**Biological Variation of Myoglobin in Serum**

To the Editor:

Measurement of myoglobin in serum provides essential information that aids in both early diagnosis and treatment of patients with myocardial infarction or skeletal muscle damage [1, 2]. However, information on the biological variability of myoglobin is lacking, a fact that may seem surprising when one considers the many reports on myoglobin as an indicator of muscular damage.

To investigate the biological variation of this analyte, we took four blood specimens from each of 10 apparently healthy laboratory workers (5 men and 5 women, ages 25–50 years) on the same day once a week for 4 weeks. In accordance with the Helsinki II Declaration, the design and execution of the experiment were explained thoroughly to the subjects, and informed consent was obtained. They were urged to continue their dietary habits or activities, and their weight remained stable within ±1.0 kg. Furthermore, none took any medication or consumed substantial quantities of alcohol.

After the volunteers fasted for 12–14 h and refrained from any morning exercises or smoking, venous blood was obtained while they were in the sitting position between 0800 and 0900 for 1–5 min with minimal stasis by the same phlebotomist with the use of reduced-pressure blood collection tubes (Sarstedt). Serum specimens, separated by centrifugation (4000 g for 15 min), were aliquoted and stored at −25 °C until analysis. When all the specimens were available, they were thawed, mixed, and centrifuged for analysis in a single run in duplicate, in random order. Myoglobin was measured with an automated fluorogenic ELISA (Opus myoglobin assay, Behringwerke AG) as described previously [2].

Biological within-subject variance was estimated from the total within-subject variance minus within-run analytical variance. The latter was estimated from replicate analyses of specimens from the subjects themselves. Biological between-subject variance was estimated from the total variance of the set of duplicate data from the assay performed on each subject minus analytical and within-subject components [3]. All of the components of variation were then transformed to the relevant CV with the use of the overall mean.

The mean values of myoglobin and estimated components of variation [analytical variation (CVA), intraindividual variation (CVi), and interindividual biological variation (CVg)] are shown in Table 1.

The means and the intraindividual variances did not differ significantly (P = 0.55 and P = 0.76, respectively) between sexes. The data on analytical and biological variation allowed the calculation of the analytical goal for imprecision (≤1/2CVA, i.e., ≤5.6%) and inaccuracy [≤0.25(CVA^2 + CVI^2)^1/2, i.e., ≤4.4%], the critical difference required for serial results from an individual to change significantly [2.77(CVA^2 + CVI^2)^1/2, i.e., 35%], the number of specimens that should be collected to estimate the homeostatic set point of an individual to within 5% [1.96(CVA^2 + CVI^2)/25, i.e., 25] [4], and the index of individuality (CVi/CVg, i.e., 0.8).

This last calculation shows that population-based reference intervals can be of some value in assessing patients’ results, with the caveat that results for some individuals may be unusual for them even if they lie within the population reference interval [5]. In confirmation of this, Tucker et al. [6] recently described a substantial number of patients whose myocardial infarction can be diagnosed early by observing significant changes in serial myoglobin results, with the concentrations of this marker still within the reference interval.

Among other things, this study shows that the Opus precision assay does not meet the goal based on biological variation. Improvement in the precision of the measurement is therefore required if this assay is to be offered on a routine basis. However, the performance of at least two replicate analyses on the same spec-imens should be mandatory. Derived data shown here cannot be used by other laboratories working with assays with different imprecisions.

---

**Table 1. Mean values of myoglobin and estimated components of variation.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean conc., µg/L</th>
<th>CVA, %</th>
<th>CVi, %</th>
<th>CVg, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>17.0</td>
<td>6.0</td>
<td>11.1</td>
<td>13.8</td>
</tr>
<tr>
<td>Male</td>
<td>17.5</td>
<td>10.3</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>16.5</td>
<td>12.0</td>
<td>16.0</td>
<td></td>
</tr>
</tbody>
</table>

---

References


Mauro Panteghini*
Franca Pagani

* Author for correspondence.

---

**Interference with Nephelometric Assay of C-Reactive Protein and Antistreptolysin-O by Monoclonal IgM-κ from a Myeloma Patient**

To the Editor:

The serum concentrations of C-reactive protein (CRP) and antistreptolysin-O (ASO) are measured by any of several rapid and reliable nephelometric and immunoturbidimetric methods. However, the presence of pathological concentrations of mono-
clonal immunoglobulin (Ig), glycosylated IgM, rheumatoid factor (RF), and anti-xeno Ig antibody [1–4] can interfere with their determination by certain methods with clinically important consequences.

