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Non-protein bound dienogest in serum and salivary dienogest in women taking the oral contraceptives Certostat® and Valette®

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Dienogest (17α-cyanomethyl-17-hydroxy-4,9-estradien-3-one) is the progestagen component of the oral contraceptives Certostat® and Valette®. In contrast to other 19-norsteroid progestagens like levonorgestrel, norethisterone, gestodene and 3-ketodesogestrel, dienogest does not bind to sexual hormone binding globulin (SHBG). The absent binding to SHBG results in a high portion of free, non-protein bound dienogest in serum. In female volunteers taking the oral contraceptives Certostat® and Trisiston®, the part of non-protein bound dienogest and levonorgestrel, respectively, in serum was determined by the method of centrifugal ultrafiltration. The portion of free dienogest was found to be 9.55 ± 0.95% ($m \pm s_D$, $n = 13$) of total serum dienogest. Free levonorgestrel constituted 0.97 ± 0.14% ($n = 12$) of total serum levonorgestrel. In an investigation with 47 female volunteers taking Certostat®, serum total dienogest was quantified by a specific radioimmunoassay and free dienogest in serum by centrifugal ultrafiltration. In the serum samples with dienogest concentrations in the range of 4.1–57.7 ng/ml, the part of free, non-protein bound dienogest was found to be 8.90 ± 0.54% of serum total dienogest. There is a high correlation between serum total dienogest and free dienogest ($r = 0.989$). In another investigation with 20 female volunteers taking the contraceptive Valette®, serum total dienogest and salivary dienogest were quantified by radioimmunoassay and free dienogest in serum by centrifugal ultrafiltration. In the serum samples with dienogest concentrations in the range of 7.5–50.6 ng/ml, the part of free, non-protein bound dienogest was 8.78 ± 0.77% of serum total dienogest. Salivary dienogest constituted 7.99 ± 0.94% of serum total dienogest showing a high correlation with serum free dienogest ($r = 0.953$) and serum total dienogest ($r = 0.958$). The high portion of non-protein bound compound in serum is a characteristic pharmacokinetic feature of dienogest.

1. Introduction

Steroid hormones in blood interact with binding proteins such as sexual hormone binding globulin (SHBG) and corticosteroid binding globulin (CBG), and also with albumin [1]. The steroid protein binding has considerable influence on the pharmacokinetics of a compound. It protects against a rapid biotransformation and elimination and can hinder the penetration of the compound into tissues and organs. The free, non-protein bound fraction of steroids in blood has been regarded for a long time as the bioavailable form of the compound which rapidly can distribute into tissues. Nowadays, there is increasing evidence that the steroids bound to albumin are likewise fairly bioavailable [2].

The majority of the synthetic 19-norsteroid progestagens like levonorgestrel, norethisterone, gestodene, and 3-ketodesogestrel, is known to be bound in blood by SHBG and albumin [3]. Only a few percent of these compounds is present in serum in a free, non-protein bound form [4–7]. First evidence that the new 19-norprogestagen dienogest (17α-cyanomethyl-17-hydroxy-4,9-estradien-3-one) is not bound to SHBG was obtained by gel electrophoresis under steady state conditions of plasma samples incubated with ^{3}H-dienogest. With this method and by competition experiments it was also demonstrated that dienogest cannot displace testosterone from its binding to SHBG [8]. Juchem et al. confirmed the absent binding of dienogest to SHBG in competition studies, and they also found no binding of dienogest to CBG [9].

Equilibrium dialysis studies according to Rivarola et al. [10] gave first evidence that dienogest is present in serum to a considerable part in a free form. This method had the disadvantage to work with diluted serum. The method of centrifugal ultrafiltration with membranes of a definite pore size allows to use undiluted serum and conditions which do not affect the equilibrium [11]. The Centrifree® devices provided by Amicon/Millipore are highly practicable and stand out by small sample volumes, a rapid separation and a low self-adsorption. This method was applied to determine the free, non-protein bound dienogest in serum samples of women taking the oral contraceptives Certostat® and Valette®. Since salivary steroids have been discussed to be highly correlated with the free steroid fraction in serum [12], in the Valette® group, salivary dienogest concentrations were simultaneously determined by radioimmunoassay.

