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EFFECTS OF SERUM-CLOT CONTACT TIME ON SECOND-TRIMESTER PRENATAL SCREENING MARKERS AND THEIR STABILITY IN SERUM

UTICAJ DUŽINE KONTAKTA KOAGULUMA SA SERUMOM NA VREDNOSTI BIOHEMIJSKIH MARKERA PRENATALNOG SKRININGA DRUGOG TRIMESTRA I NJIHOVA STABILNOST U SERUMU

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Summary: Alpha-fetoprotein, human chorionic gonadotropin and unconjugated estriol are screening markers for fetal trisomies and structural disorders of the neural tube defect type. Determination of these biochemical markers is performed in laboratories that receive samples, serum or blood, and sample preparation as well as transport conditions may affect analyte stability and test results. The effect of serum-clot contact time prior to centrifugation (30, 60, 90, 120 minutes and 24 hours) was examined for serum values of alpha-fetoprotein, human chorionic gonadotropin and unconjugated estriol, as well as their stability in serum samples over periods of up to five days at 2–8 °C, and 30 days at –20 °C. No statistically significant difference was found for analyte values in serum obtained by centrifuging blood samples after serum-clot contact times of 30, 60, 90 and 120 minutes at room temperature and 24 hours at 2–8 °C, i.e. there is a 24-hour tolerance for prolonged serum-clot contact. Alpha-fetoprotein and free estriol serum values do not show statistically significant changes over a period of 5 days at 2–8 °C. Similarly, serum freezing does not affect alpha-fetoprotein and free estriol values. As for human chorionic gonadotropin values showing statistical differences where serum is stored at both +4 °C and at –20 °C, their levels are concentration-dependent. Free estriol serum values at +4 °C over the time period of 5 days showed statistically significant differences.

Keywords: alpha-fetoprotein, chorionic gonadotropin, free estriol, screening, serum-clot, stability

Kratak sadržaj: Alfa-fetoprotein, humani horioni gonadotropin i nekonjugovani estriol koriste se za *skrining* trizomija fetusa i strukturnih poremećaja tipa defekata neuralne tube tokom drugog trimestra trudnoće. Za određivanje ovih biohemijskih markera u laboratorije se šalju uzorci, serum ili krv, a način pripreme uzoraka, kao i uslovi tokom transporta mogu da utiču na stabilnost ispitivanih analita i rezultate testa. Ispitivan je uticaj dužine kontakta koaguluma sa serumom pre centrifugiranja (30, 60, 90, 120 minuta i 24^h) na vrednosti alfa-fetoproteina, humanog horionog gonadotropina i nekonjugovanog estriola u serumu, kao i njihova stabilnost u uzorcima seruma tokom 5 dana na 2–8 °C i 30 dana na –20 °C. Utvrđeno je da nema statistički značajne razlike za vrednosti ispitivanih analita u serumu dobijenom centrifugiranjem uzorka krvi posle kontakta sa koagulumom u trajanju od 30, 60, 90 i 120 minuta na sobnoj temperaturi i 24^h na 2–8 °C, odnosno da postoji tolerancija od 24^h za produženi kontakt seruma sa koagulumom. Alfa-fetoprotein i slobodni estriol ne menjaju se statistički značajno u serumu tokom 5 dana na 2–8 °C. Zamrzavanje seruma takođe ne utiče na vrednosti alfa-fetoproteina i slobodnog estriola. Vrednosti humanog horionog gonadotropina, čuvanjem seruma na +4 °C i na –20 °C, menjaju se statistički značajno, ali različito u zavisnosti od koncentracije. Za slobodni estriol u serumu na +4 °C, tokom 5 dana, dobijene su statistički značajne razlike.

Ključne reči: alfa-fetoprotein, horioni gonadotropin, slobodni estriol, skrining, koagulum, stabilnost

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Introduction

Second trimester prenatal screening biochemical markers, alpha-fetoprotein (AFP), total hCG (hCG) and free estriol (uE3), are determined in maternal serum mostly between weeks 14 and 18 (1–9). Based on the determined values of these biochemical markers,

personal data and gestational age related data, the risk of trisomy (G21 and 18), neural tube defect (NTD) or similar structural disorder of that type in a fetus is assessed. In case such risk is increased, the pregnant woman is referred to other diagnostic procedures (amniocentesis, CVS, cordocentesis) (10, 11), which are costly and present a certain risk to both mother and fetus. Unlike such diagnostic procedures, biochemical and ultrasound prenatal screening are non-invasive and relatively inexpensive, available to most pregnant women, and thus today present a part of routine procedures for pregnancy monitoring.