We describe here interference by IgM-κ from a patient with myeloma in the laser nephelometric assay for CRP and ASO. The patient, a Japanese woman, age 35 years, was referred to our hospital for treatment of IgM-κ-type myeloma. The results of serological tests at the time of her admission (and reference range) were as follows: CRP, 0.465 g/L (0–0.003); ASO, 4.9 × 10⁶ IU/L (0–166 000); IgG, 19.5 g/L (10.9–17.4); IgA, 3.78 g/L (1.63–3.25); and IgM, 70.0 g/L (1.31–2.83). The CRP, ASO, IgG, IgA, and IgM were measured by a Behring nephelometer II automated analyzer (Behringwerke AG); the value for the patient's serum was within the reference range.

To exclude the possibility of non-specific binding between the patient's IgM and latex particles, an RF assay was performed by nephelometry with the use of latex particles coated with human IgG (Behringwerke AG); the value for the patient's serum was within the reference range.

To verify the results obtained, we tested with the use of an equivalent mixture of purified patient's IgM (10 g/L) and normal human serum (NHS). As the control, IgM isolated from the sera of other myeloma patients with high IgM and high or low CRP values, was mixed with NHS. Two different methods (nephelometry and SRID or RR) for the quantitative determination of CRP and ASO were used to assay the mixture. Only the mixture of IgM of our patient and NHS interfered with the measurement of CRP and ASO by the nephelometry, yielding high values for CRP and ASO (Table 1).

Three possible hypotheses might explain the high values for CRP and ASO caused by the patient's IgM: the presence of IgM–CRP and ASO complex, the occurrence of a nonspecific reaction between IgM and latex particles, and the occurrence of non-specific or specific reactions between IgM and anti-CRP antibody or streptolysin-O antigen coating the latex particles. The first hypothesis was ruled out by the finding that CRP and ASO were detected in the normal IgM fraction by Sephadex G-200 gel filtration. If CRP and ASO were bound to IgM, these fractions should show a deviation. CRP and ASO assay methods that used no latex particles (SRID, RR) also did not show high values for CRP and ASO. The second hypothesis was ruled out by the finding that, in the RF assay by nephelometry using the same latex particle methodology (CRP latex and ASO latex), the RF value of the patient's serum was normal. However, our findings did not rule out the third hypothesis.

The results thus suggested that the IgM from the present patient may have bound to the latex particles coated with anti-CRP antibody and to those coated with the streptolysin-O antigen used in this study. We were unable to determine which part of the complex of the latex particles and anti-CRP antibody or streptolysin-O antigen had bound to the patient's IgM. This appears to be the first report of the occurrence of false-positive values for CRP and ASO,

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>CRP, g/L</th>
<th>ASO, IU/L</th>
<th>IgM, g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.S.</td>
<td>+ saline solution</td>
<td>0.393</td>
<td>3 950 000</td>
<td>64.90</td>
</tr>
<tr>
<td>P.S.</td>
<td>+ anti-IgM</td>
<td>0.047</td>
<td>553 000</td>
<td>13.80</td>
</tr>
<tr>
<td>M.S. (1)</td>
<td>+ saline solution</td>
<td>0.163</td>
<td>54 000</td>
<td>40.50</td>
</tr>
<tr>
<td>M.S. (1)</td>
<td>+ anti-IgM</td>
<td>0.179</td>
<td>88 000</td>
<td>2.18</td>
</tr>
<tr>
<td>IgM</td>
<td></td>
<td>&lt;0.003</td>
<td>&lt;58 000</td>
<td>52.00</td>
</tr>
<tr>
<td>N.S.</td>
<td>+ P.S. IgM</td>
<td>0.012</td>
<td>647 000</td>
<td>4.88</td>
</tr>
<tr>
<td>N.S.</td>
<td>+ M.S. (2) IgM</td>
<td>&lt;0.001</td>
<td>40 000</td>
<td>4.14</td>
</tr>
<tr>
<td>N.S.</td>
<td>+ M.S. (3) IgM</td>
<td>&lt;0.001</td>
<td>41 000</td>
<td>4.85</td>
</tr>
<tr>
<td>N.S.</td>
<td>+ saline solution</td>
<td>&lt;0.001</td>
<td>42 000</td>
<td>0.62</td>
</tr>
</tbody>
</table>

"CRP, ASO, and IgM were all measured by a Behring nephelometer II using latex methodology.

"Serum was mixed with an equal volume of the treatment solution.

P.S., patient's serum; N.S., normal human serum; P.S. IgM, purified IgM from patient's serum; M.S. (1), myeloma patient's serum containing high CRP; M.S. (2) IgM and M.S. (3) IgM, purified IgM from serum of myeloma patients with high or low CRP.
but not of RF, in the presence of monoclonal IgM-κ from a patient with myeloma as determined with latex methodology, i.e., CRP latex, ASO latex, RF latex measured by the laser nephelometric method.

Accordingly, when high serum CRP and ASO are found in patients with myeloma in the absence of signs or symptoms of inflammation or infection, assays for these substances may require the removal of Ig from the serum sample to avoid interference in the nephelometric assay and the resulting falsely increased values.

