Determination of Trace Elements in Blood Serum of Patients with Behçet Disease by Total Reflection X-Ray Fluorescence Analysis

Pakize Dogan, Mehmet Dogan, and Reinhold Klockenkämper

The distribution of trace elements Fe, Cu, Zn, Se, and Rb was determined in sera from patients with the complete type of Behçet disease in the active stage and from healthy controls. Total reflection x-ray fluorescence was used for quantitative analysis; in general, atomic absorption spectrometry was used only for control of the Se values. Zn, Se, and Rb concentrations were significantly ($P < 0.0001$) lower for patients than for control subjects. No significant difference was determined in the Fe content. However, there was a significant increase in Cu in the patients so that the ratio Cu/Zn, Cu/Se, and Cu/Rb were significantly higher for the patients than for the control subjects ($P < 0.0001$).

Indexing Terms: iron - copper - zinc - selenium - rubidium.

Behçet disease, first described by the Turkish dermatologist Hulusi Behçet, is a multisystemic vasculitis disorder with unknown etiology. Recurrent oral and genital ulcerations and eye and skin lesions are the original findings. Other important manifestations may include recurrent synovitis, cutaneous pustules or nodules, meningoencephalitis, and thrombosis of the arteries and veins (1).

The presence of individual and regional differences in signs and symptoms shows that genetic, viral, and environmental factors are probably involved in the pathogenesis of the illness (2). Various authors report that Behçet disease is the most prevalent endogenous uveitis in Japan (3, 4); the disease is also recognized in Mediterranean countries, including Turkey (5).

In one study, Shimizu et al. (6) measured the environmental pollutants Cu, Cl, Zn, Cr, and Br in ocular attacks in Behçet disease and reported that only Cu has a strong relationship to the ocular attacks. In another study, Delilbaşı et al. (7) found that Se is significantly lower in sera of patients than in control subjects, suggesting that Se may affect the prognosis for these patients.

Here, we determined the distribution of Fe, Cu, Zn, Rb, and Se in sera of patients with the complete type of Behçet disease and compared this with that in healthy controls. We primarily used total reflection x-ray fluorescence (TXRF) analysis, the suitability of which for multielement determinations in whole blood and human blood serum has already been shown by Yap (8) and by Prange et al. (9). The method requires small sample volumes (<0.1 mL), uses internal standardization for simple and reliable quantification, and has low detection limits (down to 20 μg/L).

Materials and Methods
Sample Material and Preparation

Subjects. Patients with the diagnosis of Behçet disease, attending the Department of Dermatology and Department of Obstetrics and Gynaecology, Erciyes University, Faculty of Medicine, were examined. These 40 patients were 14 males and 26 females, ages 14 to 54 years. Twelve apparently healthy persons, five men and seven women, ages 20 to 50 years, served as control subjects.

To test the accuracy of the analytical method, we analyzed a reference material of freeze-dried human serum. This material had previously been examined by various analytical methods by Versieck et al. (10, 11).

Serum preparation. Blood samples were drawn from the antecubital vein into disposable plastic syringes, and serum was obtained by centrifugation. Hemolysed samples were excluded from the study. The samples were stored at −20 °C until analysis. Samples transported from Turkey to Germany were carried in an ice box at 0 °C by plane. Serum used for element analysis was thawed and mixed before assay. For the determination of trace elements by TXRF, we used four different techniques of sample presentation: (a) direct analysis of the sera, (b) digestion of the sera under normal pressure with an acid mixture of HNO₃ and HClO₄, (c) digestion of the sera under high pressure by HNO₃ alone, and (d) digestion of the sera under normal pressure by HNO₃ alone. Only the third and fourth method led to reliable results so only these methods will be described here in detail.

For digestion under high pressure with HNO₃, we transferred 1 mL of serum sample to a special quartz digestion tube, added 2 mL of concentrated HNO₃, and capped the tube with Teflon foil and a quartz block. The tube was closed by the special apparatus of a high-pressure asher and then put into an oven. The pressure was adjusted to 13 MPa and the temperature was increased to 280 °C during 1 h. After cooling, we added 50 μL of a 100 mg/L Ga solution as an internal standard and adjusted the final volume to 5 mL with distilled water. A 20-μL aliquot was transferred onto a quartz glass carrier and dried for analysis by TXRF.

