Selenium and Other Elements in Human Maternal and Umbilical Serum, As Determined Simultaneously by Proton-Induced X-Ray Emission

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Using PIXE (proton-induced x-ray emission), we simultaneously determined the concentrations of Se, Ca, Fe, Cu, Zn, Br, and Pb in blood serum from 56 pregnant women, 25 healthy controls, and 31 others with twin pregnancy or some complicating condition (diabetes, hypertension, epilepsy, hepatitis gravidarum, pre-eclampsia, small baby), and in cord-blood serum from 21 newborns. Pellets, pressed from the serum samples after addition of yttrium as an internal standard, mixing, and evaporating at 30 °C with or without reduced pressure (<1 kPa), were bombarded by 2.2 MeV protons from a Van de Graaff accelerator in the air and the induced X-rays collected by a Ge(Li) detector. Relative to mean Se values for early six- to 12-week pregnancy (0.045 ppm), those for 35–42 week pregnancy (0.028 ppm) were lower (p < 0.001). Umbilical cord serum showed even lower values (0.016 ppm, p < 0.001)—findings in harmony with the incidence pattern of Keshan cardiomyopathy. Pb crossed the placenta; values for cord serum were significantly different from those in pregnancy serum. Cu, Zn, Fe, and Ca showed the significant expected patterns in the different groups. Compared with the late-pregnancy controls, Fe was high in mothers of small-birth-weight babies (1.70 ppm, p < 0.02). Br was high in pre-eclampsia (3.59 ppm, p < 0.05) and mothers with twins (3.61 ppm, p < 0.05).

Additional Keyphrases: early and late pregnancy · Keshan cardiomyopathy · Fe, Cu, Zn, Br, Pb, Ca

For healthy intra-uterine development the fetus requires adequate amounts of major, minor, and trace elements, which are only obtainable from the maternal blood via the placenta (1, 2). For example, during the nine months of human pregnancy the fetus accumulates 30 g of calcium (3). Not only essential elements but toxic elements as well, such as lead, may also cross the placenta. In this study we have used proton-induced x-ray emission analysis (PIXE) to measure simultaneously the trace elements Fe, Cu, Zn, Se, and Br, the major element Ca, and the toxic element Pb in blood serum from women in early and late normal pregnancy, in late pregnancy complicated by disease, and in serum from the umbilical cord of babies, collected at the time of delivery.

The particular advantage of the PIXE method we used is that it gives simultaneous results for some biologically essential and toxic elements with little sample preparation, thus minimizing risks of contamination. Our utilization of an internal standard (Y) and proton bombardment of the sample in the air with use of an exit foil (Kapton®) allows accurate results to be obtained with quick and simplified sample changing.

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Theory

Under proton bombardment the target atoms of a sample lose inner-shell electrons. Outer-shell electrons then fill the inner-shell vacancies and the transitions are accompanied by emission of characteristic X-rays corresponding to the energy difference between the two shells. Thus the target atoms can be identified and their relative amount determined on the basis of the corresponding peak areas.

The cross section for production of an inner-shell vacancy increases sharply as a function of particle energy, approaching a maximum when the particle velocity is equal to the average velocity of the electron in that shell. This has been demonstrated experimentally and various theories also describe the situation (see, e.g., 4). When the energy of the bombarding particle is increased further, the cross section starts a slow decrease.

When both the variation in the cross section for characteristic X-rays and various background effects caused either by secondary electron bremsstrahlung or bremsstrahlung due to the deceleration of the incident charged particles are taken into account, 2–4 MeV is the optimum proton energy range.

Methods

Experimental Arrangement

The measurements were performed in the air at the 2.5-MV Van de Graaff accelerator of the University of Helsinki. The proton beam (E0 ~ 2.2 MeV) was brought into the air with use of a system described earlier (5, 6). To facilitate measurements, a Kapton foil, polyimide (C22H16O3N2), 7.5 μm thick, was used as the exit foil. This foil could withstand a beam current of 0.3–0.5 μA for longer than 10 h, because it was supported on a perforated carbon disc with about 200 exit holes, each 0.3 mm in diameter. The beam size was ~ 2 × 2 mm2.

The samples were positioned at 45° to the incident beam, and the X-rays were detected with a Ge(Li) detector (Seforad PGP 50-6 OFB, active area 50 mm2 and depth 6 mm) positioned at an angle of 90° to the beam, 12 mm from the sample surface. The FWHM (full width at half maximum peak height) of the FeKα peak (6.4 keV) was 167 eV during data collection.