**References**


Koji Yamada¹
Atsuhito Yagihashi¹
Sayoko Ishii¹
Kuniko Tanemura¹
Takashi Kida¹
Naoki Watanabe²
Yoshiro Niitsu²

¹ Dept. of Lab. Diagnosis
² Fourth Dept. of Intern. Med.
Sapporo Med. Univ.
School of Med.
South-1, West-16, Chuo-ku
Sapporo, 060, Japan

* Author for correspondence.

**Incomplete Removal of Labile Fraction When Measuring Hemoglobin A₁c with Bio-Rad Variant Analyzer**

To the Editor:

When glucose reacts with hemoglobin (Hb), the precipitated product is a Schiff base. This Schiff base rearrangement to produce stable Amadori (Hb), the first products are Schiff bases that slowly undergo Amadori rearrangement to produce stable ketooamines (glycohemoglobin). The relative fraction of the ketooamines to total Hb can be measured as hemoglobin A₁c (Hb A₁c), which constitutes ~60% of the bound glucose [1]. The Schiff base (or labile fraction) is unstable and is fairly readily hydrolyzed, especially at acid pH. Effective removal of the labile fraction is essential for the accurate determination of Hb A₁c because the labile fraction cannot be separated from the true (ketooamine) fraction by simple ion-exchange procedures and because its concentration varies acutely with the plasma glucose concentration [2]. Partial and variable removal of the labile fraction adds to assay imprecision and reduces the correlation [3] between measured Hb A₁c and mean plasma glucose.

The Bio-Rad Labs. Variant analyzer is a dedicated HPLC system designed to measure variant hemoglobins or Hb A₁c. For Hb A₁c the hemolyzing reagent is a pH 5.0 citrate buffer that also serves to remove the labile fraction. The Bio-Rad Instrument manual advises users to “let samples stand at 18–28 °C for at least 10 min to allow Schiff base removal” (this was increased recently to 15 min). The manual also describes how to run the instrument essentially in batch mode. At startup, or on restarting after running samples, the recommended protocol is an 8-min wash, a 3-min prime with hemolysate, followed by 3-min runs with two calibrators and two controls (total 23 min) before patients’ samples are processed. With this protocol, patients’ hemolysates are tested after variable periods of incubation at room temperature and at 8 °C (the temperature of the sample tray). A stat sample could be prepared, incubated for 10 min at room temperature and placed on the already started analyzer for almost immediate sampling, or at the other extreme, a second sample could be at position 100 and thus spend 10 min at room temperature followed by ~300 min at 8 °C before analysis.

Soon after we started to use the Variant and attempted to offer a quicker turnaround time for diabetes clinic patients, we received complaints from the diabetologists that some results were “unreliable” and “too high” compared with what they expected. Their expectations were based on observations of weekly mean clinic values (50–60 patients per week) and cumulative records for individual patients measured quarterly with our previous HPLC method [4] over a 12-year period. Yet the Bio-Rad Lyphocheck quality-control samples, Bilevel Diabetes Controls 1 and 2 (with claimed values of 5.6% and 9.7% of total Hb), run several times each day gave excellent precision with respective values for mean, SD, and CV of 5.6%, 0.11%, and 1.9% (n = 200) and 9.4%, 0.14%, and 1.5% (n = 209). We initially attributed the diabetologists’ concerns to unfamiliarity with the Variant results, which we assumed included a slightly different fraction of Hb as Hb A₁c compared with that given by our previous in-house HPLC method [4]. However, discussions with Randie R. Little of the University of Missouri–Columbia suggested that the labile fraction might be incompletely removed with a 10-min incubation at room temperature; thus, we decided to test the effectiveness of the Bio-Rad procedure.

We tested samples from inpatients and clinic patients at The Hospital for Sick Children, Toronto: a tertiary-care, university-affiliated hospital. We varied the incubation time for hemolysates prepared at room temperature (21–23 °C) and 4–8 °C. For the latter, we rapidly prepared hemolysates by using hemolyzing reagent straight from the refrigerator at 4–8 °C and immediately placed the prepared hemolysate in the Variant instrument with the sample tray at 8 °C. For room temperature experiments, we prepared several hemolysates from 2 or 3 patients at periodic intervals, marking the time of preparation on the hemolysate tubes. We then left them
for various lengths of time at room temperature before loading them on the Variant, one at a time into a sample tray position that was to be sampled within the next minute. The incubation time was determined from the time of injection noted on the Variant printout and the time of preparation marked on the hemolysate tube. Graphs were prepared of measured Hb A₁c against time (Fig. 1). Complete removal of the labile fraction was assumed when the Hb A₁c had fallen to a plateau. Extrapolating the curve back to zero incubation time gave the total value for “true” Hb A₁c plus the labile fraction.

