When Is an Indole Not an Indole?

To the Editor:

An indole is not an indole when it is a naphthalene derivative!

We routinely measure 5-hydroxyindoleacetic acid (5HIAA) by a long-established method using nitrosodimethylaniline, as first described by Udenfriend in 1955 [1], but with various modifications to improve specificity [2]. The profession’s elder statesmen may well recall the method of Hanson and Serin [3] using Erlich’s reagent.

Although the Udenfriend method is very specific for 5-hydroxyindoles, it is subject to interference, probably the most documented being that from acetaminophen and its metabolites [4]. Recently, a urine was received for 5HIAA analysis from a 52-year-old male patient complaining of hot flushes. The result was considerably increased before and after the drug-free period (442 and 387 μmol/24 h, respectively) as measured by the Udenfriend method but not by the HPLC method (19 and 30 μmol/24 h).

After discussion, the consultant, general practitioner, and patient all agreed to withhold Relifex for 1 month, repeat the 5HIAA collection, recommence Relifex, and repeat the 5HIAA analysis 1 month later. The 5HIAA result was normal (25 μmol/24 h, measured by the Udenfriend method only) when the patient was not taking the drug and was greatly increased before and after the drug-free period (442 and 387 μmol/24 h, respectively) as measured by the Udenfriend method but not by the HPLC method (19 and 30 μmol/24 h).

In conclusion, we think it essential that all positive 5HIAA analyses be confirmed by a specific method such as HPLC.

References

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Whole-Blood Folate Values in Subjects with Different Methylenetetrahydrofolate Reductase Genotypes: Differences Between the Radioassay and Microbiological Assays

To the Editor:

Traditionally, serum and erythrocyte folate were analyzed by microbiological assay [1]. Because of the original technical difficulty of such assays, more and more laboratories switched over to the radiometric competitive binding assays (radioassays) as they became available. However, it was subsequently found that these newer assays were problematic, particularly with respect to erythrocyte folate estimates [2]. These difficulties are said to be caused by the different versions of the radioassay, and it is now believed that both assays are measuring similar folates, albeit by a different principle, and that the assays give equivalent results [3].

The C677T genetic variant results in a thermolabile and less active form of the folate-dependent enzyme 5,10-methylenetetrahydrofolate reductase and has a homozygous prevalence of between 5% and 15% depending on the population studied [4]. This variant is of interest clinically because it has been found to be more prevalent in people with neural tube defects [4–6] and also in those with increased plasma homocysteine, a new risk factor, or at least marker, for cardiovascular disease [7]. One group has published [8] and to our
knowledge two other groups have found that subjects homozygous for the variant (TT) have higher erythrocyte folate values compared with those heterozygous (CT) or those without the variant wild-type (CC). All three groups have used radioassays for these determinations. It has been our consistent finding with the microbiological assay, however, that the converse is true; we find lower erythrocyte folates in those homozygous for the variant compared with those who are heterozygous or wild-type [9]. Although the populations studied by us and others are different, it is difficult to understand why those with the thermolabile variant should have higher concentrations of folate in some populations [8] and lower in other populations [9]. An obvious explanation is that the method of folate assay used in the former case was radioassay [8] and in the latter [9], microbiological assay.

To resolve this issue, we carried out comparative assays on the three different genotypes for samples selected at random from a normal group of women (group I). Because van der Put et al. [8] had used children with neural tube defects and their parents in their studies, we also selected a group of the three genotypes from such subjects (group II). All procedures were approved by the relevant ethics committees. The microbiological assay was carried out on microtiter plates with the chloramphenicol-resistant strain of Lactobacillus casei [10], and the radioassay was performed with the Dualcount Solid Phase Boil assay (Diagnostic Products Corp.), which was that used by van der Put et al. [8]. We have expressed the results as whole-blood folate for reasons of clarity because these values are converted in the same way to erythrocyte folate values by using the packed cell volume. Serum or plasma folates were not analyzed because the radioassay [8] and microbiological assay [9] give similar results: lower folate values for TT homozygotes.