To reduce the proportion of low-energy X-ray quanta ascribable to Cl, Ar, K, and Ca, we used absorbers made of five layers of Kapton, each 125 μm thick. The beam was regulated to keep the count rate at ~1000 counts/s.

The current was typically 0.3 μA and a charge of 100–200 μC collected on the target during average run times of 5–10 min. The charge was measured directly from the sample holder, which was insulated from the exit foil support.

The X-ray spectra were stored in a PDP 9/L computer and plotted with use of the VIPUNEN program (7) in a Burroughs 7800 computer. The peaks were analyzed by the VIPUNEN program and checked manually.
Sample Preparation

One-milliliter serum specimens were obtained from the First Department of Obstetrics & Gynaecology of the Helsinki University Central Hospital. The glass tubes used were first carefully washed with detergent and doubly distilled de-ionized water, then in 0.01 mol/L HCl, and finally extensively rinsed in doubly distilled de-ionized water and allowed to dry in an enclosure at atmospheric pressure. Blood was sampled into the glass tubes from women in normal early (six to 12 weeks) and late pregnancy (35 to 42 weeks) and from women with pregnancies complicated by various conditions. The main complicating conditions were diabetes, hepatosis, pre-eclampsia, hypertension, twins, and pregnancies resulting in small babies (weight <2.5 kg). A further expectant mother suffered from epilepsy.

Serum was obtained from mixed arterial and venous cord blood collected into glass tubes at delivery. The samples were collected by allowing the blood to drip into the tube from the cut umbilical stump, avoiding contact between the tube and the tissues to prevent contamination.

Serum was obtained from the blood specimens by centrifugation and stored at 20 °C until the targets were prepared.

Using pooled serum from normal volunteers and standard procedures described, we obtained 10 P XE spectra. We saw no random variation in the P XE spectra beyond the limits expected from counting statistics, indicating that the tubes were similarly clean. Water blanks could not be run because protein (which should be normal serum protein because of matrix effects) was required to form the pellets.

Samples were prepared by both pipetting and weighing: 50 μL of an aqueous yttrium solution (YCl3·6H2O) was added to the samples as an internal standard, and the pipetting was checked gravimetrically. The yttrium concentration was about 15 ppm (mg/kg) of the wet weight of the samples. After vigorous vortex-mixing, the sample was added dropwise, in several steps, to aluminum cups and dried in an oven at 30 °C. (The temperature was kept this low to avoid volatilization of elements.) For the selenium calibration, the sample preparation was further simplified by evaporating serum water at 30 °C in an oven under reduced pressure (<1 kPa).

The dried samples (weight loss in drying −93% for each of the two different methods) weighed 75 to 80 mg and were, if necessary, first crushed with a mortar and pestle before being pressed into pellets. Dried serum is very fragile as a pellet, so we pressed the pellets into the center of a supporting collar of Whatman filter paper (6.5 mm i.d., 13 mm o.d.) so that the dried serum pellet was formed in the center of the filter-paper "doughnut." (During measurements the beam contacted only the sample itself.) We used a pressure of 5.9 MPa (60 kg/cm²). The masses of the samples supported by the collars were ~25 mg, their thickness was about 0.5 mm, and their surface area 130 mm². The smooth surface of the pellets ensured that the measuring geometry stayed constant from run to run, with no shadowing caused by possible unevenness of the sample surface. Use of the collar made the pellets quite durable, and they could easily be used for several runs and (or) stored for further measurements.

In the absolute determination of calcium, iron, copper, zinc, selenium, and bromine concentrations we used standard solutions (Titrisol®, Merck, Darmstadt, F.R.G.) diluted in water. We added various volumes of each of these standard solutions to replicate samples of pooled normal serum and supplemented them with yttrium in the same way as the unknown samples. The quantities added were measured both volumetrically and gravimetrically.

The preparation procedure was the same for both calibra-

tion standards and samples. This standardization procedure gives the concentrations in terms of wet weight. The resulting small concentrations of added elements cause no significant changes in the self-absorption of the serum samples (8). The smallest concentrations of added Ca, Fe, Cu, Zn, and Br in the standards were chosen to be of the same order as the concentration of the respective elements already present in serum samples; multiples of this amount were added so that at least five calibration standards were obtained for each element. We investigated the characteristics of the system for measurement of selenium, using concentrations in the range 0.050 to 50 ppm. SeKα X-ray yields were linearly related to the corresponding added concentrations; the correlation coefficient was 0.996 for 10 points.

