Introduction

The AACB Working Party on Biogenic Amines [BAWP] was formed over 20 years ago when urine biogenic amines became an EQA program of RCPA Quality Assurance Programs Pty Ltd [1]. Since then, many AACB members have contributed to this expert group and all are to be thanked for their participation and time spent on its various activities. In particular, mention must be made of Dilo Pillai and John Earl, who have been driving forces and long-standing members of the group.

An important role of the BAWP has been to monitor the overall analytical performance of urine catecholamines and their metabolites for laboratories enrolled in the Biogenic Amines Urine EQA Program. When the program was first offered in the early 1990s, it was taken up initially by a small number of laboratories in the Australasian region. As has occurred in other EQA programs, the number of enrolments has increased over time, and reached 50 in 2013.

With the development of plasma free metanephrines as a new screening test for phaeochromocytoma in the early 2000s, an EQA program was created in 2008 to cover these analytes [2]. Initially, laboratories used HPLC with electrochemical detection for their analyses, but in the last few years tandem mass spectrometry has become the dominant analytical method to achieve the required analytical sensitivity and specificity for their measurement [3].

In a period of over 20 years, it is to be expected that laboratory methods will evolve and adapt to new technologies. While changes in analytical methods can be tracked via EQA enrolment method classifications supplied to the RCPA EQA office each year, no details are provided. In 2011, members of the BAWP, with the assistance of scientific staff of the Chemical Pathology group of RCPA EQA, decided to design and circulate a survey to all laboratories enrolled in 2012 in the Biogenic Amines Urine Program. Much of the survey design was worked out by Lambro Johnson and the survey was released for completion and return in March 2012. To increase the response rate to the survey, a follow-up of non-responding laboratories was carried out from the QAP office in April-May 2012, so that the final proportion of survey responders was 62%. A summary of some of the findings of the survey was presented at the AACB Annual Scientific meeting in Melbourne in 2012 [4].
Survey Design

Although early methods for biogenic amines and their metabolites used colorimetry and fluorimetry, they were non-specific and were replaced in the 1980s by HPLC to achieve chromatographic separation (“fractionation”) of analyte groups, such as the catecholamines (noradrenaline, adrenaline, dopamine) and the metadrenalines or metanephrines (normetanephrine, metanephrine, 3-methoxy-tyramine) [5].

HPLC introduces many variables, both in sample chromatography and pre-analytical factors. The survey form was divided into several sections to capture as much relevant information as possible on both pre-analytical and analytical factors. The main sections for urine biogenic amines covered:

- Sample collection conditions (preservative, temperature);
- Range of analytes tested (catecholamines, metanephrines, acid metabolites);
- Frequency of analysis and workload;
- Sample preparation techniques (extraction types);
- Source of calibrators and QC materials;
- Type of HPLC and detector in use;
- Reference ranges and source.

Survey responses were collated and grouped to produce summary statistics so that laboratory confidentiality was maintained.

Survey Results

Points of Harmony
Laboratories were consistent in the following areas, with the majority:

- Collecting 24-hour urine for adults in an acid container, with dietary restrictions especially for 5HIAA analysis;
- Using reverse-phase column conditions with HPLC coupled to an electrochemical detector;
- Performing one, or sometimes two, analytical runs per week to cover their workload;
- Purchasing commercial liquid calibrators in preference to preparing in-house calibrators from weighed-out chemicals;
- Purchasing and using commercially-available urine quality control materials instead of preparing them in-house;
- Measuring 3 analytes (noradrenaline, adrenaline, dopamine) for catecholamine testing, but only 2 analytes (normetanephrine, metanephrine) for metanephrine testing when performed.

Points of Disharmony
There were 3 areas where laboratories differed greatly in their approach to the analysis of biogenic amines. They were:

- The range of analytes covered by the laboratory;
- The details of urine sample preparation prior to HPLC analysis;
- Reference ranges used for reporting and interpreting results, especially for spot urines from children.
The number and type of analytes offered by each laboratory service is principally a business decision based on the pattern of test requests. In this respect, it has been noted previously that despite expert recommendations that urine metanephrines are superior to catecholamines for the diagnosis of phaeochromocytoma, clinical requests for urine catecholamines still far exceed those for metanephrines [6]. In this survey, there were twice as many laboratories performing urine catecholamine analyses as there were performing metanephrines. Clearly, further education of clinicians is required to comply with the expert recommendations derived from evidence-based medicine [7,8].