Table 1 shows that, overall, the microbiological assay gives higher values than the radioassay, as reported in the literature [2,11]. After subdivision of the data by genotype and reanalyzing either as individual groups or combined, there was a decrease in whole-blood folate for the wild-type (CC) and heterozygous (CT) groups (statistically significant for group I \(P = 0.0005\) and \(0.017\), respectively) when carried out by the radioassay compared with the microbiological assay. However, in the homozygous (TT) groups, the whole-blood folate concentrations measured by radioassay were higher, and the difference was statistically significant \((P = 0.026)\) in the combined analysis. Thus, in two different populations, subdivided only with respect to this genotype, the assays are measuring components that change with respect to genotype.

Explanations could be that in those with the homozygous (TT) variant, a species of folate or a degradation product accumulates because the enzyme is compromised. This form could be bound in the radioassay and thus appear active and measurable but might not be microbiologically active. It is also possible, but less likely, that the microbiological assay is underestimating the concentration of some active folate. Alternatively, a folate may accumulate that is estimated in both assays but gives values quantitatively different from the forms of folate normally present. We are at present searching for such putative derivatives. The nature of such derivatives showing either accumulation of known folates or the

<table>
<thead>
<tr>
<th>All genotypes</th>
<th>Radioass.</th>
<th>Microbiol.</th>
<th>Difference between methods (radioassay–microbiol.)</th>
<th>(P), Wilcoxon paired test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (n = 115)</td>
<td>128 ± 54</td>
<td>137 ± 72</td>
<td>−9 ± 50</td>
<td>0.007</td>
</tr>
<tr>
<td>Group II (n = 89)</td>
<td>147 ± 63</td>
<td>150 ± 58</td>
<td>−4 ± 70</td>
<td>0.482</td>
</tr>
<tr>
<td>Combined I + II (n = 204)</td>
<td>136 ± 59</td>
<td>143 ± 67</td>
<td>−7 ± 59</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Wild-type (CC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I (n = 38)</td>
<td>136 ± 56</td>
<td>164 ± 96</td>
<td>−28 ± 57</td>
<td>0.0005</td>
</tr>
<tr>
<td>Group II (n = 30)</td>
<td>140 ± 50</td>
<td>152 ± 54</td>
<td>−1 ± 43</td>
<td>0.092</td>
</tr>
<tr>
<td>Combined I + II (n = 68)</td>
<td>138 ± 52</td>
<td>159 ± 80</td>
<td>−21 ± 52</td>
<td>0.0001</td>
</tr>
<tr>
<td>Heterozygotes (CT)</td>
<td></td>
<td></td>
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<tr>
<td>Group I (n = 41)</td>
<td>114 ± 44</td>
<td>125 ± 53</td>
<td>−11 ± 37</td>
<td>0.017</td>
</tr>
<tr>
<td>Group II (n = 30)</td>
<td>140 ± 48</td>
<td>160 ± 56</td>
<td>−21 ± 60</td>
<td>0.128</td>
</tr>
<tr>
<td>Combined I + II (n = 71)</td>
<td>125 ± 47</td>
<td>140 ± 57</td>
<td>−15 ± 48</td>
<td>0.006</td>
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<tr>
<td>Homozygous variant (TT)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Group I (n = 36)</td>
<td>137 ± 60</td>
<td>123 ± 54</td>
<td>+14 ± 36</td>
<td>0.195</td>
</tr>
<tr>
<td>Group II (n = 29)</td>
<td>160 ± 86</td>
<td>138 ± 63</td>
<td>+22 ± 93</td>
<td>0.112</td>
</tr>
<tr>
<td>Combined I + II (n = 65)</td>
<td>147 ± 73</td>
<td>130 ± 58</td>
<td>+18 ± 71</td>
<td>0.026</td>
</tr>
</tbody>
</table>

* Group I was selected at random from a group of normal women; group II was selected in equal proportions from children with spina bifida, their mothers, and their fathers.
existence of a known or as yet unknown naturally occurring degradation product may be of interest with respect to the etiology of neural tube defects or the homocysteinemia that is associated with this variant. At a more practical level, the comparative evaluation of the two assays reported here indicates that folate concentrations are not being measured accurately by one of the assays in the 5% to 15% of people who are homozygous for the thermolabile variant.