2. Investigations, results and discussion

2.1. Comparison of non-protein bound dienogest and levonorgestrel in serum of female volunteers taking the oral contraceptives Certostat® and Trisiston®

The method of centrifugal ultrafiltration with the devices of Amicon/Millipore has proved to be a convenient procedure which is marked by its quickness and accuracy and by the fact that undiluted serum can be used. By incubating the serum samples with a definite quantity of the radioactive-labelled steroid, the tracer distributes between free and protein-bound steroid according to the present binding relation of the unlabelled steroid in the sample. By the process of ultrafiltration, only the free steroid can pass the membrane of the device and appears in the ultrafiltrate. Centrifugation occurs within few minutes and warrants favourable separation conditions. Relating to the total tracer in the system, the percent free, non-protein bound steroid is calculated. In reference to the total serum steroid concentration determined by radioimmunoassay, the free fraction in ng/ml can be derived.

In Table 1, single values of percent non-protein bound dienogest and levonorgestrel, respectively, in serum samples of female volunteers taking the oral contraceptives Certostat® and Trisiston® are listed. The blood samples of the volunteers were taken at different days of the cycle and at different times after intake of the dosage form.

Table 1: Free, non-protein bound dienogest and free levonorgestrel determined by centrifugal ultrafiltration in serum samples of female volunteers taking the oral contraceptives Certostat® and Trisiston®, respectively

Volunteer	Certostat® Group		% free Dienogest
	Cycle Day	Hours p.a.	
1	10	12	8.88
2	11	20	11.60
3	11	9	9.74
4	17	9	9.30
5	11	9	10.35
6	14	8	10.30
7	7	12	10.38
8	2	5	11.18
9	11	15	9.34
10	15	3	8.84
11	1	22	8.60
12	10	15	8.36
13	8	23	8.31

Free, non-protein bound Dienogest: 9.55 ± 0.95% ($m \pm s_D$; $n = 13$)

Volunteer	Trisiston® Group		% free Levonorgestrel
	Cycle Day	Hours p.a.	
14	8	22	0.96
15	5	12	0.96
16	3	20	1.13
17	18	20	0.78
18	17	24	1.11
19	11	21	0.92
20	17	22	0.84
21	18	22	0.87
22	11	24	0.83
23	21	23	0.98
24	10	24	1.28
25	8	23	0.94

Free, non-protein bound Levonorgestrel: 0.97 ± 0.14% ($m \pm s_D$; $n = 12$)

The free, non-protein bound dienogest constitutes 8–11% of total serum dienogest and differs significantly from the low levels of free levonorgestrel which have been found to be only about 1% of the total serum levonorgestrel. The data clearly demonstrate the significant difference in the portions of free, non-protein bound dienogest and levonorgestrel, respectively.

2.2. Serum total dienogest and free, non-protein bound dienogest in female volunteers taking the oral contraceptive Certostat®

In an other group of female volunteers taking the oral contraceptive Certostat®, total serum dienogest was determined by radioimmunoassay and the free, non-protein bound dienogest by centrifugal ultrafiltration. The total dienogest concentrations in the serum samples comprised the range of 4.1–57.7 ng/ml. Free dienogest constituted 8.90 ± 0.54% (range 7.80–10.07%) of total serum dienogest ($n = 47$). Fig. 1 shows the high correlation of serum total and free dienogest ($r = 0.989$).

