The recently commercially produced specific enzyme phenylalanine dehydrogenase offers many possibilities for applications in screening programs (7) and for follow-up of dietary therapy (8). Moreover, automatization adds speed and precision. In fact, results are available in about 0.5 h after blood extraction, with an accuracy similar to that of ultraviolet (8) or visible spectrophotometric methods involving the same enzyme. Finally, the automated method seems more precise than these manual methods.

We greatly appreciate the skillful technical assistance of J. Moreno and M. Quintana.

References


Biochemical Markers for Detecting Bone Metastases in Patients with Breast Cancer
Luigi Moro,^1 Carlo Gazzarrini,^1 Diana Crivellari,^2 Enzo Galligioni,^2 Renato Talamini,^2 and Benedetto de Bernard^1

A study was carried out to assess the best use of biochemical bone markers to exclude metastases in patients with breast cancer. Urinary galactosyl-hydroxylysine and serum alkaline phosphatase were used to monitor bone resorption and deposition, respectively. Hydroxyproline was also measured. In a selected population of patients, possibly affected by metastases on the basis of scintigraphic examination, which is highly sensitive but poorly specific, we assessed the efficiency of the markers by a double statistical analysis. In this group, the only marker able to predict metastases was galactosyl-hydroxylysine.

Additional Keyphrases: galactosyl-hydroxylysine • urine • bone • alkaline phosphatase • hydroxyproline • receiver-operating characteristic curves

In Western countries, breast cancer is the main cause of death in women between ages 35 and 45 years (1); moreover, metastases are present in 50-85% of these cases (2). Methods that can identify such a complication early are therefore of great interest.

Roentgenography is the most common method for this, but it is not sensitive enough to detect small lesions. Only when metastases are ≥1-2 cm in diameter and ~30% of bone mineral has been lost are lesions detectable by this technique (3). Bone scintigraphy is much more sensitive but has poor specificity, giving positive findings also for benign tumors and other non-malignant conditions, e.g., Paget disease, dysplasia, and osteomyelitis (4-6). Follow-up of patients with positive scintigraphy findings and negative roentgenograms shows that only 52% will develop bone metastases (7). It is, therefore, important to find other bases for the diagnosis.

Investigators have explored the use of biochemical-bone markers for this purpose. Recently, we compared (8) the predictive capacity of two markers of bone resorption, galactosyl-hydroxylysine (GHYL) and hydroxyproline (HYP), in two groups of patients affected by breast cancer, one with (M+) and the other without (Mo) scintigraphic and radiological evidence of bone metastases. We have now extended this study to 101
patients, who were divided into three groups according to their scintigraphic and radiological data; we have included serum total alkaline phosphatase (ALP; EC 3.1.3.1) as an index of bone formation, in an attempt to increase the efficiency of the diagnosis.

**Patients and Methods**

**Patients**

Three age-matched groups of women with breast cancer were selected for this study: 28 patients with clinical, radiological, and scintigraphic evidence of osteolytic, osteoblastic, or mixed bone metastases (M+), and 41 without such evidence (Mo). A third group of 32 patients (Mx) had positive scintigraphic results without clinical or radiological evidence of metastases. The patients had no previous history of any skeletal or hepatic disease and were not receiving drugs that might influence skeletal metabolism, e.g., corticosteroids, calcitriol, diuretics, or vitamin D metabolites. After mastectomy, the patients were investigated every 3 months with clinical, radiological, and laboratory tests. Bone scintigraphy with $^{99m}$Tc was repeated yearly.

**Procedures**

**Urine collection and measurements.** A 24-h urine sample was collected from each patient every 3 months for measurements of creatinine, HYP, and GHYL. The collection was started after 3 days of a collagen-free diet. Borate (1 g/L) was added to the urine samples to prevent bacterial growth. The samples were stored at $-30^\circ C$ until analyzed.