References

4. van der Put NM, Eskes TK, Blom HJ. Is the existence of a known or as yet un
identified mutation in the methyl-
tetrahydrofolate reductase gene a risk fac-
10. Molloy AM, Scott JM. Microbiological assay for serum, plasma, and red cell folate using cryo-

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Simplified Simultaneous Assay of Total Plasma Homocysteine and Methionine by HPLC and Pulsed Integrated Amperometry

To the Editor:

Measurement of total plasma homocysteine (tHcy) can be a useful adjunct in the diagnosis of cobalamin or folate deficiency and is emerging as an independent predictor in many vascular-occlusive diseases [1]. As clinical interest in this metabolite grows, the demand for simple and efficient methods of determination has increased. In some situations, a methionine-loading test may be conducted to evaluate homocysteine catabolism, but methionine is rarely measured concomitantly, because it usually requires a different assay methodology altogether. In homocystinuria caused by cystathionine beta-synthase deficiency, circulating methionine is often increased, whereas homocystinuria resulting from a relative deficiency of the remethylation pathway is characterized by hypomethoni
nemia [2].

In our previously reported serum assay for tHcy [3], we used the DX-500 Ion Chromatograph ( Dionex Canada), outfitted with two pumps (in parallel), valves, and two columns (a 4 x 50 mm OmniPac PCX-500 precolumn and a 4 x 250 mm OmniPac PCX-500 analytical column) plumbed in series to permit “heart-cut” trapping of tHcy [4]. However, with the ED40 electrochemical detector set for pulsed inte-

grated amperometry (PIA) mode, any compound with a reduced sulfur atom, including methionine, will generate a signal proportional to concentration [5].

In our initial procedure, the disulfide reduction procedure with sodium borohydride (NaBH$_4$) [6] was the most labor-intensive step and constituted a substantial source of assay error. Here, we report a simplified protocol for the tHcy assay that permits accurate simultaneous quantification of methionine.

As suggested by Gilfix et al. [7], we used tris(2-carboxyethyl)phosphine (TCEP) as a reductant instead of NaBH$_4$. To 300 mL of plasma we added 30 mL of 100 g/L TCEP (Pierce Chemical Co.) and gently mixed with a rotating stirrer at room temperature for 30 min. Then, we added 1170 mL of mobile phase (150 mmol/L NaClO$_4$, 100 mmol/L HClO$_4$, and 50 mL/L CH$_3$CN) and centrifuged the mix at 10 000 g for 5 min. The supernatant was passed through a C$_{18}$ solid-phase extraction cartridge, as described before [3], and 50 mL of filtrate was injected directly. Altering the valve-switch times to 1 min and 2 min generated a larger “heart-cut” of the eluting peaks, with homocysteine eluting at 7.9 min and methionine at 11.3 min.

With our plasma control, we found that TCEP reduction is complete within a minute or so at room temperature (Fig. 1A). Reduction of Hcy by borohydride at the same temperature was still incomplete at 30 min, and even at 50 °C required at least 15 min to approach completion. Moreover, use of the TCEP reductant significantly decreased between-run variation (CV = 3.1%, n = 10, P < 0.05, n test for comparison of variances) in comparison with reduction of the same sample with NaBH$_4$ and use of our initial protocol (CV = 7.4%, n = 10). Omission of the urea denaturant resulted in a 4% increase of our target tHcy value for the control sample, but the chromatographic profile without urea was less noisy and the assay variation (within-run CV) was correspondingly decreased from 4.8% (n = 16) to 3.8% (n = 16).

Assay of 58 patients’ samples with