Melanogenuria: Laboratory Evaluation of the Qualitative Thormählen and Ferric Chloride Tests and Their Clinical Utility

Richard E. Scott,1 Virginia L. Ward, Gregory F. Grinstead,2 Bernadine S. Stevens, and David M. Wilson

Malignant melanoma, a disease that is increasing in occurrence and medical concern, is characterized by the excretion of melanogens. Two qualitative tests are recommended for melanogen detection, the Thormählen test and the ferric chloride test. We evaluated the laboratory and clinical performance of these tests by subsequently re-evaluating 201 urine samples that had been submitted for routine melanogen analysis. We used (a) Thormählen, (b) ferric chloride, (c) small-scale thin-layer chromatography, and (d) spectrophotometry. Nearly 30% of Thormählen test results were equivocal. The ferric chloride test was of no value in itself or in categorizing equivocal Thormählen results as positive or negative. The small-scale chromatography was irreproducible. Prompt scanning of the Thormählen reaction product was helpful in classifying equivocal results. History review of 121 histopathologically diagnosed melanoma patients indicated that these qualitative assays were of no clinical value in the diagnosis or monitoring of melanoma patients.

Additional Keyphrases: malignant melanoma · cancer · melanogen detection · test performance

Malignant melanoma (1), once a rare tumor, is becoming more common. Its prevalence has doubled in the last decade, having shown a steady increase for over four decades, and the mortality rate for melanoma is increasing faster than for any other cancer except lung cancer (2). Biochemically, melanoma is characterized by the increased urinary excretion of melanogens, colorless phenolic and indolic precursors of melanin, of which several have been identified (3, 4). According to their reaction in the Thormählen test (5), melanogens are termed Thormählen positive (indolics) or Thormählen negative (phenolics).

The qualitative Thormählen and ferric chloride (von Jaksch) (6) tests are still considered the most sensitive and specific tests generally available for detecting melanogenuria (7–9). We routinely perform the Thormählen test to determine if a sample is positive or negative. When results are uncertain, the ferric chloride test is then used. However, we have found that a large proportion of samples give equivocal results, necessitating a final (and subjective) judgement by a supervisor. A doubling in the number of melanogenuria test requests during the last year prompted us to re-evaluate our current procedures.

Materials and Methods

Sample Handling

For routine melanogenuria testing of clinic patients, freshly voided untimed urines were collected in plastic containers, with no added preservative. The sealed containers were transported to the laboratory and analyzed within 30 min to 4 h of sampling. For reference-laboratory patients, similarly collected samples were frozen before transport to the laboratory, where they were thawed, equilibrated to ambient temperature, mixed, and analyzed within 30 min to 1 h. If the samples could not be re-evaluated during these time intervals, sealed samples were stored frozen at −20 °C.

Laboratory Evaluation

Two hundred and one urine samples submitted for routine melanogenuria testing were each re-evaluated for this study by the four methods detailed below. In addition, we obtained a control group of 50 urines from non-melanoma clinic patients; each was analyzed by the Thormählen test. Test results were evaluated by at least two individuals not involved with the initial routine testing procedure, and history reviews were performed retrospectively. Of the re-evaluated samples, 50 were from clinic patients whose histories were reviewed for any correlation between a positive or equivocal melanogenuria test result and melanoma type, stage of disease, or treatment regimen. The remainder were from our reference-laboratory patients, whose histories were unavailable for review. A history review was also completed for those control group patients whose samples gave equivocal Thormählen results.

Urines positive for amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, or opiates were screened with the Thormählen test to determine if specific drug groups were responsible for equivocal results.

The tests used for re-evaluation of samples were:

- **Thormählen test:** To 1 mL of urine in a test tube, add 0.2 mL of sodium nitroprusside (20 g/L; prepared weekly, stored sealed at 4 °C) and mix. Add 0.2 mL of a 100 g/L solution of sodium hydroxide and mix. This produces an amber to brick-red color that soon begins to fade. Wait 1 min, then add 0.2 mL of glacial acetic acid and mix. Note the initial color: blue is positive (5, 10), greens are equivocal (see Results) and are referred to supervisory staff, amber is negative. A positive control (positive melanoma urine, stored at −70 °C for up to six months) or indole (10) (200 mg/L; Sigma Chemical Co., St. Louis, MO) is run with each series of samples.