For digestion under normal pressure, we added 1 mL
of concentrated HNO₃ to 500 μL of sample (or 400 μL of concentrated HNO₃ to 200 μL of sample) and heated at 160 °C for 60–90 min. After cooling to room temperature, a Ga standard solution was added as above and thoroughly mixed. We then pipetted 20 μL of the mixture onto a sample carrier and evaporated the solvent for analysis by TXRF.

All chemical reagents and acids used for preparation were “Suprapure” (Merck, Darmstadt, Germany). The water was “ultrapure” (Millipore system, ASTM type 1; Millipore, Bedford, MA).

Instrumentation

TXRF. The excitation unit EXTRa II used consisted of an x-ray generator, a line-focus x-ray tube, and a multiple-reflection low-pass filter (Rich. Seifert & Co., Germany). It was joined to a energy-dispersive spectrometer with a Si(Li)-detector, a multichannel analyzer, and a computer (formerly Link Analytical Ltd., High Wycombe, UK; now Oxford Analytical Instr. Ltd., Abingdon, UK). The x-ray tube with a Mo anode was operated at a voltage of 58 kV and a current between 10 and 30 mA. The Si(Li)-detector provided a resolution of 160 eV for Mn Kα. Polished, siliconized quartz glass discs were used as sample carriers, from which the primary beam was totally reflected under a glancing angle of only 4° of arc. The fluorescence radiation of the sample was detected under a viewing angle of 90° (Figure 1). Integration time was set between 100 and 500 s. As many as 12 different elements were determined simultaneously in the samples: S, Cl, K, Ca, Mn, Fe, Cu, Zn, Se, Br, Rb, and Sr (Figure 2).

Atomic absorption spectrometry (AAS). Se was determined by the AAS-hydride technique. Briefly, 0.5 mL of sample serum was digested under normal pressure with an acid mixture of HNO₃ and HClO₄ at 210 °C as described previously by Alt et al. (12). After cooling the digested sample to room temperature, we added 1 mL of 5 mol/L HCl and incubated this in a water bath at 95 °C for 15 min to reduce Se from oxidation state VI to IV. We then added 4 mL of water and placed the sample in a hydride generator connected to a spectrometer. The operational settings of the spectrometer were as follows: wavelength, 196.0 nm; background correction, deuterium lamp; spectral slit width, 2 nm; Se lamp (electrodeless discharge), 6 W; absorbance measurement, peak height.

Hydride system. Se hydride was obtained by reaction with a freshly prepared solution of 3 g of NaBH₄ in 100 mL of 0.01 mol/L NaOH. The reaction flask, the connecting tubes, and the quartz cuvette were purged (~30 s) with inert gas (purified nitrogen, flow rate 5–6 L/min). Then, the NaBH₄ solution was added to the sample solution in the reaction flask by means of a peristaltic pump. Se hydride was flushed into the heated quartz cuvette (900 °C) by nitrogen and the hydrogen that was formed simultaneously.

The hydride was thermally decomposed in the cuvette and the elemental Se was determined by AAS. Quantification was done by means of standard solutions, freshly prepared for each analytical series. The mass of Se chosen for calibration was between 10 and 50 ng.

Results

Four different techniques were applied to the analysis of trace elements in serum samples. However, only the third and fourth techniques—digestion by HNO₃ under high pressure and normal pressure—led to appropriate sample preparations for TXRF. The results of four independent analyses (digestion plus TXRF determination) of the human serum reference material are reported in Table 1. The small deficiency of Fe observed is insignificant for the later statements. The measured values for Cu, Zn, Rb, and Se show a good agreement with the certified values and prove the accuracy and precision of the TXRF method, including sample preparation.

The results of nine parallel determinations obtained for a particular serum (no. 23) from a Behçet patient are summarized in Table 2 for five different elements. The relatively poor precision for Rb and Se (18.8% and 29.0%, respectively) is caused by assay of low concentrations, close to the detection limit of the method.

The results obtained for sera of all patients and controls are summarized in Table 3. The individual values for Fe, Cu, Zn, Rb, and Se are in accordance with values of the literature (9, 13), although the Se concentration was fairly low. Because the Se concentration is near the detection limit of TXRF, we also determined Se by AAS, which gave far better precision (CV = 3.1%). However, the mean Se value by AAS was nearly equal to that by TXRF (see Table 3).

