onset of chest pain. None had heart failure, severe renal or hepatic dysfunction, aortic stenosis, hypotension, or increased heart rate, and none was taking cardiovascular drugs.

Venous blood was sampled every 4 h for the first 24 h of admission, and then every 8 h for the next 24 h. Each specimen was centrifuged as soon as the clot formed, and the serum was separated and stored at −20 °C until analysis.

We measured iron in a Rotochim II parallel centrifugal analyzer (V.A. Howe Co. Ltd., London, U.K.), using Ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] in an acetic buffer containing thioglycolic acid. Serum ferritin was measured with reagent kit sets (Amerham International plc, Amersham, U.K.).

TIBC was measured by saturation of the binding sites with ferric chloride, removal of excess iron with magnesium carbonate, and assay of iron as described above. Transferrin was measured immunometrically in the Rotochim II analyzer with use of antibody from SAPU, Law Hospital, Carlake, Scotland.

The imprecision (SD) of the iron, ferritin, TIBC, and transferrin measurements, judged from duplicate analysis of patients' specimens, were: 1.51 μmol/L (n = 110), 21.1 μg/L (n = 143), 2.97 μmol/L (n = 73), and 0.04 g/L (n = 157), respectively.

We assayed the activity of the myocardial isoenzyme (CK-MB) of creatine kinase (EC 2.7.3.2) in a DuPont aco 3 discrete analyzer. All subjects had an increase in CK-MB to more than 100 U/L, showing that all had suffered a myocardial infarction (normal reference range: up to 10 U/L).

Table 1 summarizes our analyses. Although hyperferrremia does develop after myocardial infarction, it is insignificant until 24 to 32 h after onset of symptoms. Interestingly, iron concentrations increased slightly during the first 12 h, although not significantly. This phenomenon has been seen after surgery (4), but was attributed to autotransfusion of blood and the resulting traumatization of erythrocytes. Perhaps mild hyperferrremia does in fact occur before hyperferrremia in all the stressful events that result in low iron concentrations (5), although there is some evidence that iron concentrations fall after surgery within 6 h (6).

Ferritin concentrations increased significantly after 40 to 48 h, as it does after surgical stress (4, 7, 8). TIBC values did not change significantly during the 48 h after infarction but, in contrast to the short-term changes after surgery (6), transferrin concentrations declined significantly only at 32 to 40 h.

We conclude that specimens for investigation of iron status in relation to myocardial infarction must be taken within 12 h of the onset of chest pain. Moreover, the decline in serum iron, which has been termed "rapid" (2), is unlikely to occur early enough to be a useful adjunct to existing tests. Our present findings support our earlier data (3) that values for serum iron and TIBC remain essentially unchanged shortly after stress. Our finding that a significant decrease in serum iron occurs before a significant increase in ferritin does not support suggestions (4, 9) that increased ferritin synthesis is the primary event that leads to retention of labile iron and a decrease in the early phase of iron release.

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References

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25-Hydroxyvitamin D2 and 25-Hydroxyvitamin D3 as Measured by Liquid Chromatography and by Competitive Protein Binding

To the Editor:
Measurement of 25-hydroxyvitamin D2 [25(OH)D2] and 25-hydroxyvitamin D3 [25(OH)D3] has been reported by several laboratories that measured absorbance of the effluent from a retrograde "high-performance" liquid chromatography (1, 2). We have had a problem quantifying 25(OH)D2 because a large absorption peak appears between the two peaks that represent 25(OH)D2 and 25(OH)D3. This peak appeared frequently in chromatograms of control samples that had been stored at −60 °C and assayed within two weeks. We undertook to determine the extent to which this peak interfered with measurement of 25(OH)D2 and 25(OH)D3 by absorbance during liquid chromatography as compared with competitive protein binding (CPB) assay, and to develop reliable techniques for assaying these metabolites in plasma containing the interfering peaks.

We isolated column effluent corresponding to the 25(OH)D region from plasma, using a modification of the method described by Lambert et al. (3). We extracted aliquots of plasma with methanol:methylene chloride (2:1 by vol) and resolved the 25(OH)D-containing fractions by chromatographing these plasma extracts on 0.8 × 15 cm columns of Sephadex LH-20 (Pharmacia Inc., Piscataway, NJ), using the solvent system of hexane:chloroform:methanol (9:1:1 by vol). Some fractions were further resolved by passage through a second such LH-20 column, with use of the equilvolume solvent

| Table 1. Concentrations of Iron, Ferritin, TIBC, and Transferrin in 21 Patients at Various Times after Myocardial Infarction |
|---|---|---|---|---|
| Time, h | Iron, μmol/L | Ferritin, μg/L | TIBC, μmol/L | Transferrin, g/L |
| 0–4 | 13.2 | 1.4 | 195 | 38 | 55.0 | 1.9 | 2.76 | 0.08 |
| 4–8 | 13.5 | 2.7 | 160 | 30 | 62.3 | 3.6 | 2.77 | 0.10 |
| 8–12 | 14.3 | 3.1 | 149 | 31 | 56.7 | 2.8 | 2.76 | 0.09 |
| 12–16 | 14.1 | 3.2 | 219 | 37 | 57.9 | 3.5 | 2.74 | 0.06 |
| 16–20 | 12.1 | 2.3 | 248 | 60 | 55.9 | 1.9 | 2.74 | 0.11 |
| 20–24 | 10.8 | 1.3 | 236 | 48 | 54.7 | 2.9 | 2.57 | 0.08 |
| 24–32 | 9.1* | 0.9 | 242 | 51 | 53.6 | 2.8 | 2.55 | 0.07 |
| 32–40 | 8.1* | 1.7 | 316 | 84 | 50.1 | 3.2 | 2.45* | 0.08 |
| 40–48 | 6.2* | 0.8 | 404* | 77 | 48.5 | 3.6 | 2.36* | 0.08 |

