Sulphates of 3β-hydroxy-5-ene Steroids in Women with Epilepsy

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Abstract: Epilepsy is associated with various reproductive disorders and some antiepileptic drugs also influence the steroid metabolism. There is only limited data concerning the role of steroid sulphates in human epilepsy. Moreover, the substitution treatment with therapeutic substances also improves cognitive functions in humans. Therefore, we evaluated the balance between free and Δ^5 sulphated steroids in women with epilepsy on various antiepileptic drugs. The study included 28 patients (17.0–51.0 years), with generalized (n=16) or catamenial epilepsy (n=12) followed in the follicular (FP) and luteal (LP) phases of menstrual cycle. Fifteen patients were on monotherapy and 13 were on polytherapy with 2 or 3 drugs. RIA was used for the steroid analyses. Statistical evaluation was done by Mann-Whitney tests and multivariate regression with reduction of dimensionality (Orthogonal Projections to Latent Structures, O2PLS). The final O2PLS model found a single significant predictive component extracting the variability shared between carbamazepine therapy, age of the subjects, and steroid levels and correlating with the variables as follows pregnenolone sulphate (PregS)-FP: R= –0.844, $p<0.01$; DHEAS-FP: R= –0.923, $p<0.01$; PregS-LP: R= –0.876, $p<0.01$; DHEAS-LP: R= –0.902, $p<0.01$; carbamazepine therapy: R=0.441, $p<0.01$; age of the participants (R=0.584, $p<0.01$). Carbamazepine significantly decreased DHEAS in both FP ($p=0.02$) and LP ($p=0.003$) and PregS in LP ($p=0.03$) and tended to decrease the PregS

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levels in FP (p=0.10), while primidone decreased DHEAS in both FP and LP (both p=0.05) and did not significantly change the levels of PregS. In conclusion, carbamazepine and primidone therapies significantly suppressed the sulphated steroids in serum.

Introduction

Epilepsy in women is associated with various reproductive disorders like hypogonadotropic hypogonadism (Herzog, 1991a, 1991b; Meo and Bilo, 2003; Morrell, 2003), lower fertility (Morrell, 1999; Pimentel, 2000), abnormalities in luteotropin secretion (Stoffel-Wagner et al., 1998; Morrell, 1999; Tauboll et al., 2003), higher frequency of polycystic ovary syndrome (PCOS) (Herzog, 1991a; Isojarvi, 2003; Meo and Bilo, 2003), higher frequency of anovulatory cycles and with disturbances in the formation of ovarian steroids (Stoffel-Wagner et al., 1998; Morrell, 1999). Some antiepileptic drugs (AED) have also profound impact on steroid metabolism. Carbamazepine, phenytoin and phenobarbitone induce the hepatic P450 cytochrome enzyme system and stimulate steroid clearance. Carbamazepine therapy results in decline of serum TSH, DHEAS and LH in the follicular phase (FP) of menstrual cycle, and estradiol in the LP, otherwise in increase of the cortisol, SHBG and prolactin levels (Isojarvi, 1990; Stoffel-Wagner et al., 1998; Morrell, 2003). Phenytoin treatment lowers circulating adrenal and ovarian steroids as DHEAS and estrogens and mounts circulating cortisol and 5α-reductase activity (Soory and Suchak, 2001; Morrell, 2003). Valproate treatment suppresses cortisol levels (Aydin et al., 2005) and induces a metabolic syndrome with centripetal obesity, hyperinsulinemia, lipid abnormalities, and polycystic ovaries/hyperandrogenism in women with epilepsy. These valproate side effects can be reduced by substituting valproate with lamotrigine (Isojarvi et al., 1998). Testosterone levels and free androgen index are mostly higher in valproate-treated women (Isojarvi et al., 2005). It is probable, that some of these hormonal changes could influence the course of epilepsy. Lamotrigine appeared to have minimum effect on gonadal steroidogenesis, however minor changes in ovarian morphology or alterations in other tissues were observed in lamotrigine treated female rats. Although serum estradiol was significantly reduced, testosterone, FSH, LH, insulin and progesterone remained unchanged in the lamotrigine treated animals (Roste et al., 2003). Concerning hormonal changes after topiramate use, there is no human data available.