Calculation of Results

For Ca, Fe, Cu, Se, and Br, we used the Kα peak areas for calculating the concentrations. Zn was calculated from the ZnKα peak, which is free from the contribution of Cu, in contrast to the ZnKα peak. For lead the PbLα peak was used.

We calculated element concentrations in serum manually by comparing the sample peak areas per yttrium count with use of a calibration curve plotted as peak area per yttrium count (ordinate) vs concentration of added standard (abscissa).

A peak was assumed to be detected if its area was ≥ 3√Ntbd, where Ntbd is the area of the background under it.

With standard run times of 5 to 10 min the detection limits for Ca, Fe, Cu, Zn, Pb, Se, and Br (in ppm) were 0.95, 0.025, 0.20, 0.130, 0.010, 0.010, and 0.070, respectively.

The reproducibility (CV) of measurements within the same run lies in the range of 1 to 5%, depending on the areas of the peaks. In the case of Pb and Se values falling near the limit of detection, the reproducibility fell to 8−12%. As seen from Figure 2b, this involved 10 Se and seven Pb values for babies (of 21 measured) and eight maternal Pb values (of 52 measured).

The reproducibility of measurement was also tested by using duplicate pellets from the same serum sample. These gave similar results.

Statistical Analysis

In analyzing statistical differences between groups, we used Student's unpaired t-test where distributions were approximately normal. In other cases, we used Wilcoxon's rank sum test.

For determining the means for parametric statistical analyses, we set values falling below the limit of detection as being at the limit of detection. This may have increased the mean Pb value and the mean Se value for cord-blood serum.

Results

Figure 1a shows an X-ray spectrum of a patient's serum, a case of twin pregnancy. In addition to peaks due to K, Ca, Fe, Cu, Zn, and Br, clear peaks arising from Pb and Se are seen. The spectrum was obtained by using a beam current of 0.3 μA and collecting a charge of 200 μC.

Figure 1b shows an X-ray spectrum of a cord-blood serum. The collected charge was 200 μC as in the previous case. In contrast to blood sera from pregnant women, the ratio of Cu to Zn is reversed in all cord-blood sera.

Data on element concentrations in serum are summarized in Table 1. The distributions of the values for Fe, Cu, and Zn in the different major groups are plotted in Figure 2a with the mean concentration values for each group. The distributions of selenium concentrations are shown in Figure 2b in normal early and late pregnancy, babies, and complicated
late pregnancy, divided into groups according to the diagnosis.

Selenium concentrations during normal early pregnancy were significantly higher than in normal late pregnancy (p < 0.001, Wilcoxon's rank sum test) and the values for babies were significantly lower than in either normal early pregnancy (p << 0.001) or normal late pregnancy (p < 0.002). Selenium concentrations in pregnancies of diabetics were lower than in normal late pregnancy (p < 0.06).

Calcium concentrations in normal early pregnancy, normal late pregnancy, and complicated late pregnancy were significantly lower than in normal healthy non-pregnant individuals (p < 0.01). Values for the babies were significantly higher (p < 0.01) than those for pregnant women.

Values for iron in cord serum were significantly higher than in pregnancy serum (cf. Figure 2a). Samples showing hemolysis had been excluded.

The values for copper in normal early pregnancy were significantly lower (p < 0.02) than in normal late pregnancy (Figure 2a). Those for cord-blood serum were highly significantly lower (p << 0.001) than for pregnancy serum.

Zinc concentrations during normal early pregnancy were significantly higher than during normal late pregnancy (p < 0.05) and were significantly higher for cord-blood serum.
than for normal late pregnancy ($p < 0.05$).

Br concentrations in pregnant women and babies were similar. In pre-eclampsia and twin pregnancies these values were significantly higher ($p < 0.05$) than in normal late pregnancy. In mothers who had small-birth-weight babies the Fe values were significantly higher ($p < 0.02$) than in normal late pregnancy. The same was true, at a lesser significance level ($p < 0.06$), for cases of pre-eclampsia and twin pregnancies.

**Discussion**

The main advantage of PIXE is that one can measure many elements of medical and biological interest in a quick single run, with little sample preparation and rather automatically. Methods such as X-ray fluorescence, neutron activation analysis—where decay times are often difficult to match for convenient elemental profiling—and secondary ion mass-spectrometry are also multi-elemental but currently do not seem to be as favored as PIXE, at least for trace elements. As yet, atomic absorption spectrophotometry is insufficiently multi-elemental to compete as an element-profiling technique, although it is very useful in analyses for individual elements and in simultaneous analysis for Cu and Zn.

