Measurement of Apolipoprotein B by Radial Immunodiffusion

To the Editor:

The observed (1) association of atherosclerotic heart disease or peripheral vascular disease with increased concentration of plasma apolipoprotein B (apo B) has prompted interest in assay of this apolipoprotein in clinical laboratories. The goal of current research in this area is to determine the validity of apo B as an independent risk factor (2). Several methods for selective measurement of low-density lipoprotein (LDL)-associated apo B in whole plasma have been used, generally involving exclusion from the gel of very-low-density lipoprotein (VLDL)-associated apolipoprotein by including a higher concentration (15 g/L) of agarose in the gel. Alaujovic et al. (3) and Havekes et al. (4) have used electro-immunodiffusion to determine apo B of LDL. However, the method used in most laboratories is a rate determination with radial immunodiffusion. Apo B concentrations are measured after a 16-h incubation by comparing the ring diameters of samples with those of standards.

We have used commercial immunodiffusion plates (Nor-Partigen; Behring Diagnostics, Montreal, Canada) to measure apo B in plasma and in purified LDL fractions (isolated by ultracentrifugation between d 1.020 and 1.050). We found that if the procedure is conducted according to the manufacturer's instructions (five-day diffusion time) the relationship between the diameter and LDL apo B concentration is nearly linear. However, when the diameter is measured after a shorter diffusion time (15–20 h) the relationship is nonlinear over the range 0.50–2.00 g/L (Figure 1).

In patients' plasma we found a marked difference between values at 16 h and at five days in samples with apo B concentrations greater than 0.90 g/L (Figure 2). It is unlikely that the differences were due to apo B in VLDL, because very few of the patients' plasma examined had increased triglycerides. We also used previously frozen plasma, which minimizes the contribution of apo B from the VLDL (4).

The largest discrepancy between the rate and the end-point determination was in patients heterozygous for familial hypercholesterolemia, who have above-normal concentrations of apo B. One might speculate that in these individuals the discrepancies are due to the presence of "heavy LDL"—a particle that has a greater proportion of apo B than does normal LDL (5). The effect of particle-size differences on radial immunodiffusion has been explored previously (6).
In conclusion, we advocate caution in interpreting concentrations of plasma apo B from rate determinations modified from commercially available methods for plasma apo B. Purified LDL standards should be used to assess performance characteristics and to prepare standard curves. The endpoint determination appears to distinguish well between normal and abnormal populations for apo B concentrations. Whether this is because of the presence of abnormal particles in the plasma of individuals with high apo B remains to be determined.

**References**


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Organic Aciduria Associated with Isovaleric Acidemia

To the Editor:

Metabolites excreted in large amounts in urine from infants with isovaleric acidemia were shown by Tanaka and colleagues to be isovalerylglutamic (1) and 3-hydroxyisovaleric acid (2). More recently, several unusual organic acids have been identified in urine from such infants, including methylsuccinic, 4-hydroxyisovaleric, mesaconic, and 3-hydroxyisohexanoic acids and isovalerylglutamate (3–6). The fact that these abnormal constituents were not observed in the original studies of isovaleric acidemia (7) casts some doubts as to whether these biochemical changes are a consistent feature of the disease. With this question in mind, we wish to report our studies of a case of isovaleric acidemia.

A female infant, born in a country hospital, became lethargic on the third day of life and her condition deteriorated progressively, necessitating transfer to King Edward Memorial Hospital for Women in an aerial ambulance. Gas chromatography of ethyl acetate extracts of the patient’s urine demonstrated the presence of organic acids confirming the diagnosis of isovaleric acidemia, as will be discussed in detail later. Initial treatment by intravenous plasma infusion led to a slight improvement in blood pressure. However, despite another exchange transfusion, the patient continued to deteriorate and died on the eighth day.

For quantifying the organic acids in urine, we added 100 μg of 2-hydroxymethylacetic acid to 1 mL of urine as saturated with NaCl and acidified by addition of two drops of 6 mol/L HCl. The urine was then extracted three times with 2-mL portions of ethyl acetate. The ethyl acetate phases were combined and dried over 1 g of anhydrous sodium carbonate for 2 h, then divided into two aliquots and evaporated. One aliquot was silylated with 50 μL of N,O-bis(trimethylsilyl)acetamide (70 °C, 30 min); the other was methylated with diamethane (8). Both aliquots were injected onto a 1.8 m × 2 mm (i.d.) glass glass-chromatographic column packed with 3% SE-30 on Chromosorb W AW-DCMS coupled to a Varian MAT 311 mass spectrometer. The temperature program started at 60 °C, then, after a 10-min isothermal delay, increasing by 4 °C/min to 250 °C. Acids were quantified by measuring peak areas obtained by triangulation relative to that of the internal standard, taking into account extraction efficiencies and detector responses.

The profile of trimethylsilyl derivatives of organic acids in the infant’s urine is shown in Figure 1. Peak identity was assigned on the basis of mass spectra identical to those already published for authentic compounds (4). Methyl ester derivatives were necessary for the initial identification of isohexanoic acid (5) and isovalerylglutamic acid (6), which were not detected in silylated extracts. The concentration of leucine metabolites in an untimed urine specimen collected on day 7, at a time when the infant was already in a coma and deteriorating rapidly, is shown in Table 1. The diagnosis of isovaleric acidemia based on the demonstration of isovalerylglutamic in the infant’s urine was further substantiated by a marked inability of cultured skin fibroblasts from the infant to metabolize DL-[2,4-14C]leucine to 14CO2 (9). The infant’s fibroblasts had only 1.5% of the capacity of normal skin to metabolize leucine (17 vs 1163 ± 594 pmol/10⁶ cells in 3 h).

Previous reports of the presence of products of ω and ω-1 oxidation of isovaleric acid in urine from individuals with isovaleric acidemia have involved studies of a severe form of the disease resulting in the death of the baby within two to three weeks after birth, following an intractable illness (3, 4), a description that fitted our patient also. Because she showed no sustained recovery before her death, we could not study the effect of sympot remission on urinary profiles. We feel, however, that the profile of organic acids in our patient’s urine, which was very similar to that reported by Truscott et al. (4), may be typical of cases of the severe form of isovaleric acidemia.

We thank Dr. S. P. Wilkinson for help with mass spectrometry, Dr. W. Carey (Adelaide Children’s Hospital) for the confirmation of skin fibroblast studies, and Dr. J. R. Tompkins for olfactory skills which first led to suspicion of the diagnosis.

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**Table 1. Excretion of Leucine Metabolites in the Infant’s Urine**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc, mol/mol creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Hydroxyisovaleric</td>
<td>1.15</td>
</tr>
<tr>
<td>4-Hydroxyisovaleric</td>
<td>0.11</td>
</tr>
<tr>
<td>Methylsuccinic</td>
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<tr>
<td>3-Hydroxyisohexanoic</td>
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<td>Mesaconic</td>
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<td>Isovalerylglutamic</td>
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<tr>
<td>Isovalerylglutamate</td>
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