Various adrenal and gonadal steroids can surpass the blood-brain barrier. Besides the binding to intracellular receptors in brain, some steroids, their metabolites as well as locally produced brain steroids (which are known as neurosteroids) can bind to active sites of neuronal membrane receptors and influence the ion transport and neuronal activity (Bixo et al., 1997; Joels, 1997; Edwards et al., 1999b; Morrell, 1999; Pimentel, 2000; Beyenburg et al., 2001; Rupprecht, 2003). The neurosteroids and steroid neuromodulators of peripheral

origin are known as neuroactive steroids. The first group of neuroactive steroids increases neuronal activity and consequently the cognitive abilities and memory. Alternatively, some of these substances may also increase the neuronal excitability and frequency of epileptic seizures. Estradiol influences synaptic connectivity and increases the neuronal excitability (Edwards et al., 1999a, b) but could act also as a neuroprotective substance like dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA) and its 7-hydroxy and 7-oxo metabolites (Mao and Bager, 1998; Bastianetto et al., 1999; Jellinck et al., 2001; Morfin and Starka, 2001; Wise, 2003; Leskiewicz et al., 2008). Pregnenolone sulphate (PregS) may be either excitotoxic or neuroprotective, depending on the type of neurotransmitter receptor-associated channels (Weaver et al., 1998; Shirakawa et al., 2005).

The second group of neuroactive steroids consisting of progesterone (Klein and Herzog, 1998) and some of its reduced metabolites (Frye, 1995) increases during the luteal phase (LP) of the menstrual cycle and possesses anticonvulsive, hypnotic, anxiolytic and sedative effects. Besides the aforementioned neuroactive steroids, some reduced C19-steroids (Mao and Barger, 1998; Bastianetto et al., 1999; Jellinck et al., 2001; Morfin and Starka, 2001; Wise, 2003; Reddy, 2004) (which are less dependent of the phase of menstrual cycle) (Kaminski et al., 2005) also exert these effects, which, however, may not be a monotonous function of concentration (Backstrom et al., 2003). Sulphation of NS or hydrolysis of their polar conjugates can invert the neuromodulatory effects of the original substances (Park-Chung et al., 1999). Some authors reported that women with epilepsy had reduced progesterone levels in the LP (Murri et al., 1986; Bonuccelli et al., 1989; Herzog, 1991a) and others showed a lower ratio of the inhibitory 3α-pregnanolone isomers (PI) to its competitors (3β-isomers) on type A γ-aminobutyric acid receptors ($GABA_A$-r) (Stoffel-Wagner, 2001; Backstrom et al., 2003; di Michele et al., 2003; Lundgren et al., 2003). Progesterone deficiency may be associated with the lack of its inhibitory metabolites 3α-PI, which may correlate with a higher frequency of epileptic seizures. Several studies indicated a connection between the catamenial epilepsy and the disturbances in the biosynthesis of progesterone and its reduced metabolites (Galli et al., 2001; Backstrom et al., 2003; Grosso et al., 2003). A number of studies in women with epilepsy evaluated the role of estradiol and progesterone balance (Murialdo et al., 1998, 2009; Stoffel-Wagneret al., 1998; Galimberti et al., 2009) in the pathogenesis of the disease. Progesterone and its derivatives are also suggested as anticonvulsant therapy (Herzog, 2009; Reddy, 2009; Reddy and Rogawski, 2009). However, there is only limited data available in the literature concerning the role of steroid sulphates in human epilepsy. Therefore, the goal of the present study was to evaluate the alterations in the balance between free and Δ^5 sulphated steroids in women with epilepsy who were treated with valproate, carbamazepine, lamotrigine, primidone, topiramate and phenytoin.

Material and Methods

Subjects

The study was cross-sectional and included 28 women (17.0–51.0 years of age) either with generalized epilepsy (n=16) or with catamenial epilepsy (n=12). All subjects s were followed in both FP and LP. The study subjects did not use any drug known to interfere with the steroid biosynthesis and metabolism and did not have any other endocrine disorder, were non-smokers and did not consume alcohol. Sixteen of the women had irregular menstrual cycle and 12 had eumenorrhoea. Epilepsy onset occurred between 1.5 and 37 years of age and lasted from 1 to 50 years. Concerning the therapy with AEDs, 15 patients were treated by monotherapy (4 with valproate, 7 with carbamazepine, 2 with lamotrigine, 1 with primidone and 1 with phenytoin) and 13 women were on polytherapy with 2 or 3 drugs in various combinations of above named medicaments (Tables 1–4).

Table 1 – Levels of hormonal and epilepsy indices in the follicular and luteal phase of the menstrual cycle in patient on polytherapy, valproate-taking group vs. patients not using valproate

