\[ n = \text{the number of measurements for each calibration point} \]
\[ \bar{x} = \text{the mean values of all calibrator concentrations} \]

The resulting detection limits for normal- and low-range applications are given in Table 1. Improvements were 12.5-, 45-, 15-, 32.5-, and 22.5-fold for ApoA-I, A-II, B, C-III, and E, respectively.

The assays were linear up to 65 mg/L for Apo A-II and 8 mg/L for Apo C-III and ApoE. For ApoA-I and ApoB, a high-dose hook effect attributable to antigen excess was observed starting at 275 mg/L (ApoA-I) and 150 mg/L (ApoB), respectively. However, because of the wide overlap between the detection limit of all normal-range assays (Table 1) and the linearity range given for the low-range applications (Fig. 1C), these problems could be circumvented by the simultaneous use of both applications for each marker, which is recommended to detect unexpected outliers. Because high salt concentrations also hamper antibody reactivity, we checked the recovery of all five apolipoproteins in high ionic strength environments. Potassium bromide solutions with densities up to 1.25 g/L are used to isolate lipoprotein fractions by sequential ultracentrifugation. Within the range 1.0–1.25 g/L, no decrease of recovery was observed for samples at the lower and upper ends of the linearity ranges of the respective applications (data not shown). At higher salt concentrations, samples had to be desalted before analysis because the recovery progressively decreased.

The comparison of low-range turbidimetric assays with ELISA methods are shown in Fig. 1B. Immunoturbidimetry slightly overestimated apolipoprotein concentrations except for ApoB. The most critical discrepancy again was observed for ApoE, which exhibited a regression coefficient of 0.869. In contrast, immunoturbidimetry underestimated ApoB by 10%. Correlation coefficients of all method comparisons performed underscored the good agreement of both methods.

Taken together, our results indicate that for apolipoprotein concentrations below the physiological range, our immunoturbidimetric methods offers features that are comparable to other more time-consuming, costlier techniques such as ELISA. Taking into consideration that both the low- and normal-range applications can be set up easily and in parallel on most currently used random-access analyzers, the proposed applications might be a helpful alternative to previous methods, even when there is a demand for the precise determination of low apolipoprotein concentrations. We therefore conclude that the described methodology is appropriate for rapid determination of apolipoproteins in research and routine diagnostic determinations.

References

Serum Tractate-resistant Acid Phosphatase 5b Is a Specific and Sensitive Marker of Bone Resorption, Jussi M. Halleen,1* Sari L. Alatalo,2 Anthony J. Jancikila,2 Henning W. Woitge,3 Markus J. Seibert,4 and H. Kalervo Viinämäinen1 (1) Institute of Biomedicine, Department of Anatomy, University of Turku, Kiuamyllynkatu 10, 20520 Turku, Finland; 2 Special Hematology Laboratory, Veterans Affairs Medical Center, Louisville, KY 40206; 3 Department of Medicine, University of Heidelberg, 69115 Heidelberg, Germany; * author for correspondence: fax 358-2-3337352, e-mail jushal@utu.fi

Bone-resorbing osteoclasts and activated macrophages express high amounts of type 5 tartrate-resistant acid phosphatase (TRAP; EC 3.1.3.2) (1). Osteoclasts secrete TRAP into the circulation, and serum TRAP has been considered a potentially useful marker of bone resorption. Several technical problems have prevented the use of serum TRAP as a specific marker of bone resorption. Early enzymatic assays lacked specificity because of the presence of interfering acid phosphatases derived from

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platelets and erythrocytes (2, 3). Serum TRACP can be determined by use of fluoride (4) or by immunoassays with antibodies specific for TRACP (5–9). However, there are two forms of TRACP in the serum, namely TRACP 5a and TRACP 5b. Of these, TRACP 5b is derived from osteoclasts, whereas TRACP 5a originates from other, as yet unidentified sources (10–12).

