studies either  $[M+H]^+$  or  $[M+NH_4]^+$  was chosen as precursor ion for tandem mass spectrometric experiment. Now, unlike in negative ion MS/MS, there were differences between the fragmentation patterns of the steroid glucuronides.

The optimal collision energy varies between 15-30 V, and it should be determined separately for each compound. Fragments can be divided in three groups (Fig. 3):

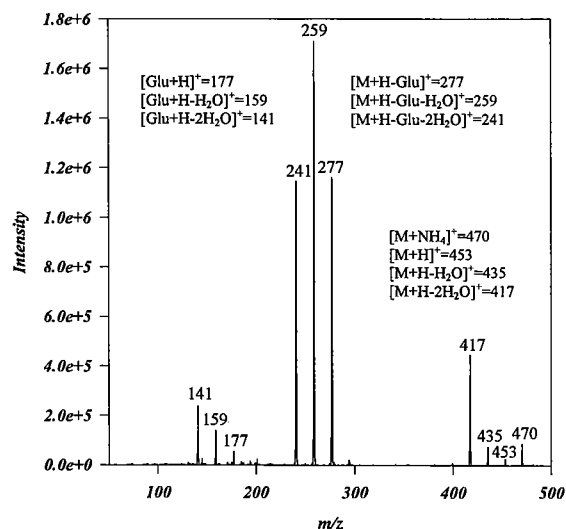
- 1) ions formed by primary eliminations of one and two water molecules in addition to ammonia,
- 2) ions formed by the loss of a glucuronide group and following losses of one and two water molecules, and
- 3) ions originating from the glucuronide moiety.

In the case of 4-ene-3-one structured steroids the glucuronide moiety is cleaved without primary losses of water molecules, which was the case with other steroid glucuronides (Table 4). Our results are in good agreement with earlier studies [6] concerning the behavioral difference of testosterone and epitestosterone glucuronides. It is suggested that the strain in the structure of ETG induces the cleavage of the glucuronide moiety already at the lowest level of collision energy (offset voltage 5 V). As an interesting detail, metabolites 5 $\alpha$ -MTG and 5 $\beta$ -MTG did not show any sign of the steroid aglycone at any level of collision energy, but very strong ions of the aglycone minus one and two water molecules instead ( $m/z$  289 and 271, respectively). The third group of product ions yields from the breakdown of the glucuronide moiety. Although the abundance of these ions is increased at higher collision energies, they are not diagnostic for the steroid structure and thus of minor interest.

## CONCLUSION

The yield of the enzyme-assisted synthesis is only moderate so far. Despite of that, it is still method worth while to consider when producing glucuronide conjugates of anabolic steroid metabolites, because of the stereo-specificity of the enzymes. Positive ion ESI ionisation provides more characteristic mass spectrometric behavior of anabolic steroid glucuronides than negative ion ESI, which still can produce additional data for future screening methods. Positive ion MS/MS is preferable in the development of direct LC-MS/MS methods, because of the formation of structure specific product ions. Positive ion mode multiple reaction monitoring (MRM) has been used in preliminary studies of micro-LC-MS/MS. Yet the work is incomplete and the

detection limits of the method are not defined, LC-separation coupled with MS-detection may  $[M+NH_4]^+$  offer a good alternative for the detection of anabolic steroid glucuronides.



**Fig. 3.** Positive ion MS/MS of 5β-NG with offset voltage of 25 V.

## ACKNOWLEDGEMENTS

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## REFERENCE

- [1] Schänzer, W., Metabolism of anabolic androgenic steroids, *Clin. Chem.* **42**:7 (1996) 1001-1020.
- [2] Hum, D.W., Bélanger, A., Lévesque, E., Barbier, O., Beaulieu, M., Albert, C., Vallée, M., Guillemette, C., Tchernof, A., Turgeon, D., Dubois, S., Characterization of UDP-glucuronosyltransferases active on steroid hormones, *J. Steroid. Biochem. Molec. Biol.* **69** (1999) 143-423.
- [3] Luukkanen, L., Elovaara, E., Lautala, P., Taskinen, H., Vainio, H., Characterization of 1-hydroxypyrene as a novel marker substrate of 3-methylcholanthrene-inducible phenol UDP-glucuronosyltransferase(s), *Pharmacol. Toxicol.* **80** (1997) 152-158.