2.3. Total dienogest and free, non-protein bound dienogest in serum, and salivary dienogest in female volunteers taking the oral contraceptive Valette®

Steroid concentrations in saliva are considered to reflect the free, biologically active fraction in blood. Since saliva samples can be collected in a stress-free, non-invasive

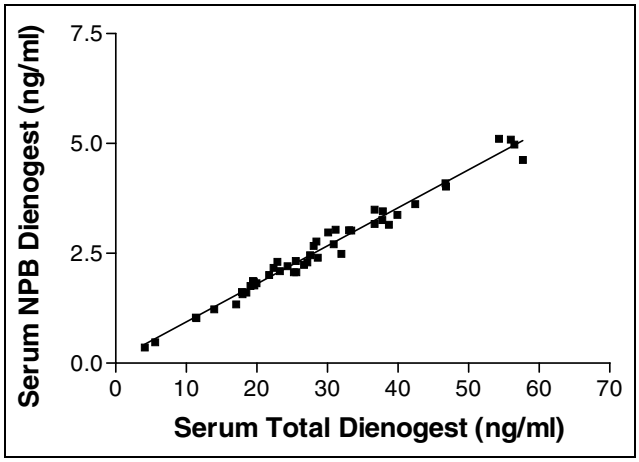

Fig. 1: Correlation between the serum concentrations of total dienogest and free, non-protein bound (NPB) dienogest in serum of female volunteers taking the oral contraceptive Certostat®

fashion, studies with this material offers substantial benefits when frequent sampling is necessary. There are various studies on salivary steroids which have demonstrated that interesting informations can be obtained with this material. Quantitation of progesterone in saliva was applied to assess ovarian function [13, 14]. Salivary testosterone concentrations were found to be better correlated with the stage of puberty than did serum total testosterone levels [15]. Salivary cortisol determinations were used to monitor fluctuations in adrenal secretory activity [16], and salivary norethisterone was useful to characterize the bioavailability of preparations and their effect on ovarian function [17]. The absence of specific and non-specific binding proteins in saliva facilitates the application of direct, non-extraction assays for quantitation.

In a group of female volunteers taking the oral contraceptive Valette®, total serum dienogest and salivary dienogest were determined by radioimmunoassay and the percent free, non-protein bound dienogest by centrifugal ultrafiltration. In Table 2, the data of 20 volunteers are compiled.

Table 2: Serum total dienogest (DNG) and salivary dienogest determined by radioimmunoassay, and serum free, non-protein bound (NPB) dienogest determined by centrifugal ultrafiltration in female volunteers taking the oral contraceptive Valette®

Volunteer	Serum DNG ng/ml	Salivary DNG ng/ml	NPB-DNG ng/ml
101	19.0	1.6 ≈ 8.4%	1.5 ≈ 8.0%
102	19.5	1.8 9.2%	1.8 9.0%
103	27.4	2.6 9.5%	2.6 9.6%
104	7.5	0.5 6.7%	0.6 8.2%
105	14.8	1.1 7.4%	1.2 7.9%
106	20.9	1.5 7.2%	1.6 7.8%
107	21.3	1.7 8.0%	1.6 7.7%
108	22.4	1.5 6.7%	1.9 8.4%
109	23.1	2.0 8.7%	1.9 8.4%
110	26.6	2.3 8.6%	2.3 8.7%
111	50.6	3.2 6.3%	4.6 9.0%
112	49.8	3.7 7.4%	4.4 8.8%
113	22.2	1.7 7.7%	1.9 8.6%
114	22.9	1.7 7.4%	2.2 9.4%
115	25.8	2.0 7.8%	2.2 8.7%
116	16.4	1.5 9.1%	1.6 9.7%
117	13.0	1.2 9.2%	1.3 9.9%
118	17.8	1.6 9.0%	1.8 10.4%
119	20.1	1.6 8.0%	1.9 9.5%
120	17.4	1.3 7.5%	1.4 8.0%