Variable	Using valproate	Not using valproate	Difference p-value
Epilepsy since (years)	n=7, 10 (7.5, 16.5)	n=20, 15.5 (7.5, 23.3)	0.332257
Epilepsy duration (years)	n=7, 16 (5, 23)	n=20, 11 (4, 21.5)	0.824294
Menarche (years)	n=8, 14 (12.8, 14)	n=18, 13 (12, 14)	0.525500
Age (years)	n=8, 27 (22.5, 34.8)	n=20, 28 (22.8, 32.5)	0.818720
	Follicular phase		
Pregnenolone sulfate (nmol/l)	n=8, 104 (69.1, 138)	n=20, 96.7 (31.9, 159)	0.799284
Pregnenolone (nmol/l)	n=5, 0.353 (0.335, 0.43)	n=14, 0.683 (0.397, 1.03)	0.095619
17-hydroxypregnenolone (nmol/l)	n=8, 2.9 (2.23, 3.6)	n=14, 3.6 (1.73, 6.28)	0.494786
DHEA (nmol/l)	n=8, 27.7 (14.1, 35.8)	n=20, 20.4 (17.3, 24.5)	0.799284
DHEAS (μmol/l)	n=8, 4.86 (2.05, 6.42)	n=20, 3.27 (1.03, 4.57)	0.203597
Progesterone (nmol/l)	n=8, 0.63 (0.483, 0.888)	n=20, 1.11 (0.715, 1.82)	0.154466
Cortisol (nmol/l)	n=6, 524 (485, 571)	n=14, 469 (312, 764)	0.457901
17-hydroxyprogesterone (nmol/l)	n=7, 1.57 (1.15, 2.11)	n=17, 1.42 (1.06, 2.07)	0.775033
	Luteal phase		
Pregnenolone sulfate (nmol/l)	n=8, 204 (172, 210)	n=20, 143 (47.8, 206)	0.309108
Pregnenolone (nmol/l)	n=7, 0.252 (0.196, 0.983)	n=16, 0.887 (0.649, 1.52)	0.094844
17-hydroxypregnenolone (nmol/l)	n=8, 4.05 (2.73, 8.93)	n=14, 5.5 (2.5, 7.53)	0.811144
DHEA (nmol/l)	n=8, 30.8 (18.1, 40.6)	n=20, 22.3 (17.8, 29.3)	0.445570
DHEAS (μmol/l)	n=8, 6.51 (2.66, 7.52)	n=20, 2.88 (1.7, 5.04)	0.093307
Progesterone (nmol/l)	n=8, 2.09 (1.1, 34.9)	n=20, 7.42 (1.65, 15.7)	0.838786
17-hydroxyprogesterone (nmol/l)	n=3, 1.72 (1.7, 1.76)	n=9, 2.04 (1.61, 4.27)	0.405381

After signing informed consent form approved by the Ethics Committee of the Institute of Endocrinology, all women underwent twice blood sampling: between the 1st–5th and 22nd–24th day of the spontaneous menstrual cycle. For evaluation of the analytes 10 ml of blood was withdrawn on fasting in the morning. Blood samples were centrifuged and stored at –20 °C until analyzed.

Steroid analysis

Pregnenolone, 17-hydroxy-pregnenolone and PregS were measured by RIA as described in our previous reports (Hill et al., 1999a, b, 2002), however a brief description of the methods is provided.

The serum containing pregnenolone underwent diethyl-ether extraction of 200 μl of serum in the first step. Although, the antiserum against steroid-19-O-(carboxymethyl)oxime-BSA was completely unspecific for the 4-ene steroid

Table 2 – Levels of hormonal and epilepsy indices in the follicular and luteal phase of the menstrual cycle in patient on polytherapy, carbamazepine-taking group vs. patients not using carbamazepine

Variable	Using carbamazepine	Not using carbamazepine	Difference p-value
Epilepsy since (years)	n=15, 13 (7, 22.5)	n=12, 14.5 (8, 18.5)	0.541347
Epilepsy duration (years)	n=15, 15 (4.5, 21)	n=12, 6.5 (4, 22.5)	0.731850
Menarche (years)	n=13, 13 (12, 14)	n=13, 14 (12, 14)	0.894040
Age (years)	n=15, 28 (23.5, 36.5)	n=13, 28 (22, 32)	0.380725
	Follicular phase		
Pregnenolone sulfate (nmol/l)	n=15, 56.9 (30.8, 113)	n=13, 134 (93.9, 184)	0.101982
Pregnenolone (nmol/l)	n=11, 0.665 (0.427, 1.13)	n=8, 0.399 (0.348, 0.706)	0.247676
17-hydroxypregnenolone (nmol/l)	n=10, 3.45 (1.83, 6.55)	n=12, 3.05 (1.85, 5)	0.620830
DHEA (nmol/l)	n=15, 18.6 (15.8, 22.6)	n=13, 33.9 (21.4, 40.2)	0.050256
DHEAS (μmol/l)	**n=15, 1.95 (1.02, 3.77)**	**n=13, 5.15 (3.47, 6.35)**	**0.022594**
Progesterone (nmol/l)	n=15, 1.28 (0.77, 1.84)	n=13, 0.73 (0.53, 1.04)	0.240128
Cortisol (nmol/l)	n=11, 518 (317, 736)	n=9, 491 (352, 576)	0.849361
17-hydroxyprogesterone (nmol/l)	n=13, 1.38 (0.69, 2)	n=11, 1.57 (1.12, 2.1)	0.582047
	Luteal phase		
Pregnenolone sulfate (nmol/l)	**n=15, 131 (39.6, 188)**	**n=13, 208 (106, 251)**	**0.028662**
Pregnenolone (nmol/l)	n=12, 0.887 (0.593, 1.43)	n=11, 0.737 (0.24, 1.18)	0.498404
17-hydroxypregnenolone (nmol/l)	n=10, 6.3 (3.65, 9.15)	n=12, 4.05 (1.3, 6.78)	0.198389
DHEA (nmol/l)	n=15, 18.6 (17.6, 30.8)	n=13, 26.4 (19.3, 33.8)	0.299983
DHEAS (μmol/l)	**n=15, 2.49 (1.25, 3.62)**	**n=13, 5.49 (4.02, 7.46)**	**0.002966**
Progesterone (nmol/l)	n=15, 5.41 (1.59, 19.1)	n=13, 7.1 (1.19, 14.6)	0.981622
17-hydroxyprogesterone (nmol/l)	n=8, 1.94 (1.36, 5.27)	n=4, 1.76 (1.71, 2.2)	0.734095