We found that the labile fraction, calculated as the difference, varied between patients. With eight poorly controlled patients whose mean Hb A₁c was 11.9% of total Hb (range 9.0–16.1%), the mean labile fraction was calculated as 1.7% of total Hb (range 0.4–2.4%). The labile fraction was only partially removed after 10 min at room temperature (mean 40% removal, range 25–58%). Even at 30 min, removal was only 79% complete (range 67–100%). This resulted in overestimation of Hb A₁c by 9.0% (range 2–13%) at 10 min and by 2.9% (range 0–5.2%) at 30 min (in comparison with plateau values = 100%). At 4–8 °C, the rate of removal of labile fraction was much less than that at room temperature. At 10 min, only 12% of the labile fraction had been removed; after 30 min, the corresponding value was 28% (n = 2).

In four routine batches of samples (n = 114) that were hemolyzed (in small groups) with either a 10-min (y) or 60-min (x) incubation with Bio-Rad hemolysis reagent, results (expressed as % of total Hb) correlated (r = 0.986), but were ~7% higher when the shorter incubation was used: y = 1.07x – 0.20.

We also tested 59 samples with an alternative procedure for removing the labile fraction [4]. Samples (100 μL) were incubated for 45 min at 37 °C with 4 mL of 50 mmol/L acetate buffer, pH 5.5, containing 113 mmol/L NaCl. Afterwards, these samples were centrifuged at 1300g for 10 min and decanted. The wet erythrocyte button was gently mixed with the residual acetate buffer and the suspension was hemolyzed, as though it were blood, with the Bio-Rad hemolyzing reagent. Samples were then transferred to the Variant and tested without additional incubation. Comparison of results for these samples (y’) with the results for those that had been incubated with Bio-Rad Hemolyzing reagent alone for at least 60 min at room temperature (x’) showed excellent correlation (r = 0.991) for Hb A₁c (expressed as % of total Hb): y’ = 1.01x’ + 0.15.

We therefore recommend that sample hemolysates to be run on the Variant be left 60 min at room temperature before sampling to completely remove the labile fraction.

References
To the Editor:

We have addressed the efficiency of Schiff base removal in the Performance Characteristics section of the current Variant Hemoglobin A1c Instruction Manual (March, 1997). Under conditions that simulate routine (batch) analysis, the reduction rate is 80–100%. The decrease in Schiff base occurs not only during the incubation time but also during the priming and calibration sequence, when the sample gradually cools from ambient temperature to the Variant’s sample storage temperature (8 °C). Much of the data reported in this letter was produced by using a limited incubation (10 min) and “stat” analysis. Under the conditions described by the authors, Schiff base removal will be less effective. The Instruction Manual currently recommends a 30-min incubation for extremely hyperglycemic individuals; this approach will yield the most accurate results in tertiary-care centers where patients with type I diabetes are treated.

Steven R. Binder
Bio-Rad Labs.
Hercules, CA

The authors of the Letter respond:

To the Editor:

We welcome these comments, but are still unclear how Bio-Rad intends the instrument to be used in tertiary-care centers (or other laboratories). Must the technologist make a decision on whether the sample is from an “extremely hyperglycemic” patient, before deciding whether to incubate the hemolysate for 15 or 30 min? How is “extreme” hyperglycemia defined? Alternatively, does Bio-Rad now recommend a 30-min approach for all samples analyzed in tertiary-care centers, in case they are from extremely hyperglycemic individuals? As we have shown, even if analyzed “stat,” samples after a 30-min incubation give results that are, on average, only 2.9% higher than the plateau values. The Variant is a very precise instrument and we see no merit in adding analytical imprecision by incomplete removal of the labile fraction.

Graham Ellis
Lebe Chang
Bessie Cogionis
Denis Daneman

Analytical Agreement and Clinical Correlates of Plasma Brain Natriuretic Peptide Measured by Three Immunoassays in Patients with Heart Failure

To the Editor:

Brain natriuretic peptide (BNP) is a 32-amino acid peptide structurally related to atrial natriuretic peptide and predominantly secreted by myocardial ventricles. Interest in this peptide has recently increased because its concentration carries prognostic value in patients with myocardial infarction [1], congestive heart failure (CHF) [2], or cardiac hypertrophy [3]. The circulating concentration of BNP is also a predictor of mortality, independently of cardiovascular disease [4]. Three commercially available immunoassay methods are designed to measure the plasma concentration of human BNP: two recent nonextraction assays and an older extraction RIA. Here, we compared these three immunoassays and correlated them to cardiac function in healthy volunteers and patients with CHF.