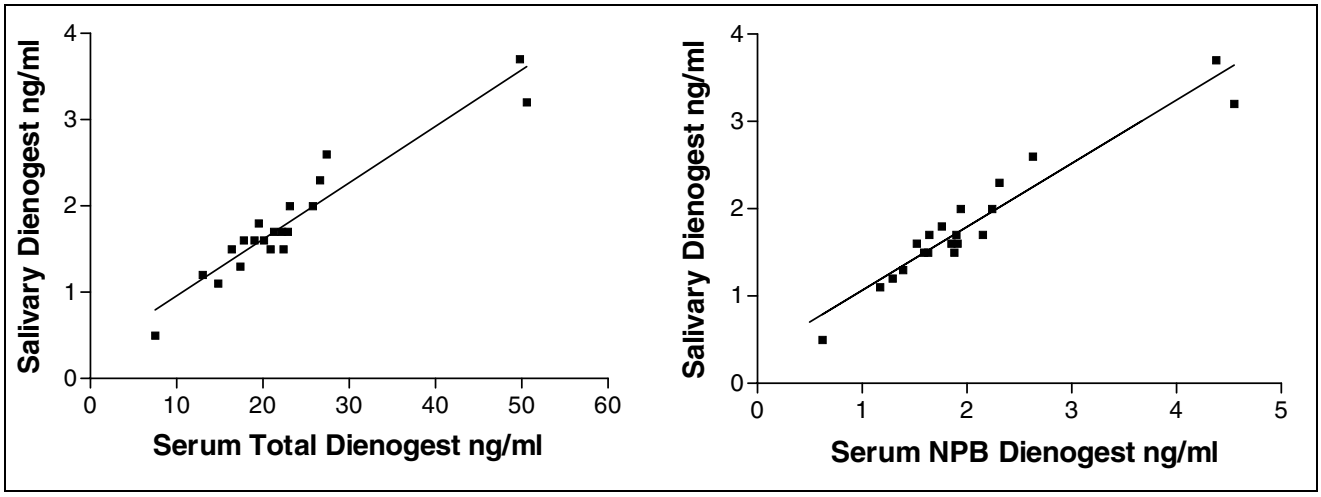

Fig. 2 : Correlation of salivary dienogest with total serum dienogest (left figure, r = 0.958) and with the free, non-protein bound (NPB) dienogest in serum (right figure, r = 0.953)

The data in Table 2 and the calculated correlations in Fig. 2 demonstrate that salivary dienogest correlates well with the serum total dienogest and also with the free dienogest in serum. The absolute concentrations of salivary dienogest fairly reflect the free, non-protein bound dienogest in serum. It is worth noting that the percentage of free dienogest in serum and also the relation of salivary dienogest to total serum dienogest cover a small range of 7.7–10.4% and 6.3–9.5%, respectively. This is not found with other steroids. In studies on salivary testosterone and progesterone, the proportion of salivary steroid concentrations to serum steroid concentrations varies significantly and increases up to 10 times when high steroid levels are present in blood. The increase in salivary steroid concentrations is discussed as a consequence when all available binding sites of SHBG and CBG, respectively, in blood are occupied [18].

This dependence of the free steroid fraction on the concentration of the available high affinity binding protein can be ruled out for dienogest, since albumin with its huge binding capacity is the main binding component. Therefore, salivary dienogest gives a good reflection of the free and also of the total dienogest concentrations in serum and will be a valuable tool for pharmacokinetic studies. In a previous investigation on dienogest pharmacokinetics, practically the same elimination half life time was obtained from the serum and salivary dienogest concentration curves [19].

The rather high portion of free dienogest in blood has been likewise found in animal species such as rat, rabbit, and dog [20]. Obviously, the absent binding to SHBG is due to the 4.9-dien-3-oxo structure of the molecule, by which dienogest differs from the other mentioned progestagens. The high portion of the free steroid and the exclusive binding to albumin makes dienogest exceptionally good bioavailable and favours the penetration into target organs. On the other hand, the absent binding to high affinity proteins facilitates the elimination and prevents an accumulation after repeated administration. Thus, the mode of dienogest protein binding contributes in different respect to the favourable effects of the compound in women.

3. Experimental

3.1. Materials

3.1.1. Certostat®

Jenapharm GmbH (Jena/Germany); 1 package includes 21 dragees each containing 50 μg 17α-ethinylestradiol + 2 mg dienogest. One dragee is taken daily from days 1–21 of the cycle.

3.1.2. Valette®

Jenapharm GmbH; 1 package includes 21 dragees with 30 μg 17α-ethinylestradiol + 2 mg dienogest. One dragee is taken daily from days 1–21 of the cycle.