analogues, the matrix effect was completely removed by inserting the HPLC separation step as follows: The dried diethyl ether extract was dissolved in 100 μl of methanol and 25 μl of the solution was applied into the HPLC system and eluted with a methanol-water mixture (methanol 68%) at 50 °C. The flow rate was 1 ml/min. The fraction containing pregnenolone (1 ml) was collected according to the retention time of the standard chromatographed before each series of samples. HPLC was usually carried out overnight taking advantage of the automatic sample processor. The standard solutions were prepared in steroid-free serum by serial dilution of the highest concentrations of pregnenolone, which was 101.1 nmol/l. Eight-point calibration curve was constructed for each steroid and processed like the samples. The dry residues of collected fractions were dissolved in 100 μl of the assay buffer (20 mM sodium phosphate, pH 7.1 in saline, containing sodium azide and BSA 0.1 g/l. To each set of tubes the corresponding radioligand

Table 3 – Levels of hormonal and epilepsy indices in the follicular and luteal phase of the menstrual cycle in patient on polytherapy, primidone-taking group vs. patients not using primidone

Variable	Using primidone	Not using primidone	Difference p-value
Epilepsy since (years)	n=5, 24 (9, 26)	n=22, 13 (6.5, 19.8)	0.287973
Epilepsy duration (years)	n=5, 17 (5, 23)	n=22, 11 (4, 20.5)	0.531185
Menarche (years)	n=5, 13 (13, 14)	n=21, 14 (12, 14)	0.839311
Age (years)	**n=5, 39 (29, 49)**	**n=23, 26 (22, 31)**	**0.024270**
	Follicular phase		
Pregnenolone sulfate (nmol/l)	n=5, 56.9 (42.2, 184)	n=23, 102 (42.4, 142)	0.928305
Pregnenolone (nmol/l)	n=5, 0.955 (0.665, 1.06)	n=14, 0.399 (0.34, 0.715)	0.064078
17-hydroxypregnenolone (nmol/l)	n=5, 4.2 (3, 7.1)	n=17, 2.7 (1.6, 4.9)	0.308304
DHEA (nmol/l)	n=5, 21.3 (17.7, 24.5)	n=23, 21.4 (15.4, 36.9)	0.833709
DHEAS (μmol/l)	**n=5, 0.99 (0.8, 1.84)**	**n=23, 3.85 (2.12, 6.06)**	**0.044484**
Progesterone (nmol/l)	n=5, 1.32 (0.78, 1.56)	n=23, 1.02 (0.595, 1.55)	0.490303
Cortisol (nmol/l)	n=3, 526 (474, 707)	n=17, 491 (346, 679)	0.427263
17-hydroxyprogesterone (nmol/l)	n=4, 1.79 (1.54, 2.58)	n=20, 1.34 (0.985, 2.08)	0.187901
	Luteal phase		
Pregnenolone sulfate (nmol/l)	n=5, 107 (80.9, 202)	n=23, 181 (73.2, 213)	0.568774
Pregnenolone (nmol/l)	n=4, 0.617 (0.403, 0.889)	n=19, 0.868 (0.55, 1.33)	0.372276
17-hydroxypregnenolone (nmol/l)	n=5, 4.4 (3.4, 5.9)	n=17, 5.1 (2.2, 8.5)	0.814128
DHEA (nmol/l)	n=5, 18.6 (18, 25.5)	n=23, 25.8 (18, 37.2)	0.352491
DHEAS (μmol/l)	**n=5, 1.27 (1.01, 1.92)**	**n=23, 4.12 (2.56, 6.44)**	**0.044484**
Progesterone (nmol/l)	n=5, 7.73 (5.41, 14.5)	n=23, 2.68 (1.34, 24.7)	1.000000
17-hydroxyprogesterone (nmol/l)	n=3, 2.04 (1.94, 2.72)	n=9, 1.72 (1.61, 4.27)	0.405381