We studied 26 patients (15 men, 11 women; ages 51–84) with depressed left ventricular function and a wide range of ejection fractions (22–57%) measured by bidimensional echo-Doppler technique. Six volunteers without evidence of cardiovascular disease were also included in the evaluation. Blood samples (14–21 mL) were collected in chilled tubes containing EDTA-sodium and apro- tinin (500 kallikrein inhibitor units/mL), and the plasmas obtained were immediately separated and stored in aliquots at −80 °C until assay. Each plasma sample was assayed for BNP with a nonextraction RIA from Peninsula Labs. (cat. no. RIAS9086), a nonextraction IRMA from Shionogi (Shionoria® BNP), and an extraction RIA from Phoenix Pharmaceuticals (cat. no. RK-011-03). For the last assay, plasma was extracted on C18 Sep-Pak (Waters) cartridges. Protocols and manufacturers’ directions were followed for all immunoassays. Plasma volumes for the determinations by the three methods were 0.1, 0.1, and 1 mL, respectively. The procedures followed were in accordance with the current revision of the Helsinki Declaration of 1975.

Figure 1 displays the agreement between each of the three immunoassays evaluated, according to the representation of Bland and Altman [5]. There was a good agreement between the two nonextraction immunoassays, though with a zero-bias difference (−97 ± 128 ng/L, mean ± 2 SD). Conversely, there was a significant divergence between either nonextraction immunoassay and the assay utilizing extraction, the difference increasing with the average BNP concentration. The extraction immunoassay gave lower BNP concentrations, on average, than either nonextraction method.

All three immunoassays showed a good clinical correlation with left ventricular ejection fraction for the patients with CHF. After log-transformation of BNP concentrations (y values), the regression analysis resulted in the following respective parameters for the Shionogi, Peninsula, and Phoenix immunoassays: slope −0.042 ± 0.01, −0.026 ± 0.01, and −0.024 ± 0.01; intercept 3.91 ± 0.22,
3.50 ± 0.13, and 2.99 ± 0.11; and \( r^2 \) 0.73, 0.75, and 0.74.

In conclusion, the three immunoassays evaluated here showed similar correlations with left ventricular function in patients with CHF \( (r = 0.73\text{--}0.75) \), but the results of the two nonextraction methods differed from the results of the extraction method.

S.M. is a fellow of the “Training and Mobility of Researchers” program from the EU.

### References


### Increased Plasma Endothelin-1 After Nicotine Consumption in Nonsmokers

**Maurizio Bevilacqua**

Tarcisio Vago

Gabriella Baldi

Guido Norbiato

Endocrine Unit

Ospedale “Luigi Sacco”

Via G.B. Grassi 74

20137 Milan, Italy

Serge Masson

Roberto Latini

Dept. of Cardiovascular Res.

Ist. di Ricerche Farmacol. “Mario Negri”

Milan, Italy

*Author for correspondence.

Increased Plasma Endothelin-1 After Nicotine Consumption in Nonsmokers

To the Editor:

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide originally isolated from the supernatant of cultured porcine endothelial cells [1]. ET-1 has not only contractile effects but also growth effects on both smooth muscle and heart muscle cells in vitro [2]. Studies have shown that some substances [1, 3], e.g., vasopressin [4], stimulate the release of immunoreactive ET-1 from cultured bovine carotid endothelial cells. Smoking is known to induce a variety of effects in the cardiovascular and hormonal systems in humans [5–10]. Administration of nicotine causes the release of some hormones [9, 10] and produces increases in blood pressure, heart rate, cardiac output, and oxygen consumption [5–8]. Recently, Yildiz et al. [11] reported that heavy cigarette smokers had higher plasma ET-1 concentrations than either light smokers or controls. In another study, Haak et al. [12] stated that after a short-term tobacco consumption the plasma concentrations of ET-1 significantly increased within 10 min of smoking.

Although tobacco smoke contains other compounds, besides nicotine and carbon monoxide, we decided to test in 10 healthy nonsmoker volunteers (all medical students, 5 men and 5 women, ages 21–24 years) the acute effect of nicotine chewing gum (Nicorette, supplied by Pierrel Pharmaceuticals) on plasma ET-1 concentrations. Each piece of chewing gum contained 2 mg of nicotine. We also measured the plasma concentrations of the vasoconstrictor peptide vasopressin, also known to act on the cardiovascular system.

On the day of the study, fasting subjects arrived between 0800 and 0900. At the time of collecting venous samples, all subjects had been kept in supine position for at least 30 min to stabilize their physical condition. Before and 5, 15, 30, and 60 min after the subjects chewed the nicotine gum, venous blood samples were collected in ice-chilled EDTA-coated tubes and centrifuged at 3000g for 30 min; the plasma obtained was frozen and stored at −70 °C until assayed. At the same intervals, the subjects’ blood pressure and pulse rate were monitored. After 1 week, all volunteers repeated the experimental study but with a placebo gum that looked the same as the nicotine gum.