3.1.3. Trisiston®

Jenapharm GmbH; 1 package includes 9 dragees with 30 μg 17α-ethinylestradiol + 50 μg levonorgestrel, 6 dragees with 40 μg 17α-ethinylestradiol + 75 μg levonorgestrel, and 6 dragees with 30 μg 17α-ethinylestradiol + 125 μg levonorgestrel. The dragees are taken from days 1–9, 10–15 and 16–21, respectively, of the cycle.

3.1.4. Centrifree® micro partition system

Millipore GmbH (Eschborn/Germany). The devices of Amicon/Millipore are ready for use for serum volumes of 0.15–1 ml. The separation border is 30 000 daltons, the retention of serum proteins is >99.9%.

3.1.5. Serum samples

Blood samples were drawn with syringes from the cubital vein of female volunteers who used Certostat®, Valette® or Trisiston® for oral contraception for a long time and came in regular intervals for consultation to the hospital. After standing for some time, the samples were centrifuged for 15 min at 3500 rpm. The supernatant serum was stored at −20° C until assayed.

3.1.6. Saliva samples

Immediately after the blood samples had been taken, saliva was collected by means of cotton plugs (Salivetten) which remained for 5 min in the mouth cavity. By centrifugation of the plugs in a special device, the saliva was obtained. After dilution with buffer, 50–100 μl were directly introduced to the dienogest radioimmunoassay without solvent extraction.

3.1.7. Volunteers

Women taking the above contraceptices and who came regularly for consultation to the hospital.

3.2. Methods

3.2.1. Dienogest radioimmunoassay

The immunoassay was developed in the former Central Institute of Microbiology and Experimental Therapy Jena (CIMET) in the 1980s [19, 21] and later on transferred to other institutions. The assay is characterized as follows:

The antiserum was raised in rabbits following immunization with dienogest-3-carboxymethyloxime bovine serum albumin conjugate. The antiserum is highly specific and has no cross-reactions with endogenous steroids. 14α,15α-^{3}H-dienogest was used as the radioactive tracer. The tracer was synthesized by a multistep synthesis by Dröscher and Römer at the former Central Institute for Nuclear Research Rossendorf near Dresden. Purification of the tracer was realized by CC (0.8 × 2 cm) on Lichroprep Si 60 (Merck, 40 × 63 μm) with $CHCl_3$/MeOH 98.5 : 1.5 v/v as eluent, and subsequent TLC on silica gel GF_{254} (Merck) in the solvent systems benzene/acetone 2 : 1 v/v and benzene/MeOH 9 : 1 v/v, resulting in a radiochemical purity of >95%.

The plasma samples (0.1 ml) were extracted with methylene chloride (1 ml) using an internal standard of 2500 dpm ^{3}H-dienogest added to the plasma sample. The extraction yield was found to be 92.6 ± 3.1% (n = 731). Later on, the extraction with methylene chloride was replaced by an ether extraction. The incubation of plasma extract, antiserum and tracer was carried out under conditions which are usual for steroid radioimmunoassays. The separation of free and antibody bound dienogest was achieved by dextran coated charcoal. The dienogest RIA procedure was found to have intra-assay variation coefficients of 2.6–7.6%, inter-assay variation coefficients of 3.9–7.2%, a good accuracy and a sensitivity of 26 pg/tube ≅ 0.26 ng/ml serum.

3.2.2. Centrifugal ultrafiltration

In glass tubes (12 × 75 mm, Sarstedt), a benzene or ethanol solution of ^{3}H-dienogest (∼ 150 000 dpm) was evaporated with a nitrogen stream in a water bath of 40 °C. Aliquots (0.5 ml) of the serum samples were added to the tubes, and dissolving of the tracer was achieved by 30 s vortexing, incubation for 30 min at 37 °C, and once more short vortexing. In two 50 µl aliquots, total serum radioactivity was measured by liquid scintillation counting with 5 ml Ultima Gold® (Canberra-Packard) in a TriCarb 1900. The residual serum is transferred to a Centrifree® micro partition device. The device is closed with a plastic cap and centrifuged for 5 min at 2800 rpm in a Heraeus Biofuge 28 RS equipped with an angular rotor prewarmed to 37 °C. From the container with the ultrafiltrate, 2 × 50 µl are taken for the radioactivity measurement with 5 ml Ultima-Gold®.
The portion of free, non-protein bound dienogest was calculated from the equation

$$\% \text{ free dienogest} = \text{dpm UF/dpm TP} \times 100$$

dpm UF = radioactivity in the ultrafiltrate; TP = radioactivity in the serum sample prior to centrifugation.