(15,000 cpm/tube) and 100 μl of the appropriately diluted antiserum (1:60,000) for pregnenolone was added and the volume was adjusted to 300 μl with buffer. The overnight incubation at 4 °C followed. Suspension (500 μl) of dextran-coated charcoal (0.025 g/100 ml Dextran T-70, 0.25 g/100 ml NORIT A) was added to the tubes, mixed, and after 10 min standing at 4 °C centrifuged at 800×g for 10 min. Radioactivity in the supernatant was measured on a 12-channel γ-counter (Berthold, Germany). Homologous radioligands were prepared by radioiodination of 19-O-(carboxymethyl)oxime tyrosine methyl ester (19-CMO-TME) derivatives of analyzed steroids by the standard chloramine T method using carrier-free Na[125I] from the Institute of Radiochemistry, Hungarian Academy of Sciences (Hungary). The antiserum was prepared as follows. The 19-CMO derivative of pregnenolone was coupled with bovine serum albumin (BSA) by a mixed anhydride method. The resulting immunogens were emulsified in a mixture of complete Freund's

Table 4 – Levels of hormonal and epilepsy indices in the follicular and luteal phase of the menstrual cycle in patient on polytherapy, topiramate-taking group vs. patients not using topiramate

Variable	Using topiramate	Not using topiramate	Difference p-value
Epilepsy since (years)	n=6, 7 (2.75, 17.3)	n=21, 16 (10, 20)	0.350086
Epilepsy duration (years)	n=4, 18 (13.3, 19.5)	n=23, 8 (4, 23)	0.412714
Menarche (years)	n=5, 13 (12, 14)	n=21, 14 (12, 14)	0.452828
Age (years)	n=5, 26 (24, 29)	n=23, 28 (22, 35.5)	0.770098
	Follicular phase		
Pregnenolone sulfate (nmol/l)	n=5, 106 (69.8, 124)	n=23, 93.9 (30.8, 150)	0.652008
Pregnenolone (nmol/l)	n=3, 0.84 (0.663, 0.897)	n=16, 0.544 (0.348, 0.805)	0.841481
17-hydroxypregnenolone (nmol/l)	n=4, 4.15 (2.28, 7.83)	n=18, 3.05 (1.73, 4.88)	0.319486
DHEA (nmol/l)	**n=5, 14.5 (13.9, 21.3)**	**n=23, 22 (17.9, 36.5)**	**0.031653**
DHEAS (μmol/l)	n=5, 3.47 (1.84, 5.99)	n=23, 3.69 (1.16, 5.18)	0.541768
Progesterone (nmol/l)	n=5, 1.32 (0.84, 2.41)	n=23, 1.02 (0.51, 1.42)	0.936568
Cortisol (nmol/l)	**n=3, 576 (567, 730)**	**n=17, 483 (346, 679)**	**0.029758**
17-hydroxyprogesterone (nmol/l)	n=4, 1.44 (0.991, 2.36)	n=20, 1.5 (1.09, 2.08)	0.230139
	Luteal phase		
Pregnenolone sulfate (nmol/l)	n=5, 195 (171, 202)	n=23, 156 (47, 213)	0.541768
Pregnenolone (nmol/l)	n=2, 1.06 (0.981, 1.13)	n=21, 0.737 (0.475, 1.34)	0.570188
17-hydroxypregnenolone (nmol/l)	n=4, 6.45 (2.6, 10.8)	n=18, 4.9 (2.5, 7.53)	0.301892
DHEA (nmol/l)	n=5, 18.3 (8.7, 27.8)	n=23, 25.5 (18.2, 33.8)	0.381345
DHEAS (μmol/l)	n=5, 4.02 (1.92, 5)	n=23, 3.12 (1.8, 6)	0.300861
Progesterone (nmol/l)	n=5, 19.1 (2.17, 30.2)	n=23, 5.41 (1.34, 14.6)	0.507146
17-hydroxyprogesterone (nmol/l)	n=1, 0.59 (0.59, 0.59)	n=11, 1.84 (1.7, 3.83)	0.192379

adjuvant-saline (1:1, v/v) and used for immunization of rabbits. Intraassay CV for pooled serum samples with low (1.91 ± 0.042 nmol/l) (mean ± SD), medium (4.37 ± 0.176 nmol/l) and high (7.98 ± 0.271 nmol/l) pregnenolone concentration were 2.20%, 4.03% and 3.40%, respectively. The interassay CV for pooled serum samples with medium and high pregnenolone concentration were 14.7% and 4.40%, respectively.