In Serbia, biochemical prenatal screening is performed in laboratories with appropriate equipment and trained personnel, where often samples from other institutions and doctor offices are tested. Due to potential preanalytic effects of sample preparation and transport, test results and their interpretation are sometimes an issue.

Laboratories determining biochemical prenatal screening markers receive serum or whole blood samples. Serum samples are either transported on the same day, or stored at 2–8 °C or frozen. Whole blood samples are transported immediately after venepunction or stored for a certain period of time at room temperature or at 2–8 °C, and serum is separated in the laboratory determining the biochemical markers. Samples are delivered by courier or delivery service. Conditions during transport differ, as well as the duration of transport itself, which may affect both the stability of assayed markers and test results.

Time period between obtaining a blood sample and receiving it at the laboratory must be no longer than 45 minutes (12) in order for the serum to be separated within one hour (13–15). Whole blood is not stored in the refrigerator (16). Blood normally coagulates at room temperature in 20 to 60 minutes. Optimal time interval between collecting and centrifuging a blood sample should be sufficient for blood clot forming, but it also must not be prolonged. Serum separation prior to completion of blood clot forming may cause incorrect results due to presence of fibrin in serum samples; minimum coagulation time for serum separation is considered to be 20–30 minutes (17). Prolonged serum-clot contact time may cause preanalytic variations (18), since biological cell activity and transmembrane diffusion may lead to changes in serum concentrations of individual analytes. It is recommended that serum or plasma be physically separated as soon as possible following centrifugation, and that serum-clot contact be prevented. Two-hour time interval is considered to be the maximum limit for serum separation following blood drawing (19). However, specific analytes are tolerant to different degrees with regard to time period for separating serum from serum-clot, and some are stable long after the two-hour period (18). With regard to stability of AFP, hCG and uE3 in separated serum, different data are provided by test-reagent manufacturers (20–22).

This paper examines the effect of time period from blood drawing to serum separation on the values of AFP, total hCG and uE3, as well as stability of the mentioned biochemical markers in separated serum. The effect of coagulation times of 30, 60, 90, 120 minutes and 24 hours prior to blood centrifuging and serum separation is analyzed. Stability of the named biochemical markers was tested in serum samples stored at +4 °C after 24 hours, 3 days and 5 days, as well as in serum samples stored for 30 days at –20 °C.

Materials and Methods

Blood samples were collected from 25 pregnant women in the second trimester of pregnancy, gestational age being between 14 and 17 weeks, who were referred to the laboratory for a routine biochemical prenatal screening. Blood samples (5 mL each) were collected in two glass test tubes previously marked (1 and 2) with no anticoagulants (BD Vacutainer System 367614) through venepuncture in the antecubital fossa (inner elbow) vein. Immediately after drawing, contents of tube no. 1 were distributed into four sterile tubes marked »30 min«, »90 min«, »120 min« and »24 hours«. The said labels refer to the time period allowed for blood clot formation. Blood samples marked »30 min«, »90 min« and »120 min« remained at room temperature until the time period indicated had elapsed, and were then centrifuged. Blood sample marked »24 hours« was stored at 2–8 °C, and after this time period it was centrifuged. Blood samples were centrifuged for 10 minutes at 1500 g, and then the serum was separated into sterile tubes where determinations were made.

Test tube no. 2 blood sample remained for 60 minutes at room temperature prior to centrifuging, and the separated serum was divided into 5 portions, where biochemical markers were determined immediately (0 day), after 24 hours (1 day), after 3 days (3 days), after 5 days (5 days) and 30 days later (30 days). Storing temperature of 1 day, 3-day and 5-day serum samples was +4 °C, and 30-day sample was stored at –20 °C until determination. Prior to analysis, the frozen serum sample was left for one hour at room temperature to defrost completely, and then it was homogenized on a Vortex.

All determinations were performed in triplicate, by the chemiluminescence method, and Siemens commercial tests were used: Immulite 2000 AFP (L2KAP6), Immulite 2000 HCG (L2KCG6), Immulite 2000 Unconjugated Estriol (L2KEF2). Determinations were carried out on automated immunochemistry analyzer IMMULITE 2000. Precision was defined for all three parameters, where AFP CV = 4.6–12.0%, hCG CV = 4.5–7.8%, and uE3 CV = 5.9–12.3%. Internal quality control for AFP and hCG was performed by commercial control preparation (TMCO, Siemens), and serum pool was used for free estriol.