*0.05 > p > 0.01. *0.01 > p > 0.001. *p > 0.001.
system hexane:chloroform (2). The fractions containing 25(OH)D were purified on a 0.39 × 30 cm column of micro-PoroSil (Waters Associates, Inc., Milford, MA) preceded by guard columns containing packed silica (Waters Associates, Inc.). The solvent system was hexane:isopropanol (98:2 by vol). We quantified the 25(OH)D₂ and 25(OH)D₃ fractions by measuring the absorbance of the effluent at 254 nm. The effluent fractions were collected at predetermined times relative to standards. Each metabolite was either collected separately (Table 1, runs 2 and 4) or the two together (Table 1, runs 1 and 3). We also quantified the effluent fractions by CBP assay, using normal rat serum as the binding protein (4). Corrections for losses were calculated from the analytical recovery of 1500 to 2000 counts/min of purified radiolabeled 25-hydroxy(26,27-methyl-³H)cholecalciferol (Amersham Corp., Arlington Heights, IL) added to the plasma aliquots before extraction. The aliquots of two normal control samples (Table 1, A and B) were measured in four separate runs with variations in the procedure as shown in Table 1.

We found that the intervening peak on HPLC could be avoided by using the solvent system hexane:isopropanol (98:2 by vol), at a flow rate of 1.5 ml/min. However, in a sample with a large intervening peak, 25(OH)D₂ and 25(OH)D₃ as measured by absorbance (Table 1, sample A, run 1) were high as compared with the values by CBP assay. We measured 25(OH)D₃ in physiological concentrations by adding a second LH-20 chromatography as used by Horst et al. (2) (Table 1, sample A, runs 3 and 4) or by decreasing the specimen aliquot from 3 ml to 2 ml (Table 1, sample A, run 2). Neither of these two strategies provided an adequate measurement of 25(OH)D₃ by absorbance as compared with the CBP value (Table 1, sample A, runs 2 and 4). In samples that do not show an intervening peak (Table 1, sample B, 25(OH)D₃—and probably 25(OH)D₂—can be quantified by any of the three procedures. But whenever large intervening absorption peaks appear in the 25(OH)D region, their effect is to increase the absorbance for 25(OH)D₂ and, to a lesser extent, for 25(OH)D₃, thus giving rise to higher values than those obtained by CBP assay. Our routine assay for 25(OH)D₂ and 25(OH)D₃ quantifies these metabolites by CBP assay of appropriate chromatographic fractions. However, an additional reversed-phase liquid-chromatographic procedure as described by Jones (5) was also found to eliminate the interfering peaks and to quantify 25(OH)D₂ and 25(OH)D₃. A similar reversed-phase chromatography also eliminates the interfering absorption peak(s) that are present in samples after prolonged storage at temperatures of −10 to −20°C (6). HPLC absorbance and CBP assay give identical results if no intervening peak appears between these two metabolites in the chromatogram.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense.

References


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Synthetic Peptides As Alternative Antigens in the Production of Antibodies against Human Apolipoproteins

To the Editor:

To quantify apolipoproteins or to study their structures and functions it is necessary to develop standardized antibodies that consistently react with determined antigenic determinants. Hybridoma technology has made it possible to regulate more rigorously both the specificity and the reproducibility of the antibody reagent. However, monoclonal antibodies may be of low avidity and low binding constant and may react with epitopes that cannot be chosen in advance on the native protein. So we are challenged to define polyclonal antibodies better in terms of their specificity characteristics and their ability to recognize the same antigen in different lipoprotein particles. Chemically synthesized short fragments of a protein can elicit antibodies that not only recognize the free peptide but also the native protein (1). We have produced antibodies to apolipoprotein C-II and E with synthetic peptides corresponding respectively to residues 46–58 of apo C-II and residues 141–165 of apo E, covalently linked to tetanus toxoid by use of glutaraldehyde. Sequences have been chosen on the basis of the probability that they will appear at the surface of the protein (2). The peptides were synthesized by a previously described solid-phase method (3). They were purified by a combination of gel filtration on Biogel and preparative liquid chromatography. We have controlled their homogeneity and amino acid composition.

Double immunodiffusion (Ouchterlony technique) of antibodies against

Table 1. 25(OH)D₂ and 25(OH)D₃ as Measured by HPLC Absorbance and CBP

<table>
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Plasma sample B

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<th>25(OH)D₃</th>
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<tr>
<td>4</td>
<td>ND</td>
<td>22.8</td>
<td>22.8</td>
</tr>
</tbody>
</table>

* 2 ml of plasma was extracted in this run, 3 ml in the others. A second LH-20 column in a solvent system of hexane:chloroform (1:1) was used in runs 3 and 4.
* ND in this column means non-detectable, <5 µg/L; in column 5, <2 µg/L.