Distinct differences in SDs were obtained for the individual patients within their group and for parallel determinations. The SD values for the different individuals (Table 3) are three- to sevenfold greater than that due to parallel determinations (Table 2) for the elements Fe, Cu, and Zn. The differences for Rb and Se seem to be insignificant.

A detailed statistical evaluation of the individual data for the five elements gives further information. The David test for normality of the data shows that only the Fe concentration seems to have a gaussian distribution within the patients and within the control group as well. Consequently, the F- and the t-test were appropriate for the Fe results and confirmed that the SD and mean

![Fig. 1. Simplified instrumental set-up for total reflection x-ray fluorescence (TXRF) - CLINICAL CHEMISTRY, Vol. 39, No. 6, 1993](image-url)
values in both groups were not significantly different (see Table 3).

A nonparametric test has to be applied for the non-Gaussian distributions of the other elements. The U-test (Wilcoxon and Mann–Whitney) shows significant differences between the two test groups with respect to Cu (P < 0.001), Zn (P < 0.0001), Rb (P < 0.0001), and Se (P < 0.01). Compared with the healthy controls, patients have a higher mean Cu concentration (1.3 times), but lower Zn (0.8), Se (0.8), and Rb (0.6). Furthermore, the distribution of SD values for Cu and Se concentrations in patients was approximately double that in the control subjects (see Table 3).

As a result, the concentration ratios of the excessive element Cu and the deficient elements Zn, Se, and Rb (summarized in Table 4) are extremely significant for patients and control subjects (P < 0.0001). For patients, the Cu/Zn ratio is 1.6-fold, the Cu/Se ratio 1.6-fold, and the Cu/Rb ratio 2.2-fold that of the healthy controls.

**Discussion**

**Preparation Techniques**

Under the conditions chosen for direct analysis, we saw no losses for trace elements that are volatile during the drying process in an acid medium. That is, Br was found to be 450–800 mg/L by this technique, but only 0.3 mg/L by the techniques involving open digestion; Cl was 1500–2000 mg/L by this technique, but only 1–5 mg/L

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**Table 1. Results Determined by TXRF for Five Different Elements in the Freeze-Dried Human Serum Reference Material Compared with Certified Values**

<table>
<thead>
<tr>
<th>Element</th>
<th>Certified values*</th>
<th>TXRF values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe, mg/L</td>
<td>2.35 ± 0.15</td>
<td>1.95 ± 0.14</td>
</tr>
<tr>
<td>Cu, mg/L</td>
<td>1.01 ± 0.04</td>
<td>0.98 ± 0.06</td>
</tr>
<tr>
<td>Zn, mg/L</td>
<td>0.87 ± 0.02</td>
<td>0.85 ± 0.01</td>
</tr>
<tr>
<td>Rb, μg/L</td>
<td>168 ± 30</td>
<td>165 ± 16</td>
</tr>
<tr>
<td>Se, μg/L</td>
<td>95 ± 5</td>
<td>100 ± 25</td>
</tr>
</tbody>
</table>

* From Vansieck et al. (10, 11).

*SD* values 4 each.

**Table 2. Concentrations of Trace Elements in a Behçet Patient's Serum Determined by TXRF**

<table>
<thead>
<tr>
<th>Element</th>
<th>Conc, mg/L</th>
<th>Mean*</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>1.48</td>
<td>0.17</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>1.20</td>
<td>0.046</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>0.73</td>
<td>0.030</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>Rb</td>
<td>0.13</td>
<td>0.025</td>
<td>18.8</td>
<td></td>
</tr>
<tr>
<td>Se</td>
<td>0.043</td>
<td>0.012</td>
<td>29.0</td>
<td></td>
</tr>
</tbody>
</table>

*SD* values 9 each.