Results

Table I shows mean values for all three analyzed parameters obtained in serum samples after different coagulation intervals. There are no significant differences between mean values of all three parameters, with $p > 0.9$ in all cases.

When the data obtained from samples collected from 25 pregnant women are examined by linear regression analysis (*Table II*) in relation to the serum sample separated after 60 minutes, the same conclusion is reached, that there is no statistically significant difference for all three parameters. AFP values in serum separated after 30 minutes are higher by 2.7%, and in serum separated after 24 hours lower by 3.1%, than the values in serum separated after 60 min, which has no statistical significance. The values in other two samples (90 minutes and 120 minutes) are almost identical to the values in the sample separated after 60 minutes. Similarly, values of hCG in sera separated after 30 minutes and after 90 minutes are higher by 5.7% and 5.6%, respectively, than in the serum separated after 60 minutes, which also has no statistical significance. In other two samples, the differences are 2.1% and 1.4%. As for uE3, the lowest value was determined in the serum separated after 30 minutes, and the difference is 3%, which is of no statistical significance. Values for other samples differ by less than 1%.

Table III presents AFP, hCG and uE3 values measured for fresh serum (separated after 60 minutes) and samples stored for 1, 3 and 5 days at +4 °C, and 30

Table I The effects of prolonged contact of sera with cells on AFP, hCG and uE3 values: mean ± SD (min – max).

	AFP (μg/L)	hCG (U/L)	uE3 (nmol/L)
30 min	38.76 ± 14.55 (18.40 – 68.00)	25580.1 ± 13924.8 (7145.3 – 69609.0)	11.27 ± 5.12 (3.53 – 21.57)
60 min	37.85 ± 14.12 (18.67 – 66.60)	25315.3 ± 13148.5 (7245.3 – 65584.0)	11.14 ± 5.26 (3.62 – 21.50)
90 min	37.75 ± 14.17 (17.93 – 66.10)	25391.0 ± 13941.5 (7537.7 – 69488.3)	11.20 ± 5.30 (3.87 – 21.70)
120 min	37.67 ± 14.01 (18.03 – 65.20)	25359.2 ± 13489.6 (8118.0 – 67240.3)	11.04 ± 5.24 (3.71 – 21.63)
24h	37.49 ± 13.72 (18.10 – 64.30)	24766,6 ± 13411.0 (6697.0 – 65159.3)	11.15 ± 5.29 (3.73 – 22.47)

Table II Linear regression analyses for effect of clot-time on values of determined analytes.

	AFP (μg/L)	hCG (U/L)	uE3 (nmol/L)
60 min vs. 30 min	r = 0.997 y = –0.107+1.027x	r = 0.998 y = –1189.5+1.057x	r = 0.996 y = 0.459+0.970x
60 min vs. 90 min	r = 0.998 y = –0.158+1.002x	r = 0.996 y = –1343.5+1.056x	r = 0.992 y = 0.062+0.999x
60 min vs. 120 min	r = 0.998 y = 0.180+0.990x	r = 0.995 y = –488.1+1.021x	r = 0.995 y = –0.009+0.991x
60 min vs. 24h	r = 0.997 y = 0.823+0.969x	r = 0.994 y = –903.0+1.014x	r = 0.992 y = 0.020+0.998x

Table III The effects of storage at 4 °C on AFP, hCG and uE3 values: mean ± SD (min – max).

	AFP (μg/L)	hCG (U/L)	uE3 (nmol/L)
start values (0 d)	38.75 ± 14.12 (18.67 – 66.60)	25315.3 ± 13148.5 (7245.3 – 65584.0)	11.15 ± 5.26 (3.62 – 21.50)
1 day	37.77 ± 13.97 (18.50 – 64.10)	25151.2 ± 14191.1 (6337.7 – 69377.3)	11.68 ± 5.81 (3.86 – 24.30)
3 days	38.79 ± 14.46 (18.10 – 64.73)	25441.7 ± 14604.7 (6434.3 – 69650.7)	11.86 ± 5.64 (3.17 – 23.50)
5 days	40.05 ± 14.47 (19.90 – 65.43	25891.2 ± 14680.6 (6902.0 – 72671.3)	11.84 ± 5.64 (4.01 – 23.80)
30 days	37.50 ± 14.60 (18.10 – 68.07)	28875.6 ± 17255.1 (6953.0 – 87929.3)	11.88 ± 5.31 (3.87 – 22.03)

Table IV Linear regression analyses for impact of storage time on values of determined analytes.