Plasma ET-1 was assayed with a specific RIA (RIC-6901; Peninsula Lab., Belmont, CA), as previously described [13]. Vasopressin was also measured with an RIA (RK-065–07 Phoenix Pharmaceuticals, Mountain View, CA). Briefly, vasopressin was extracted from samples with a C18 Sep-Pak column after acidification with 5 mL of 1 g/L trifluoroacetic acid.

![Graph of agreement between immunoassays for plasma BNP determination in healthy volunteers and CHF patients.](image)

Fig. 1. Agreement between immunoassays for plasma BNP determination in healthy volunteers and CHF patients.

*Lines represent boundaries of mean ± 2SD (n = 32 to 34 subjects).*

---

Table 1. Effect of nicotine gum on plasma concentrations (ng/L) of ET-1 and vasopressin in nonsmokers.

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Nicotine study</th>
<th>Placebo study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ET-1</td>
<td>Vasopressin</td>
</tr>
<tr>
<td>Baseline</td>
<td>8.9 ± 3.2</td>
<td>8.8 ± 2.1</td>
</tr>
<tr>
<td>+5</td>
<td>11.1 ± 4.1</td>
<td>8.2 ± 1.8</td>
</tr>
<tr>
<td>+15</td>
<td>13.8 ± 3.7a</td>
<td>9.8 ± 1.8</td>
</tr>
<tr>
<td>+30</td>
<td>11.5 ± 4.4</td>
<td>8.1 ± 2.2</td>
</tr>
<tr>
<td>+60</td>
<td>8.3 ± 2.7</td>
<td>8.5 ± 1.4</td>
</tr>
</tbody>
</table>

*Significantly different (P < 0.0001) vs values at baseline, +5, +15, and +60 min.

acid and was eluted with 4 mL of acetonitrile:water (50:50 by vol). The residue was dissolved in 0.3 mL of 50 mmol/L phosphate buffer (pH 7.4) and assayed by RIA with radioiodinated vasopressin and antiserum against synthetic vasopressin in rabbits.

For statistical evaluation, we used ANOVA, followed by Bonferroni’s correction. Results are presented as mean ± SD.

Nicotine gum was well-tolerated, but 2 subjects had local mouth irritation. All volunteers had significantly increased mean plasma ET-1 values within 15 min after chewing the nicotine gum (P < 0.0001), whereas the vasopressin values increased 5 min after this oral nicotine administration (P < 0.0002) (Table 1). Oral nicotine consumption was associated with significant increases in the mean pulse rate (P < 0.001) and systolic (P < 0.005) and diastolic (P < 0.001) blood pressure 15 min after the start of nicotine gum administration, compared with the basal values: 76.2 ± 2.5 beats/min, 125.5 ± 8 mmHg, and 86.1 ± 3.3 mmHg vs 69.7 ± 2.4 beats/min, 118.3 ± 2.5 mmHg, and 80.1 ± 3.5 mmHg, respectively.

In addition, the correlation between ET-1 and vasopressin plasma concentrations 60 min after nicotine gum administration was statistically significant (r = 0.695, P = 0.038). As reported in Table 1, plasma ET-1 and vasopressin concentrations and the hemodynamic measurements remained unchanged during the placebo experiment.

ET-1 is one of the most potent vasoconstrictors (10 times more potent than angiotensin II, vasopressin, and neuropeptide Y) [11], and its expression by endothelial cells is regulated by other vasoactive agents [1, 3, 4]. Thus, vasopressin rapidly (within 5 min) induces the expression of pro-pre-endothelin-1 mRNA and secretion of ET-1 from a cultured bovine carotid endothelial cells [14].

Our results indicate that acute nicotine consumption induces in nonsmoking healthy subjects an increase in plasma ET-1 concentrations and that this increase follows a prompt rise of plasma vasopressin. These data suggest that, at least in part, the acute consumption of nicotine stimulates the release of circulating ET-1 via vasopressin activation. Therefore, the alterations in plasma ET-1 secretion induced by nicotine consumption might contribute to the pathogenesis of several cardiovascular disorders, in view of the powerful vasoconstrictor and growth-promoting properties of the peptide. Additional studies are required to describe the effects of nicotine gum in smokers.

Biological Variation of Free and Total Carnitine in Serum of Healthy Subjects

To the Editor:

Determination of carnitine (1-β-hydroxy-γ-trimethylaminobutyric acid) in biological samples plays an important role in the diagnosis of diseases with carnitine deficiency [1]. In the past few years, and also in this Journal, several spectrophotometric methods for assaying carnitine in serum have been described [2–6]. To our

References


Claudio Letizia* Sabrina Cerci Stefano Subioli Luigi Scuro Giovanni Clemente 2nd Dept. of Intern. Med. University of Rome “La Sapienza” Rome, Italy

*Address correspondence to this author, at: 2° Clinica Medica, Policlinico Umberto I, 00185 Rome, Italy.
knowledge, however, no data have been published on the biological variation of this serum analyte.