The method for determination of 17-hydroxy-pregnenolone was analogous except the HPLC separation step. Polyclonal antiserum against 3β,17β-dihydroxypregn-5-en-20-one-19-O-(carboxymethyl)-oxime bovine serum albumin (17β-hydroxypregnenolone-19-CMO:BSA), was raised in rabbits. Its main structural determinants were the substituents on D-ring as demonstrated by its 107% cross-reaction with 17β-hydroxyprogesterone. This unspecificity was almost completely eliminated by addition of the excess of the cross-reactant directly to the analytical system. The contribution of the cross-reactant from the sample in such a system became negligible due to saturation of the populations of polyclonal antibodies recognizing the analyte as well as the cross-reactant. The possible interference of 17β-hydroxypregnenolone-3-sulfate was avoided by inserting ether extraction. The analytical system appeared to be stable to differences in cross-reactant concentrations even in samples from patients with pathologically elevated serum levels of 17β-hydroxyprogesterone. Intraassay and interassay CV for pooled serum samples were 8.30% and 15.5%, respectively.

The method for determination of PregS took an advantage of the great excess of pregnenolone polar conjugates (mainly pregnenolone sulphate) over the unconjugated steroid and unspecificity of the same antiserum, which was used for the determination of unconjugated pregnenolone. This antiserum was unspecific towards the steroid A-ring showing 42% cross-reactivity with PregS. Therefore the PregS was assayed in 100 μl of serum using the non-extraction method. Intraassay CV for pooled serum samples with low (197 nmol/l), medium (231 nmol/l) and high (497 nmol/l) PregS concentration were 4.6%, 8.0% and 7.1%, respectively. The interassay CV for pooled serum samples with low, medium and high PregS concentration were 14.6% and 19.6%, and 14.7% respectively.

17-hydroxyprogesterone was assayed by RIA kit from Immunotech, France (intra-assay CV=5.2%, inter-assay CV=6.5%), cortisol was measured using RIA kit from Orion, Finland (intra-assay CV=3.8%, inter-assay CV=4.4%), progesterone was assessed by RIA kit from Orion, Finland (intra-assay CV=7.9%, inter-assay CV=3.7%), DHEA was estimated by RIA kit from Immunotech, France (intra-assay CV=7.2%, inter-assay CV=11.9%) and DHEAS was measured by RIA kit from Immunotech, France (intra-assay CV=4.2%, inter-assay CV=7.2%).

Statistical data analysis

For the clearness, the contrasts between groups of subjects using individual AEDs and the remaining subjects not-using these AEDs were firstly assessed using

a robust Mann-Whitney test for comparison of medians between two groups or Dunn's test for multiple comparisons, however, for the more appropriate simultaneous evaluation the effects of individual AEDs in patients on polytherapy on the serum steroids, a multivariate regression with reduction of dimensionality was applied, using a method of Bidirectional Orthogonal Projections to Latent Structures (O2PLS) (Trygg and Wold, 2002; Trygg et al., 2007). The model consisted of binary variables expressing whether the individual AEDs were applied or not (value 1) or not (value 0) and age of the participants. This approach was effective in coping with the problem of severe multicollinearity within the matrixes of both dependent and independent variables. This was the case in our data being highly inter-correlated within the matrix of serum steroids (dependent variable). The O2PLS enabled us to find the serum steroids being highly correlated with the AEDs, to find the structure of the relationships between *X* and *Y* and finally to find which serum steroids are affected by individual AEDs. The O2PLS model may be expressed as follows:

$$X = T_p P_p + T_0 P_0 + E$$
$$Y = U_p Q_p + U_0 Q_0 + F$$

where: *X* is the matrix with *I* independent variables and *i* subjects, *Y* is the matrix of *m* dependent variables and *i* subjects. T_p and T_0 represent the matrixes of component scores from the predictive and orthogonal components, respectively extracted from *X*, P_p and P_0 represent the matrixes of component loadings from the predictive and orthogonal component, respectively from *X*. Similarly, U_p and U_0 represent the matrixes of component scores from the predictive and orthogonal component, respectively extracted from *Y*. Q_p and Q_0 represent the matrixes of component loadings from the predictive and orthogonal component from *Y*. *E* and *F* are error terms.

To eliminate heteroscedasticity and skewed distribution in the data and residuals, the original data was transformed (to attain Gaussian distribution before further processing) by a Box-Cox transformation using the statistical software Statgraphics Centurion, version XV from Statpoint Inc. (Herndon, Virginia, USA). The statistical software SIMCA-P+ Version 12.0.0.0 from Umetrics AB (Umeå, Sweden) was used for data analysis. The software enabled us to find the number of the relevant components utilizing the prediction error sum of squares and also allowed the detection of multivariate non-homogeneities and testing the multivariate normal distribution and homoscedasticity.