GHYL was measured by HPLC of dansylated urine samples as previously reported (8). Creatinine was measured according to the Jaffé method with a Biochemia kit (Boehringer Mannheim GmbH, Mannheim, Germany); HYP was quantified according to the procedure of Kivirikko et al. (9).

**Blood collection and measurements.** Blood samples were taken from the patients on the same day as the urine collection. Routine analysis of the resulting serum samples was performed with a Hitachi 704 automated analyzer (Boehringer Mannheim). Total ALP was determined with a Biochemia kit, in which p-nitrophenyl phosphate is the substrate.

**Statistics.** Statistical differences between the groups were evaluated by Student's $t$-test. The discriminatory power of the three markers in the different groups was estimated by calculating the $z$ scores.

To evaluate the efficiency of the tests, we fixed 10 arbitrary threshold values (10), and for each value we estimated the specificity (as the ratio between true-negatives and the sum of true negatives and false positives) and the sensitivity (as the ratio between the true positives and the sum of true positives and false negatives). The sensitivity was then plotted vs (100 - specificity) to produce a receiver-operating characteristic (ROC) curve for each assay. The diagnostic efficiency was estimated as the area under the ROC curve (10).

To evaluate the capacity of GHYL, HYP, and ALP to discriminate between Mo and M+ and between Mx and M+, we used logistic-regression analysis of the posterior probabilities for prediction from the logistic model (11).

**Results**

Because bone metastases in the patients of this study were identified by roentgenography as osteolytic (75%), osteoblastic (21%), and mixed type (4%), we also included the most used marker of osteoblastic activity, ALP, in our attempt to increase the predictive capacity of the tests. Figure 1 shows the values of urinary GHYL and HYP concentrations and serum ALP activity in the three groups of patients. All markers are significantly greater in the M+ patients than in the patients of the
Mo and Mx groups; in the latter two groups, the markers did not differ between groups. The z-score analysis confirms these observations (Table 1), and we conclude that all three markers differentiate M+ patients from the Mo and Mx patients, but none discriminates the Mo patients from the Mx patients.

In another approach to assess the value of each marker, we examined the diagnostic efficiency of the tests, drawing ROC curves separately for each marker and for combinations of the markers (Figure 2). The largest area under the curve, i.e., the best diagnostic efficiency (0.89), was provided by GHYL, followed by ALP (0.82) and HYP (0.78). However, the combination of GHYL and ALP gave an even better value (0.97), covering essentially all the area. Combining GHYL and ALP with HYP did not increase the efficiency of the tests.

We also used a weighted multivariate logistic regression to predict metastases in patients with breast cancer. We tested the logistic model's improvement for each variable (GHYL, ALP, and HYP) separately and for all the variables together. Using the data illustrated by Figure 2 in the logistic-equation model showed a significant contribution to predict metastases for the variables GHYL ($P = 0.01$) and ALP ($P = 0.02$) but not for HYP ($P = 0.53$).

We also monitored the patients in the Mx group. After 18 months, 10 Mx patients (31%) had converted to M+. We then divided the original Mx group data according to whether each subject's status remained unchanged or converted to M+, and used the ROC curve (Figure 3) and the logistic model described above to analyze the predictive capacity of the tests. GHYL provided the best efficiency (0.83) and again gave a significant contribution to predict metastases ($P = 0.03$), whereas ALP (efficiency = 0.64) was not useful ($P = 0.19$).

**Discussion**

Bone markers used in the present study appeared to agree with the data provided by the conventional means of diagnosis, because they had average normal values in Mo patients and increased values in M+ patients. Interestingly, in the Mx group, characterized only by positive scintigraphy, the concentrations of the markers were in the normal range, suggesting that metastases were either absent or quite small (below the detection limit of roentgenography).