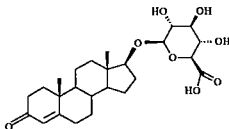
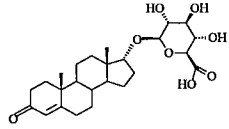
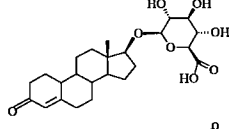
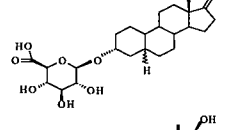
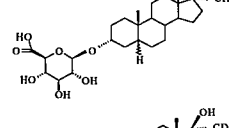
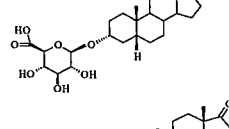
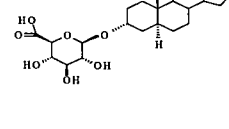
[4] Lowry, O., Rosenbrough, J., Farr, A.L., Randall, R.J., Protein measurement with the folin phenol reagent, *J. Biol. Chem.* **193** (1951) 265-275.

[5] Bowers, L.D., Sanaullah, Direct measurement of steroid sulfate and glucuronide conjugates with high-performance liquid chromatography-mass spectrometry, *J. Chromatogr. B.* **687** (1996) 61-68.

[6] Sanaullah, Bowers, L.D., Facile synthesis of [16,16,17-2H3]-testosterone, -epitestosterone and their glucuronides and sulfates, *J. Steroid Biochem. Molec. Biol.* **58:2** (1996) 225-234.

[7] Kuuranne, T., Vahermo, M., Leinonen, A., Kostianen, 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.

**Table 1.** Structures and nomenclature of steroid glucuronides.

Abbreviation	Compound	Structure	MW
TG	4-Androsten-3-one-17 $\beta$ -O-glucuronide		464
ETG	4-Androsten-3-one-17 $\alpha$ -O-glucuronide		464
NG	Estr-4-en-3-one-17 $\beta$ -O-glucuronide		450
5 $\alpha$ / $\beta$ -NG	5 $\alpha$ / $\beta$ -Estran-17-one-3 $\alpha$ -O-glucuronide		452
5 $\alpha$ / $\beta$ -MTG	17 $\alpha$ -Methyl-5 $\alpha$ / $\beta$ -androstan-17 $\beta$ -ol-3 $\alpha$ -O-glucuronide		482
5 $\beta$ -LMTG	17 $\alpha$ -CD <sub>3</sub> -5 $\beta$ -Androstan-17 $\beta$ -ol-3 $\alpha$ -O-glucuronide		485
AG	5 $\alpha$ -Androstan-17-one-3 $\alpha$ -O-glucuronide		466

**Table 2.** Negative and positive ion MS (relative abundance in parenthesis).

Compound	[M-H] <sup>-</sup>	[M+Na] <sup>+</sup>	[M+NH <sub>4</sub> ] <sup>+</sup>	[M+H] <sup>+</sup>
TG	463 (100)	487 (48)	482 (22)	465 (100)
ETG	463 (100)	487 (42)	482 (77)	465 (100)
NG	449 (100)	473 (50)		451 (100)
5 $\alpha$ -NG	451 (100)		470 (100)	
5 $\beta$ -NG	451 (100)	475 (43)	470 (100)	
5 $\alpha$ -MTG	481 (100)	505 (36)	500 (100)	
5 $\beta$ -MTG	481 (100)	505 (38)	500 (100)	
AG	465 (100)	489 (15)	484 (100)	467 (3)

Abbreviations: see Table 1.