To prepare urine samples for biogenic amine analysis by HPLC, some form of solid-phase clean-up is generally required. There are several choices of SPE materials that can be used (alumina, cation-exchange), so it is not surprising that different laboratories have validated different methods to achieve the same aim.

Of most concern, however, is the finding that reference ranges being used in the reporting of urinary biogenic amine and metabolite concentrations are widely different between laboratories, and this point deserves further examination. For spot urines collected from children, it is well known that the concentrations expressed per mol creatinine are age-related, and decrease during childhood to stable adult levels [9–11]. It is therefore necessary to divide data for children into age groups. For the purpose of this survey, 3 groups were analysed: baby (age 0-2 years); child (3-10 years); and teenager (11-20 years). Figure 1 shows the upper limit of normal (ULN) urine concentrations for VMA, HVA and dopamine, as reported by 18 laboratories in the survey, according to child age range. A very wide range of values covering up to a 5-fold difference are observed. These particular biogenic amines and metabolites are used for biochemical screening for neuroblastoma in children, and the detection of increased urinary concentrations can lead to medical imaging procedures that are not without risks. It is therefore important to harmonise reference limits so that further clinical investigations are not performed unnecessarily.
Figure 1. Upper limits of normal (ULN) in mmol/mol creatinine for reference ranges used by 18 different laboratories for children of 3 age groups (baby, child, teenager) for a. VMA; b. HVA; and c. Dopamine.
Hierarchy of Reference Interval Setting

In establishing reference intervals for any analyte, a hierarchy of 5 principles has been published and discussed, as summarised in Table 1.

Table 1. Hierarchy of Reference Intervals [12]

<table>
<thead>
<tr>
<th>Level</th>
<th>Principle</th>
<th>Reference Limits</th>
<th>Common Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Clinical Outcome</td>
<td>Based on clinical outcome</td>
<td></td>
</tr>
<tr>
<td>2A</td>
<td>Biological variation</td>
<td>2.5%-97.5% distribution of reference population</td>
<td>Literature, published studies</td>
</tr>
<tr>
<td>2B</td>
<td>Clinician Survey</td>
<td>Based on survey of clinician response to results.</td>
<td>Decision point – Rule out tumour or further investigate with medical imaging</td>
</tr>
<tr>
<td>3</td>
<td>Professional Recommendations</td>
<td>Based on Laboratory Experts.</td>
<td>BAWP Recommendations</td>
</tr>
<tr>
<td>4</td>
<td>Proficiency survey</td>
<td>Based on survey of common reference intervals used.</td>
<td>BAWP Survey 2012</td>
</tr>
<tr>
<td>5</td>
<td>State of the Art</td>
<td>Based on what is available.</td>
<td>Kit Insert</td>
</tr>
</tbody>
</table>

From the present survey, it is apparent that reference intervals for biogenic amines and their metabolites currently are at the lowest level 5, and are based on kit inserts and limited in-house data. Although this survey offers the BAWP an opportunity to raise the level to Proficiency survey (level 4), the lack of consensus between laboratories does not allow a recommendation to be made on common grounds. For the BAWP to make expert recommendations at level 3, it is necessary to seek alternative data to guide decisions. Fortunately, there have been two published studies [9,10] that meet level 2A principles, and additional criteria such as:

- Data are recent having been published within the last 5 years;
- The total study database comprised at least 800 subjects to allow stratification by age groups of at least 100 subjects;
- Analytical methods used were HPLC to allow fractionation of individual urinary amines and metabolites with accurate quantitation.