**Table 3. Concentrations of Trace Elements in Sera of Patients with Behçet Disease and of Healthy Controls by TXRF**

<table>
<thead>
<tr>
<th>Trace elements</th>
<th>Patients</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>n</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Fe, mg/L</td>
<td>1.66 ± 0.53</td>
<td>1.58 ± 0.57</td>
</tr>
<tr>
<td>Cu, mg/L</td>
<td>1.44 ± 0.32</td>
<td>1.14 ± 0.14</td>
</tr>
<tr>
<td>Zn, mg/L</td>
<td>0.92 ± 0.14</td>
<td>1.13 ± 0.20</td>
</tr>
<tr>
<td>Rb, μg/L</td>
<td>145 ± 29</td>
<td>249 ± 43</td>
</tr>
<tr>
<td>Se, μg/L</td>
<td>45.8 ± 16.5</td>
<td>62.0 ± 17.7</td>
</tr>
</tbody>
</table>

(49.6 ± 14.2)* (40) (62.5 ± 7.8) (12)

*SD* values by AAS are given in parentheses.

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As a result, the concentration ratios of the excessive element Cu and the deficient elements Zn, Se, and Rb (summarized in Table 4) are extremely significant for patients and control subjects (P < 0.0001). For patients, the Cu/Zn ratio is 1.6-fold, the Cu/Se ratio 1.6-fold, and the Cu/Rb ratio 2.2-fold that of the healthy controls.

**Table 4. Concentration Ratios Cu/Zn, Cu/Se, and Cu/Rb for Patients with Behçet Disease and Healthy Controls**

<table>
<thead>
<tr>
<th>Groues</th>
<th>n</th>
<th>Cu/Zn</th>
<th>Cu/Se</th>
<th>Cu/Rb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>40</td>
<td>1.64 ± 0.34</td>
<td>30.1 ± 11.2</td>
<td>10.5 ± 4.3*</td>
</tr>
<tr>
<td>Controls</td>
<td>12</td>
<td>1.03 ± 0.18</td>
<td>18.5 ± 3.2</td>
<td>4.9 ± 0.9</td>
</tr>
</tbody>
</table>

* n = 34.
by digestion techniques. Although the direct analysis seems to be suitable for the detection of halides (Cl, Br, and I) and Se, organic sediments and inorganic salt deposits are formed during the evaporation step, which prevents reliable quantification and reproducibility.

Digestion of serum under normal pressure with an acid mixture takes a great deal of time and effort. Although reproducible results could not be obtained by direct TXRF because of the formation of big crystals such as KClO₄, this technique seems to be suitable for the determination of Se in sera by AAS.

Digestion under high pressure allows the processing of five samples and one control in one operation; however, the processing time still takes 3 h and needs a sample volume >1 mL. The digestion in a closed chamber prevents losses of volatile elements during the digestion process, but there may be some losses at the drying step under the infrared lamp.

The digestion by HNO₃ under normal pressure also gives reproducible results for nonvolatile elements, just as the just-mentioned technique does. Moreover, small volumes of sample (200 μL) can be used for analysis. Therefore, we used both techniques to determine the nonvolatile trace elements in this study.

Biochemical Results

The Cu concentrations in the sera of Behçet patients were significantly higher than in the sera of control subjects. An increase in Cu has already been reported by others (6, 14).

Ishikawa et al. (15) showed that Cu is one of the major elements to produce experimentally a mucocutaneous-genital syndrome in animals. Moreover, among Cu, Cr, Cr, and Zn examined by Shimizu et al. (6), they found that only Cu has a strong relationship to ocular attacks and that an increase of Cu preceded the attack. They suggested that Cu as well as organophosphate and organochloride may have an etiological role as a trigger substance to produce or to lead to the development of Behçet disease. The increase in Cu in our patients’ sera may be related to increased gut absorption of Cu or perhaps to increases of ceruloplasmin, attributable to inflammation associated with the disease. This assumption is based on a previous study in which we observed higher ceruloplasmin concentrations in the sera of these same patients than in healthy controls (16).

On the other hand, concentrations of Zn and Rb were significantly lower in the sera of patients than in sera of controls. Our report shows for the first time a correlation between Rb in serum and Behçet disease. A decrease in Zn in sera has been reported by Mineshita et al. (17) and Gürkaynak and Cengiz (14). In contrast, no significant change in Zn concentration was observed during ocular attacks by Shimizu et al. (6). Our findings are in good agreement with those of Mineshita and of Gürkayınak and Cengiz.