	AFP (μg/L)	hCG (U/L)	uE3 (nmol/L)
1 day vs. 0 day	r = 0.996 y = 0.473+0.986x	r = 0.993 y = –1980.5+1.071x	r = 0.987 y = –0.491+1.092x
3 days vs. 0 day	r = 0.992 y = 0.329+1.015x	r = 0.992 y = –2383.0+1.100x	r = 0.988 y = 0.035+1.061x
5 days vs. 0 day	r = 0.991 y = 1.602+1.016x	r = 0.992 y = –2152.7+1.108x	r = 0.990 y = 0.004+1.062x
30 days vs. 0 day	r = 0.983 y = -0.991+1.017x	r = 0.983 y = –3791.3+1.290x	r = 0.994 y = 0.697+1.004x

days at –20 °C. None of the analyzed parameters showed a significant difference in mean values, although value changing trends have been observed (increase trend for hCG and uE3).

Results of regression analysis of values measured in serum samples from 25 pregnant women stored for 1, 3 and 5 days at +4 °C and for 30 days at –20 °C, in relation to the fresh serum levels, are shown in *Table IV*.

AFP values are stable over the period of 5 days, and also after storing at –20 °C, with deviations lower than ±2%. Values of hCG, however, change already on day one, where positive percentage deviation of about 10% combines with negative absolute deviation of about 2000 U/L. That is why no change is observed in samples with values between 20000 and 30000, and thus no difference between mean values is identified (*Table III*). In samples with hCG values above 30000, significantly higher values were determined when stored at +4 °C, but also at –20 °C. As for uE3, significantly higher values are determined in samples stored at +4 °C, while in samples which were stored frozen no changes have been observed.

Discussion

Correct results of the triple test require that measured AFP, hCG and uE3 values be reliable and accurate. Low serum AFP values of pregnant women in the second trimester indicate an increased risk of aneuploidies, and elevated levels point towards risk of NTD and similar structural disorders. Where AFP values measured are higher than actual ones, a number of fetuses with chromosomopathy will not be identified, whereas a number of healthy fetuses will be found to suffer from NTD. Values for hCG that are higher than actual ones will result in an increased number of false positive findings of trisomy G21 with serious consequences, including anxiety of parents and unnecessary costs of additional examinations and tests, while lower hCG values may result in false positive findings of trisomy 18, or reduce the possibility of detecting trisomy G21. Values of uE3 which are lower than actual ones will result in false positive findings of trisomies and other chromosomopathies (Smith-Lemli-Opitz syndrome), while values higher than actual ones reduce the detection rate of fetuses with the mentioned disorders.

Research has determined that there is no statistically significant difference for AFP, hCG and uE3 values in serum obtained by centrifuging blood samples after serum-clot contacts over the time periods of 30, 60, 90 and 120 minutes at room temperature, and 24 hours at 2–8 °C, and the findings are in line with the ones in similar research (18, 23–26). Research results also indicate that for determining AFP, hCG and uE3 values there is a tolerance of 24 hours for prolonged serum-clot contact time, so that it can be concluded that reduced serum-clot contact time of 30 minutes at room temperature and prolonged contact of 24 hours at 2–8 °C do not compromise triple test results, and that a period of 60 minutes may be recommended as optimum incubation time.

In case of serum stored at +4 °C after centrifuging and separation from blood clot, AFP values measured during the examined period were the same as the ones in fresh serum. Similarly, serum freezing did not affect AFP values.

As for hCG values, they show significant differences in sera stored at +4 °C or at –20 °C, but they vary in relation to the concentration. This change may be explained by secondary dissociation and degradation of intact hCG (27), which is consistent with the results of similar research (28, 29). Such an effect of freezing on hCG value might influence screening results, leading to an increased number of false positive results for trisomy G21. However, during the second trimester of pregnancy, serum hCG values in most pregnant women, primarily ones with an unthreatened fetus, are much lower, mostly between 10,000 and 25,000 U/L.

Unconjugated estriol values in serum show statistically significant differences as early on as after one day at +4 °C. Differences may be explained by hydrolysis of conjugated estriol, which is the predominant estriol form in serum during pregnancy (30). The mentioned differences, although of statistical significance, do not essentially affect test results, or the estimated risk of chromosomopathies in a fetus. As for frozen serum samples, no changes in uE3 values were identified.

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