For any new test, data on the biological variation generated from the healthy population may be used (a) in setting desirable performance standards or analytical goals, (b) in assessing the utility of conventional population-based reference intervals, and (c) in critically evaluating the significance of changes in serial results from an individual \[7\].

To investigate the analytical and biological variation of free and total carnitine, as well as of the acyl/free carnitine ratio, we collected blood samples once a week for 4 weeks from each of 14 healthy subjects (7 men and 7 nonpregnant women, ages 21–23 years), students of Mathias Belius University. The subjects agreed to maintain current dietary habits, body weight, and exercise program (if any) for the duration of the study. The criteria of the hospital Ethics Committee were respected in this experiment.

To minimize sources of preanalytical variation, venous blood specimens were drawn between 0800 and 0900 h after ~12 h of fasting. The subjects remained seated for at least 20–30 min before the blood was drawn. Usually, specimens were obtained by a single phlebotomist and with minimal stasis into Monovette Serum Gel blood-collection tubes (Sarstedt). Serum specimens for both the free and total carnitine assays were prepared and stored the same way. After clotting, each specimen was centrifuged at 1500g for 15 min and the serum obtained was eluted through Centrifree™ columns (Amicon) in an angle-head rotor at 2000g for 30 min. Aliquots (800 μL) of protein-free serum filtrates were stored frozen at −20 °C until assayed.

Free and total carnitine concentrations were determined by a spectrophotometric enzymatic assay adapted for use on the Cobas Mira analyzer (Hoffmann-La Roche). The assay uses carnitine acetyl-transferase (CAT; EC 2.3.1.7) and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) as a thiol-group color reagent. The Cobas analyzer was programed as two reagent chemistry with a primary reagent and an enzymatic start reagent. Diluted sample (80 μL) was mixed with 200 μL of the primary reagent (10 mL of 0.2 mmol/L Na₂HPO₄, pH 7.8, 0.2 mL of 10 mmol/L DTNB, and 0.4 mL of 15 mmol/L acetyl-CoA), and the reaction was started with 24 μL of sixfold-diluted start reagent [CAT from pigeon muscle (1 mL, protein 5.3 g/L, CAT 120 kU/g protein)] was diluted with 0.9 mL of phosphate buffer (0.5 mol/L, pH 7.5) to give a final concentration in start reagent of 63.6 kU/L. All specific reagents were prepared and stored the same way. After clotting, each specimen was centrifuged at 1500g for 15 min and the serum obtained was eluted through Centrifree™ columns (Amicon) in an angle-head rotor at 2000g for 30 min. Aliquots (800 μL) of protein-free serum filtrates were stored frozen at −20 °C until assayed.

With minimal stasis into Monovette Serum Gel blood-collection tubes (Sarstedt). Serum specimens for both the free and total carnitine assays were prepared and stored the same way. After clotting, each specimen was centrifuged at 1500g for 15 min and the serum obtained was eluted through Centrifree™ columns (Amicon) in an angle-head rotor at 2000g for 30 min. Aliquots (800 μL) of protein-free serum filtrates were stored frozen at −20 °C until assayed.

Free and total carnitine concentrations were determined by a spectrophotometric enzymatic assay adapted for use on the Cobas Mira analyzer (Hoffmann-La Roche). The assay uses carnitine acetyl-transferase (CAT; EC 2.3.1.7) and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) as a thiol-group color reagent. The Cobas analyzer was programed as two reagent chemistry with a primary reagent and an enzymatic start reagent. Diluted sample (80 μL) was mixed with 200 μL of the primary reagent (10 mL of 0.2 mmol/L Na₂HPO₄, pH 7.8, 0.2 mL of 10 mmol/L DTNB, and 0.4 mL of 15 mmol/L acetyl-CoA), and the reaction was started with 24 μL of sixfold-diluted start reagent [CAT from pigeon muscle (1 mL, protein 5.3 g/L, CAT 120 kU/g protein)] was diluted with 0.9 mL of phosphate buffer (0.5 mol/L, pH 7.5) to give a final concentration in start reagent of 63.6 kU/L. All specific reagents were prepared and stored the same way. After clotting, each specimen was centrifuged at 1500g for 15 min and the serum obtained was eluted through Centrifree™ columns (Amicon) in an angle-head rotor at 2000g for 30 min. Aliquots (800 μL) of protein-free serum filtrates were stored frozen at −20 °C until assayed.