Now that PIXE measurements may be performed in the air, as illustrated in our laboratory (5), sample positioning and changing has become easy, rapid, and free from complications. Once a sample is prepared according to the modified scheme given here, the measurement is relatively routine.

Our detection limits for Se and Pb when irradiation in the air is used are somewhat lower than those given by others, who have irradiated in vacuo (e.g., 9). This is probably because we used sample cooling, which prevents sample degeneration in the beam. This is technically more difficult in a vacuum, especially surface cooling. Even the mere presence of the air allows the irradiated surface to cool by convection, and thus high-beam currents can be used without sample damage.

In spectra from serum samples without added yttrium we saw the RbK$_\alpha$ peak, and estimated the concentration of rubidium to be 0.2–0.4 ppm.

Our systems have been checked by use of U.S. National Bureau of Standards Standard Reference Materials—orchard leaves and standard Florida rock—and IAEA bone standard H 5.

**Selenium** The decline in selenium concentrations in late pregnancy seems not to have been reported earlier and would suggest transfer of selenium to tissues of the developing fetus. Its concentrations in cord blood were significantly lower than in normal late pregnancy, perhaps indicating that, like Cu, Se may accumulate in the fetal liver and (or) other tissues. The pattern of Keshan disease (10, 11) in China—in which myocardial disease occurs in women of childbearing age and young children, and which can be prevented by therapy with selenium—would fit our data well if its cause is, as it seems, Se deficiency. Though in complicated late pregnancy no group had markedly lower values for Se than in normal late pregnancy, the diabetics had rather low values ($p < 0.06$), which could indicate that Se deficiency may occur sometimes in pregnant diabetics. This is worth further study in view of the higher rates of arterial and heart disease in diabetics. The low concentrations of Se in Finnish soils and in tissues and blood of Finnish adults and children have been studied extensively by Westermarck and associates (12, 13).

**Calcium:** Although the potassium K$_\alpha$ contribution under the CaK$_\alpha$ peak has to be subtracted, the error introduced into the calcium measurement is <1%. The higher calcium concentrations in infants' sera than pregnancy serum and the lower concentrations in pregnancy serum than in non-pregnant persons (14) probably can both be explained by the hemodilution that occurs relatively early in pregnancy. Our results showed no significant changes in calcium concentrations during the phases of pregnancy. We made no corrections of serum calcium concentrations for protein concentration in this study.

**Iron:** The higher values for iron in cord-blood serum are consistent with published information. In serum of neonates, values for iron are statistically higher than those for pregnancy serum but rapidly decline after birth (15). Our values for serum during pregnancy were not significantly different, although some have reported a decline in serum Fe in pregnant non-supplemented patients as pregnancy proceeds (16). Serum iron has also been reported to be high in toxemia of pregnancy (17), and our patients with toxemia had somewhat higher values for iron than in normal late pregnancy ($p < 0.06$).
Copper: The much lower copper concentrations in cord serum than in pregnancy serum are consistent with previous reports (e.g., 18). The increase in copper values during pregnancy has also been described by others (19, 20). In our late-pregnancy patients with hypertension, values for copper were lower than in normal late pregnancy (p <0.06). However, we only studied three such patients, and the possible significance of the finding is unclear.

Zinc: The fall in zinc in late pregnancy confirms the findings of others (e.g., 21, 22). The higher zinc concentrations in cord serum or neonate serum than in pregnancy serum have been described previously (e.g., 23, 24). There was no evidence of zinc deficiency in our subjects. As zinc deficiency has been reported to be associated with fetal malformations (25-27) and low birth weight (23, 28), this is reassuring in terms of population health and indicates adequate zinc nutrition in Helsinki.

Bromine: It is not clear what function, if any, bromine has in the body. In this study the significantly higher values for bromine in mothers with pre-eclampsia and those with twins are not interpretable, but would suggest further studies of such patients.

Lead: The lack of significant differences in Pb values between the groups is consistent with its simply being present as an ubiquitous environmental toxic contaminant. None of our subjects had evidence of lead poisoning and the Pb concentrations in their serum were in the mid-range of reported values for individuals not known to have been excessively exposed to lead (29-32). The extremely low plasma Pb values of Everson and Patterson (33) have not to our knowledge been confirmed yet. The similar values in babies and pregnant women indicate that lead crosses the placental barrier.

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