**Table 3.** Negative ion MS/MS with collision offset of 40 V (relative abundance in parenthesis).

Ions	TG	ETG	NG	5 $\alpha$ -NG	5 $\beta$ -NG	5 $\alpha$ -MTG	5 $\beta$ -MTG	AG
Parent ion								
[M-H] <sup>-</sup>	463 (18)	463 (11)	449 (11)	451 (52)	451 (57)	481 (89)	481 (100)	465 (59)
Product ions								
[M-H-H <sub>2</sub> O] <sup>-</sup>	445 (1)	445 (1)	431 (1)	433 (5)	433 (3)	463 (6)	463 (6)	447 (4)
[M-H-CH <sub>3</sub> COOH] <sup>-</sup>	403 (3)	403 (1)	389 (3)	391 (5)	391 (5)	421 (2)	421 (3)	405 (3)
[M-H-118] <sup>-</sup>		345 (2)			333 (4)	363 (5)	363 (4)	347 (6)
[M-H-134] <sup>-</sup>	329 (3)	329 (1)	315 (4)	317 (4)	317 (2)	347 (1)	347 (2)	331 (5)
[M-H-Glu] <sup>-</sup>	287 (22)	287 (6)	273 (32)			305 (1)		289 (6)
[M-H-Glu-2H] <sup>-</sup>	285 (15)	285 (6)	271 (23)	273 (12)	273 (3)	303 (1)	303 (4)	287 (8)
[M-H-Glu-H <sub>2</sub> O] <sup>-</sup>	269 (2)	269 (2)	255 (1)				287 (1)	271 (1)
[Glu-H] <sup>-</sup>	175 (9)	175 (4)	175 (4)	175 (2)		175 (28)	175 (15)	175 (13)
[Glu-H-H <sub>2</sub> O] <sup>-</sup>	157 (14)	157 (9)	157 (9)	157 (11)	157 (9)	157 (36)	157 (18)	157 (25)
[Glu-H-H <sub>2</sub> O-CO] <sup>-</sup>	129 (10)	129 (7)	129 (6)		129 (6)	129 (16)	129 (9)	129 (16)
m/z = 113	(92)	(88)	(75)	(75)	(55)	(90)	(59)	(100)
m/z = 95	(11)	(10)	(11)	(12)	(7)	(10)	(6)	(12)
m/z = 85	(100)	(100)	(100)	(100)	(100)	(100)	(41)	(97)
m/z = 75	(80)	(95)	(77)	(82)	(82)	(89)	(49)	(95)
m/z = 59	(6)	(9)	(6)	(6)	(5)	(12)	(3)	(7)

Abbreviations: see Table 1.

**Table 4.** Positive ion MS/MS with collision offset of 25 V (relative abundance in parenthesis).

Ions	TG	ETG	NG	5 $\alpha$ -NG	5 $\beta$ -NG	5 $\alpha$ -MTG	5 $\beta$ -MTG	AG
Parent ions								
[M+NH <sub>4</sub> ] <sup>+</sup>				470 (7)	470 (4)	500 (3)	500 (1)	484 (1)
[M+H] <sup>+</sup>	465 (95)	465 (0)	451 (100)					
Product ions								
[M+H] <sup>+</sup>				453 (7)	453 (2)			467 (1)
[M+NH <sub>4</sub> -H <sub>2</sub> O] <sup>+</sup>						482 (1)	482 (1)	
[M+H-H <sub>2</sub> O] <sup>+</sup>				435 (1)	435 (2)	465 (1)	465 (1)	449 (2)
[M+H-2H <sub>2</sub> O] <sup>+</sup>				417 (5)	417 (20)	447 (1)	447 (1)	431 (14)
[M+H-Glu] <sup>+</sup>	289 (100)	289 (88)	275 (91)	277 (19)	277 (72)			291 (23)
[M+H-Glu-H <sub>2</sub> O] <sup>+</sup>	271 (20)	271 (100)	257 (31)	259 (100)	259 (100)	289 (16)	289 (22)	273 (100)
[M+H-Glu-2H <sub>2</sub> O] <sup>+</sup>	253 (12)	253 (26)	239 (15)	241 (54)	241 (68)	271 (100)	271 (100)	255 (53)
[Glu+H] <sup>+</sup>		177 (4)	177 (1)	177 (25)	177 (7)	177 (6)	177 (2)	177 (9)
[Glu+H-2H] <sup>+</sup>	175 (4)		175 (2)	175 (1)	175 (1)		175 (1)	
[Glu+H-H <sub>2</sub> O] <sup>+</sup>	159 (5)	159 (16)	159 (7)	159 (37)	159 (15)	159 (10)	159 (3)	159 (16)
[Glu+H-2H <sub>2</sub> O] <sup>+</sup>	141 (3)	141 (8)	141 (3)	141 (56)	141 (25)	141 (12)	141 (6)	141 (22)
m/z=131	(2)	(3)	(3)					
m/z=113	(2)	(1)	(2)					
m/z=109	(9)	(3)	(2)					
m/z=97	(18)	(10)	(1)					
m/z=85	(2)	(2)	(2)					

Abbreviations: see Table 1.