Results

The contrasts between groups of subjects using individual AEDs and the remaining subjects not-using these AEDs as evaluated by a robust Mann-Whitney test are shown in Tables 1–4. The Mann-Whitney test indicated that valproate did not significantly change the levels of investigated steroids (Table 1). Carbamazepine significantly decreased DHEAS in both FP ($p=0.02$) and LP ($p=0.003$) and PregS

Table 5 – Relationships between treatment with antiepileptic drugs and relevant serum steroids in the follicular (FP) and luteal phase (LP) of the menstrual cycle in women with epilepsy as simultaneously evaluated by multivariate regression with reduction of dimensionality using the method of orthogonal projections to latent structures (O2PLS)

Variable		Comp. l.	95% CI	99% CI	Comp. l. /95% CI	Comp. l. /99% CI	R^a	Statistical significance
Dependent variables								
Follicular phase	PregS	–0.478	0.145	0.230	–3.29	–2.08	–0.844	p<0.01
	DHEAS	–0.523	0.106	0.167	–4.95	–3.13	–0.923	p<0.01
Luteal phase	PregS	–0.496	0.114	0.180	–4.37	–2.76	–0.876	p<0.01
	DHEAS	–0.511	0.137	0.216	–3.74	–2.36	–0.902	p<0.01
Predictors								
Carbamazepine		0.640	0.178	0.281	3.60	2.28	0.441	p<0.01
Age of the participants		0.770	0.203	0.322	3.79	2.39	0.584	p<0.01

Comp. l. – component loading; R^a – component loading expressed as a correlation coefficient with the predictive component; CI – confidence interval

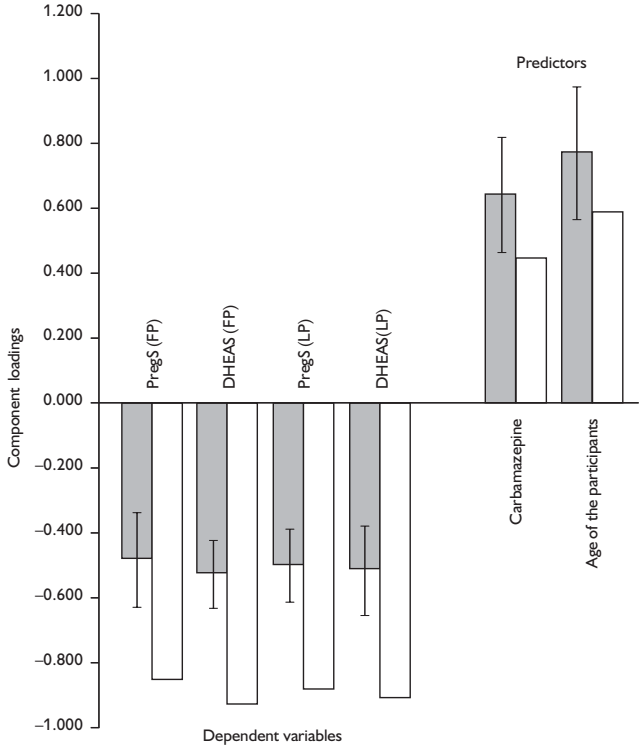

Figure 1 – Effects of carbamazepine therapy and age of the participants on the serum levels of pregnenolone sulphate (PregS) and dehydroepiandrosterone sulphate (DHEAS) in the follicular (FP) and luteal (LP) phases of menstrual cycle as evaluated using multivariate regression with reduction of dimensionality (the method of Bidirectional Orthogonal Projections to Latent structures, O2PLS). Full and empty bars represent component loadings of individual variables with the common predictive component, which are expressed as regression and correlation coefficients, respectively. The error bars represent 95% confidence intervals of the regression coefficients.

in LP ($p=0.03$). Carbamazepine also tended to decrease the PregS levels in FP ($p=0.10$) and DHEA in FP ($p=0.06$) but did not significantly change the levels of Prog, Preg17, Prog17 and cortisol (Table 2). Lamotrigine produced no significant change in the steroid levels as did phenytoin (data not shown). Primidone decreased DHEAS in both FP and LP (both $p=0.05$) and did not significantly change the levels of PregS, Prog, Preg17, Prog17, and DHEA (Table 3). Topiramate decreased DHEA ($p=0.03$) in FP but did not influence this steroid in LP. For PregS, Prog, Preg17, Prog17, DHEAS, there was no significant change in any phase of the menstrual cycle after topiramate application (Table 4).

To verify the results obtained using Mann-Whitney test and to adjust steroid levels to constant age, we have applied the O2PLS model (Table 5, Figure 1). A single significant predictive component extracting the variability shared between AEDs and steroid levels was detected. The model consisted of the relevant steroids, age of the participants and a single relevant AED – carbamazepine, which were selected using the variable importance criterion (Trygg and Wold, 2002; Trygg et al., 2007). The correlations of common predictive component with individual steroids and AEDs were as follows PregS-FP: $R= –0.844$, $p<0.01$; DHEAS-FP: $R= –0.923$, $p<0.01$; PregS-LP: $R= –0.876$, $p<0.01$; DHEAS-LP: $R= –0.902$, $p<0.01$; Carbamazepine: $R=0.441$, $p<0.01$, age of the participants ($R=0.584$, $p<0.01$). Carbamazepine significantly suppresses the levels of PregS and DHEAS, topiramate decrease DHEA concentrations in the FP, while valproate, lamotrigine, primidone, and phenytoin did not exert a significant effect. As expected, the age of the participants negatively correlated with DHEAS and PregS levels.