- **Ferric chloride test:** To 1 mL of urine in a test tube, add five drops of ferric chloride (100 g/L solution in 1.2 mol/L hydrochloric acid) and mix. Note the initial color. A gray-black color (8) or precipitate (9) is positive. Controls were as for the Thormählen test.

- **Thin-layer chromatography (TLC):** Unextracted urine samples were spotted onto separate 2.5 × 7.5 cm sheets of either non-fluorescent (polygram gel 300; Brinkmann Instruments Inc., Westbury, NY) or fluorescent cellulose (Chromagram 13254; Eastman Kodak Co., Rochester, NY).
Table 1. Thormählen Test Results for Clinic and Reference-Laboratory Patients

<table>
<thead>
<tr>
<th>Type/Stage</th>
<th>Total</th>
<th>Positive</th>
<th>Negative</th>
<th>Equivocal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastatic</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Nodular</td>
<td>8</td>
<td>0</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>SST*</td>
<td>32</td>
<td>0</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>Choroid</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Totals</td>
<td>50</td>
<td>1</td>
<td>32</td>
<td>17</td>
</tr>
</tbody>
</table>

*Percentage of total in parentheses.

Table 2. Correlation of Thormählen Test Result with Type (Nodular, SST*, Choroid, Other) and Stage (Metastatic) of Malignant Melanoma for Clinic Patients

<table>
<thead>
<tr>
<th>Type/Stage</th>
<th>Total</th>
<th>Positive</th>
<th>Negative</th>
<th>Equivocal</th>
</tr>
</thead>
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<tr>
<td>Metastatic</td>
<td>6</td>
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<td>3</td>
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<tr>
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<td>0</td>
<td>6</td>
<td>2</td>
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<tr>
<td>SST*</td>
<td>32</td>
<td>0</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
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<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Totals</td>
<td>50</td>
<td>1</td>
<td>32</td>
<td>17</td>
</tr>
</tbody>
</table>

*SST, superficial spreading type.

Table 3. Results from Spectrophotometric Scanning of Thormählen-Positive and Equivocal Reaction Products

<table>
<thead>
<tr>
<th>Result</th>
<th>Total</th>
<th>Type</th>
<th>Type</th>
<th>Type</th>
<th>Type</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Equivocal</td>
<td>59</td>
<td>1</td>
<td>15*</td>
<td>6</td>
<td>6</td>
<td>29</td>
</tr>
</tbody>
</table>

*Scan types are shown in Figure 1. *Three Type 2 samples showed slight peaking at 610 nm.

Sheets were developed with a mixture of acetone, n-butanol, water, and diethylamine (10:10:5:2, by vol), in Coplin jars. Melanogens were detected by brief, successive immersion of the sheets in Thormählen reagents.

Spectrophotometric scanning: The Thormählen test (above) was conducted on a fresh aliquot of each sample, and the solution was transferred to a 1-mL glass cuvette. The scan was completed within 5 min from the time the glacial acetic acid was added. Samples were scanned from 450 to 750 nm in a Model 35 recording spectrophotometer (Beckman Instruments Inc., Palo Alto, CA).

Clinical Evaluation

The histories of all 121 clinic patients who had had melanogenuria tests requested during 1984 were reviewed to December 1986. Melanogenuria testing (Thormählen, with the ferric chloride test as backup) had been performed as described. Test results were correlated with melanoma type, Clark level (depth of tissue invasion), and stage of disease. All patients had been examined histopathologically. Two were diagnosed as non-melanoma malignancies; diagnosis for the remainder ranged from superficial lesions to metastatic malignant melanoma.

Results

Laboratory Evaluation

Thormählen Test. Table 1 summarizes the re-evaluation data for both clinic and reference-laboratory patients. Nearly 30% of samples gave equivocal results (light-green, green, olive-green). There was a similar distribution of positive, negative, and equivocal Thormählen results for samples from both the clinic and reference laboratory. Thus, we believe that the results of the history review for the 50 clinic patients reflects what would be expected for the general population. This history review failed to show any relationship between the type or stage of malignant melanoma and results for the Thormählen test (Table 2). Further, we saw no correlation between treatment regimen and equivocal results, nor did examination of drug-positive urines reveal any specific drug-related interference with the test.

Of the 50 urines from the control group, 10 (20%) gave equivocal Thormählen test results. The history review for these 10 patients showed no previous history of malignant melanoma.