Free and total carnitine concentrations were determined by a spectrophotometric enzymatic assay adapted for use on the Cobas Mira analyzer (Hoffmann-La Roche). The assay uses carnitine acetyl-transferase (CAT; EC 2.3.1.7) and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) as a thiol-group color reagent. The Cobas analyzer was programed as two reagent chemistry with a primary reagent and an enzymatic start reagent. Diluted sample (80 μL) was mixed with 200 μL of the primary reagent (10 mL of 0.2 mmol/L Na₂HPO₄, pH 7.8, 0.2 mL of 10 mmol/L DTNB, and 0.4 mL of 15 mmol/L acetyl-CoA), and the reaction was started with 24 μL of sixfold-diluted start reagent [CAT from pigeon muscle (1 mL, protein 5.3 g/L, CAT 120 kU/g protein)] was diluted with 0.9 mL of phosphate buffer (0.5 mol/L, pH 7.5) to give a final concentration in start reagent of 63.6 kU/L. All specific reagents were prepared and stored the same way. After clotting, each specimen was centrifuged at 1500g for 15 min and the serum obtained was eluted through Centrifree™ columns (Amicon) in an angle-head rotor at 2000g for 30 min. Aliquots (800 μL) of protein-free serum filtrates were stored frozen at −20 °C until assayed.

Table 1. Biological variability statistics for serum carnitine quantities studied.

<table>
<thead>
<tr>
<th></th>
<th>Men (95% CI)</th>
<th>Women (95% CI)</th>
<th>Mean CV₁, %</th>
<th>Mean CV₂, % (range)</th>
<th>Mean CV₃, %</th>
<th>Anal. goal CV₁, %</th>
<th>Index of individ.</th>
<th>Crit. difference, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free carnitine</td>
<td>40.6 (34.8–46.4)</td>
<td>34.8 (28.8–40.8)</td>
<td>1.8</td>
<td>7.6 (2.7–13.2)</td>
<td>15.2</td>
<td>&lt;3.8</td>
<td>0.5</td>
<td>21.6</td>
</tr>
<tr>
<td>Total carnitine</td>
<td>51.8 (46.1–57.5)</td>
<td>42.6 (36.4–48.8)</td>
<td>2.2</td>
<td>10.0 (4.2–15.4)</td>
<td>9.8</td>
<td>&lt;5.0</td>
<td>1.0</td>
<td>28.4</td>
</tr>
<tr>
<td>Acyl/free carnitine</td>
<td>0.27 (0.20–0.33)</td>
<td>0.24 (0.19–0.29)</td>
<td>6.3</td>
<td>10.4 (1.6–18.9)</td>
<td>27.2</td>
<td>&lt;5.2</td>
<td>0.4</td>
<td>33.7</td>
</tr>
</tbody>
</table>

Men and women, n = 7 each; carnitine concentrations, μmol/L.

*Significantly different from the concentration in men (P < 0.02, unpaired t-test).
[11–13], probably because of different methodologies used, different physiological characteristics of the subjects, or both.

Replicate analyses for each sample were used to investigate within-run analytical variation. The $CV_A$ values of the analytical methods for free and total carnitine are lower than the respective goals, but those of the acyl/free carnitine ratio are greater. The high $CV_A$ component of carnitine assays could limit the clinical utility of the acyl/free carnitine ratio [7].

The mean $CV_I$ value for each carnitine quantity is $<$12.3%; for men and women, the acyl/free carnitine ratio is the most variable quantity. The relative distribution of free carnitine and carnitine esters varies according to fasting status, adiposity, renal function, and muscular exercise [11]. Nevertheless, these data indicate the existence of a reliable homeostatic mechanism in steady-state conditions. The $CV_G$ values indicate moderate variation in carnitine concentrations between healthy subjects. No significant differences between men and women were observed in within- or between-subject variances for any of the quantities studied.

Indices of individuality were $<$1.4 for each of the carnitine groups, meaning that individual results are more useful than population-based data [7]. For diagnosis and screening, however, free carnitine values $<$20 μmol/L and total carnitine $\geq$30 μmol/L, determined by nonradioenzymatic methods, are considered to indicate carnitine deficiency [4]. In carnitine-deficient patients, a low free carnitine concentration in serum is often associated with an increased acyl/free carnitine ratio [4, 9–11].

Finally, the critical differences obtained in this study, calculated from the mean $CV_I$ values, are also shown (Table 1). For free and total carnitine the critical difference was less than that for the acyl/free carnitine ratio. The former assays may therefore be more suitable for monitoring purposes, especially in carnitine supplementation therapy. However, the critical differences presented here are only a guide to clinical practice; other laboratories should take into consideration their own between-day imprecision of carnitine assays.

We thank Mária Dibalová for help in sample analyses and Samuel Korény for statistical assistance.

References

Roman Alberty*
Dept. of Biol.
Mathias Belius Univ.
Tajovskeho 40
974 01 Banska Bystrica
Slovak Republic

Dáša Albertová
Dept. of Clin. Biochem.
F.D. Roosevelt Hosp.
Nám. L. Svobodu 1
975 17 Banska Bystrica
Slovak Republic

*Author for correspondence.