Two diagnostic assays have been developed recently for serum TRACP 5b: a kinetic assay based on the use of specific inhibitors (13), and an immunoassay based on a highly characterized specific monoclonal antibody (12). The TRACP 5b-specific immunoassay has been shown to be a useful method for monitoring antiresorptive therapy (12). We have now further characterized the immunoassay by studying the clinical specificity and clinical sensitivity of serum TRACP 5b in various bone diseases and nonskeletal diseases.

We obtained serum samples from 303 individuals after written informed consent. At the time of sampling, none of the subjects was receiving antosteoporotic treatment. Healthy premenopausal women, healthy postmenopausal women, and breast cancer (BC) patients without evidence of bone metastases (BC−) were included as control populations. Patients with bone diseases included subjects with primary vertebral osteoporosis (OPO), osteopenia, active Paget disease of bone, and BC patients with overt bone metastases (BC+). All patients with OPO had at least one vertebral compression fracture. Patients with chronic hepatic dysfunction (HF; n = 17), chronic renal failure (RF; n = 49), and rheumatoid arthritis (RA; n = 40) were included as reference populations. All patients with RF had endstage renal disease, and they had been on chronic hemodialysis for several years (mean duration, 5 years) at the time of sample collection. The study was approved by the local ethics committees and performed in accordance with the Declaration of Helsinki.

Serum TRACP 5b activity was measured with a solid-phase immunofixed enzyme activity assay as described (12), using TRACP 5b purified from human osteoclasts as a calibrator (7). The distribution of TRACP 5b activity was within reference values in the control groups, and statistical analysis was performed using one-way ANOVA. The interassay CV was 2.2%, and the intraassay CV was 1.8%.

The reference interval (mean ± 2 SD of healthy premenopausal women) of TRACP 5b activity in serum was 0.50–3.80 U/L. Compared with healthy premenopausal women, serum TRACP 5b activity was significantly higher (P <0.001) in healthy postmenopausal women and in all groups of patients with bone diseases (Table 1 and Fig. 1A). The clinical specificity of TRACP 5b (percentage of healthy premenopausal women with TRACP 5b activity within reference values) was 95.0%, and the clinical sensitivity (percentage of individuals in a disease group with TRACP 5b activity above reference values) was 48.3% in osteopenia, 81.3% in OPO, 71.4% in Paget disease, and 80.0% in BC+ subjects (Fig. 1A).

The clinical sensitivity in BC+ was substantially higher for TRACP 5b than for other serum markers of bone resorption (Fig. 1B), including cross-linked N- and C-terminal telopeptides of type I collagen (NTX and CTX, respectively) and bone sialoprotein. Concentrations of all markers in the BC− group were within the reference interval. Reference values for all markers in this study were obtained from measurements of healthy premenopausal women and were comparable to published reference values.

Two enzymatic pathways are involved in osteoclastic bone resorption, one involving cathepsin K and releasing NTX and CTX from bone collagen, and the other involving matrix metalloproteinases without releasing NTX or CTX. The substantially lower clinical sensitivity of NTX and CTX compared with TRACP 5b in BC+ patients suggests that the increased bone resorption in BC+ patients is primarily mediated by matrix metalloproteinases.

Serum markers of bone resorption are accumulated in the circulation of patients with HF and RF (14). Mean TRACP 5b activity was not increased in patients with HF or RF, whereas mean NTX was significantly increased in both diseases (P <0.001) and were above the upper limit of normal in 58.8% of patients with HF and 93.2% of patients with RF. Total TRACP mass [measured as described previously (15)] was significantly increased (P <0.001) and above the upper limit of normal in 51.0% of the RF subjects (Fig. 1C). These results suggest that the function of the liver and the kidneys has no effect on circulating TRACP 5b activity.