3.2.3. Calculation of correlation

The correlation between the total and free dienogest concentrations in serum, and between serum free dienogest and salivary dienogest was calculated by the PadGraphPrism™ programme (GraphPad Software, Inc., San Diego, CA). The serum concentration of the free dienogest was calculated from the total dienogest serum concentration and the determined percent free dienogest.

References

1 Dunn, J. F.; Nisula, B. C.; Rodbard, D.: J. Clin. Endocrin. Metab. **53**, 58 (1981)
2 Partridge, W. M.: Endocr. Reviews, 103 (1981)
3 Pugeat, M. M.; Dunn, J. F.; Nisula. B. C.: J. Clin. Endocrin. Metab. **53**, 69 (1981)
4 Hammond, G. L.; Lähteenmäki, P. L. A.; Lähteenmäki, P.; Luukkainen, T.: J. Steroid Biochem. **17**, 375 (1982)
5 Kuhnz, W.; Pfeiffer, M.; Al-Yacoub, G.: J. Steroid Biochem. **35**, 313 (1990)
6 Kuhnz, W.; Schütt, B.; Woloszcak, R.: J. Steroid Biochem. **48**, 573 (1994)
7 Kuhnz, W.; Staks, T.; Jütting, G.: Contraception **50**, 563 (1994)
8 Hobe, G.; Schubert, K. : WHO-Project 81096, CIMET Jena, December 1982
9 Juchem, M.; Schaffroth, M.; Pollow, K.; Hoffmann, G.; Kaufmann, G.; In: Teichmann, A. T. (ed.): Dienogest – Präklinik und Klinik eines neuen Gestagens. Walter de Gruyter, Berlin – New York 1995, p. 119
10 Rivarola, M. A.; Forest, M. G.; Migeon, C. M.: J. Clin. Endocrin. Metab. **28**, 34 (1968)
11 Dowsett, M.; Mansfield, M. D.; Grigs, D. J.; Jeffcoate, S. L.: J. Steroid Biochem. **21**, 343 (1984)
12 Riad-Fahmy, D.; Read, G. F.; Walker, R. F.: J. Steroid Biochem. **19**, 265 (1983)
13 Zorn, J. R.; McDonough, P. G.; Nessman, C.: Fertil. Steril. **41**, 248 (1984)
14 Luisi, M.; Franchi, F.; Kicovik, P. M.; Silvestri, D.; Cossu, G.; Catarsi, A. L.; . Barletta, D.; Gasperi, M.: J. Steroid Biochem. **14**, 1069 (1981)
15 Samuka, E.; Terho, P.; Suominen, J.; Sautti, R.: Int. J. Androl. **6**, 319 (1983)
16 Peters, J. R.; Walker, R. F.; Riad-Fahmy, D.; Hall, R.: Clin. Endocrin. **17**, 583 (1982)
17 Turkes, A.; Dyas, J.; Read, G. F.; Riad-Fahmy, D.: Steroids **35**, 445 (1980)
18 Nahoul, K.; Rao, L. V.; Scholler, R.: J. Steroid Biochem. **24**, 1011 (1986)
19 Do Khac Hieu; Reddersen, G.; Wehrberger, K.; Hobe, G.: Pharmazie **41**, 711 (1986)
20 Hobe, G.; Hillesheim, H. G.; Küntzel, B.; Klinger, G.: Naunyn-Schmiedeberg's Arch. Pharmacol. Suppl. **347**, R44 (1993)
21 Hobe, G.; Klinger, G.; Reddersen, G.; Erdmann, A.; Klinger, G.: Pharmazie **41**, 228 (1986)

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