Discussion

The main outcome of this study is the finding that carbamazepine and primidone significantly reduce steroid sulphate levels. Pregnenolone sulphate is a potent neuroactive steroid influencing brain functions and exerting cognitive enhancing, promnesic, antistress and antidepressant effects. Besides the effect on postsynaptic receptors, at the cellular level PregS regulates the release of many important neurotransmitters such as glutamate, γ-aminobutyric acid, acetylcholine, norepinephrine and dopamine. Although these effects are mostly stimulatory, their mechanisms appear to be complicated (Zheng et al., 2009). As documented by Williamson and colleagues (Williamson et al., 2004), PregS exerts excitotoxic effects in rats and induces seizures and *status epilepticus* when administered systemically or directly into the brain (Reddy and Kulkarni, 1998; Williamson et al., 2004). PregS and DHEAS inhibit the $GABA_A$-r function, and is also moderately potent allosteric agonist at NMDA receptors (Majewska et al., 1990; Wu et al., 1991). The proconvulsant or convulsant actions of PregS are evident at high micromolar concentrations, which are 100–500-fold higher than its levels in the brain. Thus, it is highly unlikely that endogenous PregS by itself can trigger seizures. However, PregS can decrease GABAergic inhibitory transmission at physiological concentrations via

a presynaptic action (Teschemacher et al., 1997). Alternatively, allopregnanolone blocks the seizure-facilitating effects of PregS, and therefore PregS could contribute to seizure susceptibility when allopregnanolone levels are low. On the other hand, Marx et al. (2009) reported that Preg supplementation improved negative symptoms and cognitive functions in patients with schizophrenia.

DHEAS operating as a moderately potent allosteric agonist at NMDA receptors (Wu et al., 1991) is a proconvulsant steroid and can induce seizures and *status epilepticus* when administered systemically or directly into the brain (Reddy and Kulkarni, 1998). Our data show that carbamazepine and primidone decreased DHEAS levels. In addition, carbamazepine also suppressed PregS and decreased DHEA in FP and topiramate significantly decreased DHEA in the follicular phase of menstrual cycle. These results indicate that carbamazepine has a more profound effect mostly suppressing the activity of *zona reticularis*. Both carbamazepine and primidone therapy alter the balance between free and conjugated Δ^5 steroids suppressing the steroid conjugation, however the clinical importance of these changes remains to be clarified. Although, we did not observe any association between steroid levels and character of epilepsy, some authors document increased cortisol/DHEAS in more severe forms of epilepsy (Galimberti et al., 2005) and a significant reduction of the DHEAS/cortisol ratio in women with perimenstrual catamenial epilepsy (Tuveri et al., 2008). The latter results, however, indicate that the decrease of DHEAS levels induced by carbamazepine and primidone therapy may partly explain the indecent side effects of these drugs. We have also compared the levels of steroids, which were influenced by individual antiepileptic drugs, with the respective values in the group of age-matched healthy controls. In accordance with the data reported in the literature (Isojarvi, 1990), we have found lower DHEAS in both phases of menstrual cycle in women treated with enzyme inducing antiepileptic drugs such as carbamazepine and/or primidone. The DHEAS levels in controls, were 4.87 (4.4, 8.1) and 5.4 (3.83, 6.03) (medians with quartiles in μmol/l) for follicular and luteal phases of menstrual cycle, respectively (for comparison with the patients' groups see also Tables 2 and 3). The patients treated with carbamazepine exhibited comparable levels of pregnenolone sulphate like controls, while the patients, not using carbamazepine, had insignificantly higher levels of pregnenolone sulphate in the follicular phase and significantly higher levels of the steroid conjugate in the luteal phase. The levels of pregnenolone sulphate in controls, were 58.7 (49.7, 81.6) and 87.2 (68.6, 107) (medians with quartiles in nmol/l) for FP and LP, respectively (for comparison with the patients' groups see also Table 2). The levels of DHEA in controls were 20.7 (18.5, 40.7) and 18.4 (17.9, 40.8) (medians with quartiles in nmol/l) for FP and LP, respectively, and did not significantly differ from those found in patients.

In conclusion, our study shows that both carbamazepine and primidone significantly decrease the levels of sulphated steroids and so this affect can be

considered as a part of their anti-epileptic activity. When considering the connection between deficiency in these steroid sulphates and tiredness, and eventually cognitive deficits which are untoward side effects of these drugs, the possibility of the concurrent substitution treatment with these drugs could be considered.

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