Although the mean TRACP 5b activity was within the reference interval in RF patients, a subpopulation (12.5%) had TRACP 5b activity above the upper limit of normal, suggesting that they have increased bone resorption. This is not surprising because RF often is associated with secondary hyperparathyroidism and renal osteodystrophy. Greatly increased mean total serum TRACP mass in patients with RF suggests that enzymatically active TRACP 5b molecules are inactivated and degraded into fragments before they are removed from the circulation. Thus, inactive TRACP fragments may be accumulated in a manner similar to other markers. TRACP released into the uterine fluid of pigs is rapidly inactivated and degraded into fragments before they are removed from the circulation.

Table 1. Serum TRACP 5b activity in healthy women and in patients with metabolic bone diseases (mean ± SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age, years</th>
<th>TRACP 5b, U/L</th>
<th>% above normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premenopausal women</td>
<td>60</td>
<td>37.2 ± 7.2</td>
<td>2.15 ± 0.83</td>
<td>5.0</td>
</tr>
<tr>
<td>Postmenopausal women</td>
<td>40</td>
<td>71.2 ± 10.5</td>
<td>3.21 ± 1.05</td>
<td>27.5</td>
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<tr>
<td>Osteopenia</td>
<td>29</td>
<td>51.1 ± 11.1</td>
<td>3.92 ± 1.35</td>
<td>48.3</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>16</td>
<td>56.8 ± 12.4</td>
<td>4.81 ± 2.02</td>
<td>81.3</td>
</tr>
<tr>
<td>Paget disease</td>
<td>14</td>
<td>69.8 ± 7.4</td>
<td>5.51 ± 3.21</td>
<td>71.4</td>
</tr>
<tr>
<td>Breast cancer with bone</td>
<td>20</td>
<td>48.2 ± 9.5</td>
<td>6.10 ± 4.75</td>
<td>80.0</td>
</tr>
</tbody>
</table>

The clinical specificity and clinical sensitivity of serum TRACP 5b in various bone diseases and nonskeletal diseases.
tion may occur for osteoclast-derived TRACP 5b in serum, as has been suggested previously (17).

In addition to osteoclasts, activated macrophages express high amounts of TRACP (1), suggesting that TRACP released from macrophages may affect the specificity of our immunoassay. We show here that in patients with RA, a disease associated with increased activity of macrophages, total serum TRACP was substantially increased and above the upper limit of normal in 35% of the subjects (Fig. 1C). Instead, neither the resorption marker NTX nor TRACP 5b was increased, suggesting that bone resorption was not increased. These findings suggest that the observed high total serum TRACP in RA patients may be attributable to increased secretion of TRACP from cells other than the osteoclasts, possibly activated macrophages, that are not detected by the immunoassay measuring TRACP 5b.

We conclude that serum TRACP 5b is greatly increased in patients with bone diseases. The most pronounced changes were observed in patients with BC+ and OPO. Data from this study and from a previous study (12) suggest that serum TRACP 5b is a sensitive and specific marker of bone resorption that can be used in the diagnosis and follow-up of skeletal pathologies. Serum TRACP 5b may be derived exclusively from macrophages, and it may not accumulate in the circulation in RF or HF patients. Larger studies are needed to confirm the clinical relevance of serum TRACP 5b measurements and their predictive power regarding future bone loss and fractures.

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References

Comparative Study of Liquid-Liquid Extraction and Solid-Phase Extraction Methods for the Separation of Sufentanil from Plasma before Gas Chromatographic-Mass Spectrometric Analysis, Christelle Dufresne,1 Patrick Favetta,2 Chantal Paradis,1 and Roselyne Bouillieux1,2

Sufentanil (SF) is a highly potent synthetic opioid (1) used extensively in anesthesia (2, 3). Most of the techniques proposed for SF analysis in plasma samples are based on the RIA method described by Michiels et al. (4). Some methods using gas chromatography–mass spectrometry (GC-MS) have been reported (5–7). Plasma concentrations of SF in the analgesic range are extremely low, and pharmacokinetic studies require the development of sensitive GC-MS assay methods.