In the present study, we also saw a decrease of Se in sera of Behçet patients, a result in accordance with the findings of Delilbaşi et al. (7). The essential requirement of humans for Se is based on its incorporation into the enzyme glutathione peroxidase, which catalyzes the breakdown of H₂O₂ and lipid hydroperoxides in body tissues and fluids, thereby protecting against oxidative damage to body tissues. In long-term Se deficiency, all body tissues show decreased activity of this enzyme (18). In cases of the complete type of Behçet disease and in the active stage, Niwa et al. (19) reported a significant increase in the production of oxygen intermediates (H₂O₂, O₂⁻, and OH). These results and our previous findings (16) indicate a decreased activity of glutathione peroxidase and lead to the conclusion that the antioxidative mechanism of all cells is partially destroyed in Behçet disease.

When the Zn, Se, and Rb concentrations are normalized with regard to Cu content, we speculate from the present study that the changes determined for those elements will be even more apparent.

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References

Reference Intervals for 21 Clinical Chemistry Analytes in Arterial and Venous Umbilical Cord Blood

Sherry L. Perkins,1,2 John F. Livesey,1 and Judy Belcher2

Reference intervals were determined for 21 clinical chemistry analytes in umbilical cord arterial and venous blood from healthy term infants. Nonparametric analysis (rank number) was used to determine the central 95% reference interval. No significant differences were observed between male and female infants. Reference intervals for glucose, urea, creatinine, urate, phosphate, calcium, albumin, total protein, cholesterol, triglycerides, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, creatine kinase, lactate dehydrogenase, \( \gamma \)-glutamyltransferase, and magnesium all were significantly different from adult values.

Indexing Terms: pediatric chemistry • newborns

Until recently, few laboratory tests have been available to help in the assessment of fetal well-being and management of high-risk pregnancies. The combination of cordocentesis and modern biochemical analyzers produces complete biochemical profiles from small volumes of arterial and (or) venous umbilical cord blood. Other investigators have tried to correlate fetal growth indicators and clinical disorders with the amounts of specific analytes in umbilical cord blood (1-3). However, investigation of relationships between clinical outcome and laboratory tests requires reliable reference values. We have been unable to find any comprehensive reports of reference intervals for the common biochemistry analytes in arterial and venous cord blood. Many literature reports are for individual analytes, and are based on small numbers, unspecified statistical evaluation, and obsolete instrumentation. Various cord blood analytes—including mineral elements (3), cholesterol, triglycerides, apolipoproteins (4-7), creatine kinase (CK) isoenzymes (8), \( \gamma \)-glutamyltransferase (GGT) (9), and selected enzymes (10)—have been individually investigated as potential indicators of fetal and neonatal distress but none has gained widespread acceptance.

We have developed reference intervals for 21 analytes in plasma obtained from 390 umbilical cord veins and 179 umbilical cord arteries from term (\( \geq \)37 weeks of gestation) infants at delivery.

Methods and Materials

Patients and Samples

Blood was obtained from umbilical cords of 397 infants (209 girls and 188 boys) delivered between 37 and 41 weeks of gestation. Eighty-seven infants were delivered by cesarean section (33 elective), 310 were delivered vaginally. Five-minute Apgar scores were \( \geq 8 \) for all infants included in the study. After delivery of the babies but before placental expulsion, umbilical cord venous blood was drawn into heparinized 30-mL syringes and corresponding arterial blood was drawn into heparinized 20-mL syringes. Blood was transferred to 7-mL SST Vacutainer Tubes™ (Becton Dickinson, Rutherford, NJ) and transported on ice to the laboratory for centrifugation and separation of plasma from erythrocytes. Samples were stored at 4°C until analysis. Visually hemolyzed samples were not analyzed. Complete biochemistry profiles were obtained for 390 venous plasma and 179 arterial plasma specimens within 24 h of collection.

Biochemistry Analysis

Plasma samples were analyzed with a Hitachi 737 automated analyzer (Boehringer Mannheim Canada, Montreal, Canada) and the manufacturer’s standard reagents, except for glucose, CK, and urea, which were analyzed with reagents from Diagnostic Chemicals Ltd., Charlottetown, PEI, Canada.

GGT isoenzyme electrophoresis was performed with Paragon SPE gels and B-2 barbital buffer, pH 8.6 (all from Beckman Instruments, Inc., Brea, CA). Briefly, 5 \( \mu L \) of plasma was applied to the gel and electrophoresed for 25 min at 100 V. Bands were detected by incubating...