Colors for the positive samples were the characteristic dark or Prussian blue and, in one case, an emerald green/blue that was also considered positive. Equivocals ranged from light-green through green to olive-green. Negatives ranged from light yellow through amber to light-brown or brown, and in one case a pale pink.

Ferric chloride test. Results of the ferric chloride test varied greatly. For the 12 Thormählen-positive samples the color ranged from amber through light-carmine, brown, gray or black, to purple; some also showed precipitates. The Thormählen-equivocal samples varied in a similar fashion, although amber predominated, followed by purple. Thormählen negative urines showed mainly amber, although 14 purple, eight brown, one black, and one gray were noted.

TLC. The TLC method was unsatisfactory because of irreproducible Rf values, limited sensitivity, and the technician time required.

Spectrophotometric scanning. Prussian blue, the expected Thormählen test product (5), absorbs maximally at 610–620 nm (10). We scanned the Thormählen product of all 201

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samples from 450 to 750 nm, finding five basic patterns of absorbance (Types 1 to 5) depicted schematically in Figure 1. All of the 12 presumed positives showed a peak in the 610-nm region (Type 1, Table 3), but only one of the equivocals (Table 3) showed a distinct peak (Figure 1), suggesting that it should be classified instead as a positive. Of the equivocal samples with a Type 2 scan, three showed a very slight peak at about 610 nm (Table 3). The substantial absorbance in the mid range of these scans (Figure 1) may suggest an unresolved peak.

After scanning the equivocal samples, we considered four to be positive; thus, 93% of them were reclassified as negative. Even if samples yielding both Type 1 and Type 2 scans were considered presumptive positives, scanning evidently would allow 73% of the Thorméhlen equivocals to be reclassified as negative.

Clinical Evaluation

Review of the 121 clinic patient histories failed to show any correlation between the type of melanoma, stage of disease, Clark level, or treatment regimen and either a positive or negative melanogenuria test result. For the 25 metastatic patients from the group, Table 4 illustrates this lack of correlation. Only three positive melanogenuria results were reported throughout the clinical histories of these patients, not the (at least) 25 that would be expected if the test were in fact useful in detecting metastatic disease. Of these positive results, only one corresponded to the time of diagnosis of metastatic spread (choroid to liver). When metastases were to regional or even distal lymph nodes, results of the melanogenuria test invariably were negative.

Discussion

At least three current, respected clinical chemistry texts recommend the qualitative Thorméhlen and ferric chloride tests for detection of melanogenuria (7–9). However, from our study and a review of the literature, we believe that these tests give results that are unreliable and difficult to interpret, and that they are of no clinical value in the diagnosis, follow-up, or detection of occult (advanced) disease. Beeler and Henry (11) came to similar conclusions.

The original descriptions for positive qualitative melanogenuria tests were: a black precipitate for the ferric chloride (6), and a Prussian blue color for the Thorméhlen test (5). Over the years these endpoints have been changed. Thus, for the ferric chloride test (in hydrochloric acid) a brown, dark-brown, or black color (8), or a gray-black precipitate or dark-gray supernate (but not dark brown) (12), is positive; and for the Thorméhlen test an olive, brownish-green, green, greenish-blue, blue, bluish-black, or black color have all been considered positive (7–9). It is not surprising that these tests are difficult to interpret.

In the Thorméhlen test, the rationale for a green color being considered positive stems from the logic that a yellow urine and a weakly blue positive reaction will yield green (8). However, we believe that this is pushing such a qualitative test well beyond its intended limits and is perhaps the principle source of the confusion that commonly accompanies its use. Furthermore, we have shown that such equivocal results can occur in 20% of non-melanoma patients. The ferric chloride test adds only further confusion to an already difficult qualitative interpretation.

Spectrophotometric scanning appears to be a useful aid in interpreting Thorméhlen tests. Scanning of the Thorméhlen reaction product showed the presence of peaks centered on 610 nm (Type 1 and equivocal, Figure 1), not simply an increased absorbance at this wavelength (as in Types 3 and 5 scans, Figure 1). If an increased absorbance at 610 nm had been used as the sole criterion, 60% of equivocal samples (Table 3) might have been incorrectly identified as positive. However, as seen above, scanning alone does not supply unequivocal results; e.g., Type 2 scans may contain a hidden peak (Figure 1). Furthermore, we noted that even the timing of reagent additions can markedly affect the final color. For example, one sample gave a light-green, dark-green, or blue color, depending on how long after alkalization the glacial acetic acid was added. And a greenish hue could be imparted to some “negative” samples by vigorous shaking.