The present investigation confirms the data we already published (8) and shows the utility of using the combination of a specific bone resorption marker (GHYL) with a marker of bone deposition (ALP). Given an efficiency of 0.97 for this combination, the rate of false positives and negatives is almost negligible. Our findings also confirm the poor specificity of HYP, which did not increase the efficiency when combined with the other markers.

**Table 1. Discriminating Power of Each Biochemical Marker in the Three Groups of Patients**

<table>
<thead>
<tr>
<th></th>
<th>GHYL</th>
<th>HYP</th>
<th>ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo vs Mx</td>
<td>1.5</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Mo vs M+</td>
<td>8.2</td>
<td>3.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Mx vs M+</td>
<td>7.0</td>
<td>2.6</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*Based on data shown in Fig. 1.*
When we focused on the evolution of the patients of Mx group, we found that the efficiency of the tests in this group was slightly lower for GHYL (0.83), and lower for ALP (0.64) and that the interaction between GHYL and ALP did not increase the area under the ROC curve (Figure 3). Moreover, the multivariate logistic regression analysis showed that the only predictive variable for metastases was GHYL \( (P = 0.03) \). We believe this apparent discrepancy reflects the different populations of patients that form the Mx and the Mx group. The Mx group is not as homogeneous as the Mx group, because some patients in the former group may indeed have metastases, even if at the moment these could be detected only by scintigraphy. Because scintigraphy is much more sensitive than the biochemical markers, the rate of false positives (true metastases despite the biochemical markers being in the normal range) will affect the efficiency of the tests when compared with their performance for the Mx and M+ groups.

In conclusion, the ROC curve and the multivariate logistic regression analysis show that also in the Mx group, some of whom converted to the M+ group, the only predictive marker for metastases remains GHYL.

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References

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Plasma and Urinary Oxalate and Glycolate in Healthy Subjects

L. Hagen, V. R. Walker, and R. A. L. Sutton

High-performance ion chromatography (HPIC) has been widely used for oxalate analysis and, more recently, for glycolate analysis. We describe a procedure for sample preparation in which the plasma ultrafiltrate is acidified during harvesting with a cation-exchange resin, and the chloride is removed before the ion chromatography, which is performed with a newly developed AS10 column. The same ultrafiltrate sample is analyzed for glycolate. For plasma oxalate, the mean recovery of sample in eluted fractions was 95–96%, and intraassay CV was 6.2–8.1%. The reference interval (mean ±2 SD) for men was 0.8–3.2 μmol/L and for women, 1.0–2.6 μmol/L. For urinary oxalate, the reference interval for men was 175–560 μmol/day and for women, 107–432 μmol/day. For plasma glycolate, the mean analytical recovery was 96–98%, and the intra-assay CV was 2.4–6.2%. The reference interval for men was 1.9–7.5 μmol/L and for women, 1.4–7.4 μmol/L. For urinary glycolate, the reference interval for men was 0–1400 μmol/day and for women, 91–1001 μmol/day.

Additional Keyphrases: high-performance ion chromatography · reference interval

The measurement of oxalate and, more recently, glycolate in plasma has been associated with several difficulties, resulting in variable and inaccurate determinations, often with an overestimation of plasma concentrations (1–4). A high-performance ion chromatography (HPIC) system has been used to quantify plasma oxalate (1, 2, 5). Basically, this system involves production of an ultrafiltrate of plasma (or serum), which may or may not be cooled and acidified; dilution (usually) with boric acid; and injection onto an HPIC A54A ion-exchange column for elution with a carbonate/bicarbonate buffer. However, there have been problems with the recovery of oxalate from the sample and with the prevention of oxalate generation before the assay. Indirect isotopic studies indicate that oxalate concentrations in plasma are very low (6, 7) and, thus, great sensitivity is required in the assay. For glycolate, overestimation of plasma concentrations is the major problem (4, 8, 9).

Here we describe an improved sample-processing technique for determining plasma oxalate. For glycolate, we used a simple chromatographic method adapted from Wandelilak et al. (10).