Using these two recent studies published in the medical literature as an evidence base, combined reference range data are summarized in Figure 2 according to child age. The two studies are in good agreement, particularly for HVA and dopamine, and show the rapid decline in urinary excretion over the first 5 years of life to reach stable adult concentrations expressed as mmol per mol creatinine.
Figure 2. Age-related reference intervals for selected spot urinary biogenic amines and their metabolites in children from two large published studies of 1,737 subjects (9,10).
When the laboratory survey data are compared to literature data, as summarized in Table 2, it is apparent that laboratories taking part in the BAWP survey have a much wider range of ULN concentrations than would be expected for all 3 urine analytes in all 3 child age groups.

Table 2. Comparison between ranges of laboratory-survey and literature-based values of the ULN (in mmol/mol creatinine) for urine VMA, HVA and dopamine for children in 3 age-groups.
Survey ULN concentrations were collected from 18 laboratories, while literature-based values were taken from 2 published papers [9,10] with data from 1,737 children.

<table>
<thead>
<tr>
<th>Urine Analyte</th>
<th>Source of ULN</th>
<th>Baby (0-2 y)</th>
<th>Child (3-10 y)</th>
<th>Teenager (11-20 y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMA (HMMA)</td>
<td>Lab survey</td>
<td>5 to 25</td>
<td>4 to 13</td>
<td>3 to 9</td>
</tr>
<tr>
<td></td>
<td>Literature</td>
<td>9 to 13</td>
<td>4 to 9</td>
<td>3 to 6</td>
</tr>
<tr>
<td>HVA</td>
<td>Lab survey</td>
<td>10 to 25</td>
<td>5 to 20</td>
<td>6 to 9</td>
</tr>
<tr>
<td></td>
<td>Literature</td>
<td>17 to 23</td>
<td>10 to 15</td>
<td>4 to 10</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Lab survey</td>
<td>0.9 to 2.3</td>
<td>0.4 to 1.4</td>
<td>0.2 to 0.6</td>
</tr>
<tr>
<td></td>
<td>Literature</td>
<td>1.2 to 2.3</td>
<td>0.6 to 1.0</td>
<td>0.3 to 0.6</td>
</tr>
</tbody>
</table>
Discussion

In line with the efforts of many international clinical chemistry bodies, the AACB has been focusing on the harmonisation of laboratory testing, and harmonised reference intervals and decision limits are an important aspect of this endeavour (13,14). Several AACB professional activities have been devoted to harmonisation, including the 2012 annual scientific meeting and harmonisation workshops in 2012 and 2013. In the recent 2013 workshop, it was proposed that there were two main questions that laboratories need to answer when asking whether they can harmonise on the same reference intervals or clinical cut-off. They are:

1. Methodological – is the laboratory method accurate or is there a bias?
2. Own population – does the reference interval/decision limit apply to their patient population?

In the case of urine biogenic amines, data from the QAP of RCPA Pty Ltd show that nearly all laboratories are performing to set allowable limits of performance to maintain accurate measurement within analytical variation of up to 20%. This is not surprising as the survey has found that HPLC is the common analytical technique in use across all laboratories with widespread adoption of the same commercial calibrators and quality control materials.

With respect to the patient population being tested, urine biogenic amines are screening tests that are designed to rule out the presence of neuroendocrine tumours. For phaeochromocytoma detection in adults, the excretion of urine catecholamines is known to be influenced by factors such as the presence of hypertension and some of the drugs being used to treat this condition [15]. Some laboratories have different reference intervals for adult patients with and without hypertension. For neuroblastoma detection in children, there may be small genetic and dietary influences on the normal level of urinary excretion of biogenic amines and their metabolites. However, the main confounding variable affecting excretion is age, with a curvilinear relationship between urine concentrations and age as shown in Figure 2. Even after allowing for age, some laboratories in the BAWP survey are using decision limits (ULN) that appear to be too extreme when compared to current literature values. A low ULN will lead to false positive test results, while a high ULN will result in false negatives. Neither of these outcomes is desirable in tumour screening. The BAWP therefore recommends that all laboratories that perform testing for urine biogenic amines and their metabolites should review the decision limits (ULNs) that appear on their pathology reports, taking into account data published from recent large studies, particularly from children. The source of reference limits should be included as part of the documentation and validation of the laboratory method, as this is a NATA requirement.

Acknowledgement

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References