From the clinical viewpoint, Eppinger (13) suggested in 1910 that melanogenuria arose only in those patients with hepatic metastases. The summary of our history review for metastatic patients (Table 4) suggests that even this is not consistent. For the metastatic patients previously diagnosed with nodular melanoma, one positive result was for a patient with an amelanotic nodule and amelanotic involvement of the lymph nodes. The second positive result was associated with a patient whose disease was considered to be regressing, or at least quiescent, by other criteria (physical examination, x-rays), and the physician noted that the urine melanogen test was “positive for no apparent reason.” The only other positive test appeared at the time of diagnosis for metastatic disease to the liver. Unfortunately, detection of melanogenuria at this advanced stage of the disease is only of academic interest and of no benefit to the patient.

Alternative means of detecting malignant melanoma are primarily quantitative Thorméhlen assays (10, 14), 5-S-cysteinyldopa assays (15), or tyrosinase assays (14, 16). Their clinical utility is questioned (17, 18). Immunological detection of melanoma-specific proteins and circulating immune complexes has received some attention (19, 20), but again their diagnostic utility is uncertain (21, 22). We thus conclude that the laboratory currently cannot offer a suitably specific assay to assist the clinician in reliably diagnosing or following the treatment or progression of malignant melanoma. In accordance with others (11), we also conclude that the qualitative Thorméhlen and ferric chloride tests are difficult to interpret in the laboratory and are of no value in clinical diagnosis and management of malignant melanoma. It is now a century since these qualitative tests were introduced. They clearly are outmoded, and their use should be discontinued.

References
Immunoprecipitation Method for CK-MB Analysis Re-Evaluated: Influence of CK-BB and Macro-
CK on Blank Activities

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We evaluated the analytical performance of the immunoprecipitation technique for quantification of creatine kinase (CK; EC 2.7.3.2) isoenzyme MB, focusing on specimens with increased blank activities. The samples we studied had blank values that were equal to or greater than either 15 U/L or the uncorrected CK-MB value. Of 134 specimens selected, 16 and 21 contained macro CK type 1 and CK-BB, respectively. All samples containing macro CK type 1 gave negative CK-MB values, even though four contained significant quantities of CK-MB. Correcting the blank value for the fluid displacement of the second antibody suspension eliminated all but three of the negative results. However, 45 specimens, negative for CK-MB by electrophoresis, gave CK-MB values ranging from 1 to 21 U/L. Furthermore, 11 of the specimens containing macro-CK type 1 were in this group. Thus, the presence of macro-CK type 1 definitely interferes with the immunoprecipitation technique. Our results indicate the need to re-evaluate the diagnostic accuracy of the immunoprecipitation technique, especially when the un-adjusted blank activities are increased.

The clinical utility of measuring the MB isoenzyme of creatine kinase (CK; EC 2.7.3.2) as an aid in the diagnosis of myocardial injury is well established (1-3). Antibody-based activity assays, which give results more rapidly than do the conventional techniques of electrophoresis and column chromatography (4, 5), measure residual B-subunit activity after inhibition with anti-CK-M antibody. Thus, these systems are subject to interference by CK-BB and any "CK"-like activity not inhibited by the antisera—i.e., atypical CK and (or) adenylate kinase (EC 2.7.4.3) (4-7).

In the immunoprecipitation (IPT) technique of Roche Diagnostics, a blank tube is included to obviate the influence of any non-inactivated "CK" activity (6). After inhibiting with the antibody to CK-M subunit, an anti-antibody to CK-M, bound to an inert substance, is added. Separation of all isoenzymes containing CK-M is then effected by centrifugation. Residual "CK" activity—e.g., CK-BB, atypical-CK, adenylate kinase—is then measured. The blank activity is subtracted from the test result for the sample, and a correction factor is applied to yield the "true" activity of CK-MB.

The lack of CK-BB or atypical-CK influence on the analytical specificity of the IPT method has been reported in several studies (6, 8-11). However, we found specimens having discordant results, e.g., blanks with values exceeding test values or high blank activities. The fact that the blank value is high may indicate the presence of CK-BB or

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