Reliable and reproducible isolation of SF from plasma is the most important and most critical step in pharmacokinetic analysis. Among different extraction methods currently used in pharmaceutics, the use of solid-phase extraction (SPE) has grown dramatically. However, according to the literature, liquid-liquid extraction (LLE) is the technique used most frequently for isolating morphinomimetic compounds (4–8), whereas only one method using SPE with RIA has been reported (9).

We performed comparative studies on the isolation of SF and alfentanil (AF; used as an internal standard) from plasma using LLE and SPE methods, and extracts were assessed using GC-MS. The method was applied to drug monitoring in intensive care patients.

SF citrate and AF hydrochloride were purchased from Janssen Pharmaceutica. Lyophilized plasma (Lyphochek®) was purchased from Bio-Rad Laboratories. Deionized water was prepared using a MilliQ® Water System (Millipore). Methanol (Uvasol® grade), dichloromethane (Uvasol grade), isoamyl alcohol (analytical grade), propan-2-ol (analytical grade), 13.2 mol/L ammonia, and sodium hydroxide (Normapur®) were purchased from Merck. n-Heptane (analytical grade), 18 mol/L sulfuric acid, and ammonium phosphate monobasic were purchased from Carlo Erba. Acetic acid and Sigmacote® were purchased from Sigma Aldrich. Oasis® MCX 60-mg SPE columns (Waters Corporation) were used, and the vacuum manifold for semiautomatic processing of the columns was purchased from Supelco. The Oasis MCX is a mixed-mode, polymeric sorbent (30-μm particle size) with strong cation-exchange sulfonic acid groups located on the surface of a poly(divinylbenzene-co-N-vinylpyrrolidin) copolymer.

Analyses were carried out using a Hewlett Packard (HP) 5890 Series II gas chromatograph (Agilent Technology) interfaced to a HP 597A MS Engine quadrupole mass selective spectrometer (Agilent Technology). The gas chromatograph was equipped with a HP 6890 autosampler (Agilent Technology) and a split-splitless capillary inlet system. The injector, containing a quartz deactivated liner, was operated in splitless mode. The chromatographic separations were achieved on a PTA-5 base deactivated, fused-silica capillary column [30 m × 0.25 mm (i.d.); 0.50-μm film thickness; Sigma Aldrich]. Data acquisition was performed using a HP Chemstation 59940 (HP-UX series).

Injections of 3 μL were made in splitless mode with GC+grade helium (Air Product) as the carrier gas. The column-head pressure was 16.3 psi at 200 °C (flow rate, 1 mL/min; linear velocity, 38.3 cm/s). The optimum ion purge time was 2 min, and the inlet and septum purge were set at 50 and 1 mL/min, respectively. The oven temperature was held at 200 °C for 220 min, increased to 280 °C at 25 °C/min, maintained at this temperature for 17 min, and then increased to 300 °C at 25 °C/min. The optimum injector and transfer line temperatures for SF, based on maximum peak areas, were 280 and 260 °C, respectively.

The mass spectrometer was operated in the electron impact mode (ionizing energy, 70 eV; trap current, 300 μA) with selected-ion monitoring. The ion source and quadrupole temperatures were set at 220 and 110 °C, respectively, and the source pressure was held at >6.6 × 10⁻¹⁴ Pa. The mass spectrometer detector was tuned to optimum sensitivity for m/z 264, 288, and 314 of the perfluorotributylamine certified standard and programmed to monitor the m/z 289 main fragments for SF and AF, as well as the m/z 140 and 268 characteristic fragments of SF and AF, respectively (dwell time, 100 ms). For quantification, the m/z 289 fragment was used for SF and AF, and the characteristic fragment of each compound (m/z 140 for SF; m/z 268 for AF) was used as the qualifier.

Stock solutions of SF (pKₐ 8.0) and AF (pKₐ 6.5) were prepared at a concentration of 100 mg/L in water.