Review Article

Advances in Biochemical Screening for Phaeochromocytoma using Biogenic Amines

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Abstract
Biochemical testing for phaeochromocytoma is performed in diagnostic laboratories using a variety of tests with plasma, serum or 24-hour urine collections. These tests include catecholamines and their methylated metabolites - the metanephrines, either individually or in combination with their sulfated metabolites. High-performance liquid chromatography (HPLC) continues to be the dominant analytical method for biogenic amine quantitation. Chromatographic techniques are changing, with improvements in sample preparation procedures, column technology and more specific analyte detection using tandem mass spectrometry. Enrolments in quality assurance programs indicate that there are still many more laboratories in Australasia analysing urinary catecholamines than metanephrines. Nevertheless, clinical evidence and expert opinion favour metanephrines as the analytes with highest sensitivity for the detection of phaeochromocytoma. Practical issues such as better chemical stability and easier specimen collection also favour metanephrines over catecholamines. For these reasons, it is likely that laboratories increasingly will replace urine catecholamine testing with either plasma or urine metanephrines. However in interpreting positive results, the need remains to consider issues such as pre-test probability and use of potentially interfering medications.

Introduction

For decades, the detection and monitoring of neuroendocrine tumours has been greatly assisted by laboratory measurement of the biogenic amines and their metabolites. With the development of new technologies, biochemical methods for the quantitation of biogenic amines have improved. To select the best diagnostic tests from the many available there is a growing evidence-base of clinical studies. This review focuses on some recent analytical advances for the assay of catecholamines and metanephrines in biological fluids. The biochemical and biological rationale for these assays is discussed along with the application of these methods in clinical studies.

Origins and Clinical Features of Phaeochromocytoma

Phaeochromocytomas are rare neuroendocrine tumours that produce catecholamines. The name phios – dusky, chroma – colour, and cytoma – tumour refers to the colour of the tumour cells when stained with chromium salts. Catecholamine-producing neuroendocrine cells are consequently known as chromaffin cells and are usually found in the adrenal medulla and other ganglia of the sympathetic nervous system.[1] Some authors restrict the use of phaeochromocytoma to tumours of the adrenal medulla, while others use the term more broadly to include paragangliomas. The defining characteristic of phaeochromocytoma in clinical practice is the autonomous production of catecholamines.[2]

Felix Frankel is credited with the first clinical description of phaeochromocytoma.[3,4] This case has been made more interesting by a subsequent study tracing descendants of the original patient and undertaking genetic testing.[5] The investigators demonstrated a *RET* gene germ-line mutation in the kindred, thus diagnosing multiple endocrine neoplasia type 2 (MEN-2) 121 years after the original presentation. This study elegantly illustrates the importance of germ-line mutations in patients with phaeochromocytoma and this possibility should always be considered.

Phaeochromocytoma may cause problems for patients in two ways: firstly, due to adverse effects caused by the autonomous production of catecholamines and secondly, due to malignancy and metastatic spread. Case series report 5-20% of phaeochromocytomas as malignant.[6-8] The five-year survival of

malignant phaeochromocytoma is <50% and first-line treatment is surgery.[9-11] Chemotherapy and radionuclide treatments are also used, but data is limited due to the rarity of the condition and the reported experience has been disappointing.[10,11] Trials are underway of potentially more effective treatments, particularly using new radioisotope therapies, but currently surgery remains the mainstay of treatment.[12]

The consequences of catecholamine excess are many and varied. The classical clinical triad of hypertension, headache and sweating is of limited clinical utility, as it is neither sensitive nor specific for phaeochromocytoma.[13-16] Patients may have symptoms related to any adrenoreceptor agonist effect or may have no symptoms at all. In the past, patients were most often diagnosed during investigation of hypertension or unexplained spells. More recently, with the widespread use of CT and MRI scanning, phaeochromocytoma is increasingly diagnosed during investigation of adrenal mass lesions identified incidentally by abdominal imaging studies carried out for other reasons.[15-20] Patients thus come to attention in three main ways: "difficult" hypertension, unexplained spells, or by incidental radiological identification of an adrenal mass. A fourth, familial screening, may soon be added to this list.[21,22] For patients with hypertension or spells, the presence of additional features suggestive of catecholamine excess should be considered prior to biochemical investigation.[23-26] For those with adrenal mass lesions, biochemical investigation is nearly always indicated.[15-20,27] Biochemical testing for possible phaeochromocytoma is the primary focus of this review.

Genetic Syndromes of Phaeochromocytoma

The genetic syndromes commonly associated with phaeochromocytoma are shown in Table 1.[8,21,22,28-32] These vary in age of onset, location, secretory product and propensity for malignancy. The mean age of diagnosis of phaeochromocytoma in Von Hippel Lindau syndrome is about 20 years, whereas in Neurofibromatosis Type 1 it is about 42 years, similar to sporadic cases.[28,29] The incidence of malignancy in phaeochromocytoma associated with succinate dehydrogenase complex, subunit B (*SDH-B)* mutations is about 1 in 3, whereas with *SDH-D* they are nearly all benign.[30-32] Genetic testing is not considered further in this discussion and for more information readers can refer to recent reviews.[33-36]

Catecholamine Synthesis and Metabolism

Catecholamines are synthesised from the amino acid tyrosine, which is hydroxylated and decarboxylated to dopamine, as shown in Figure 1. Dopamine is then converted to noradrenaline (also called norepinephrine), which is methylated at the amino group to form adrenaline (also called epinephrine). The rate-limiting step in catecholamine biosynthesis is catalysed by the mono-oxygenase enzyme tyrosine hydroxylase,[37] which requires tetrahydro-biopterin as a co-factor in the reaction.[38] This knowledge is applied therapeutically when metyrosine, a competitive inhibitor of tyrosine hydroxylase, is used to treat catecholamine excess.

Catecholamines are produced in neurons of the central nervous system, in sympathetic nerves and in chromaffin cells of the adrenal medulla. They are taken up and stored in vesicular granules, where they exist in a highly dynamic state with passive leakage outwards balanced by active transport inwards.[39] Monoamines share the storage granule matrix with peptides and proteins, such as chromogranin A.[40] When released into the extracellular space, catecholamines are taken up by neuronal and non-neuronal transporters to rapidly terminate signal transmissions and are cleared from the bloodstream with a half-life of less than three minutes.[41-43]

Table 1. Selected characteristics of genetic syndromes associated with phaeochromocytoma.

Syndrome/Gene	Locus	Age at diagnosis Mean (range)	Characteristics of phaeochromocytoma
MEN-2/*RET*	10q11.2	35 (18-50)	bilateral benign adrenergic
NF1	17111.2	40 (25-70)	similar to sporadic
VHL	3p25-25	20 (5-50)	bilateral benign noradrenergic
SDH-B	1p36.1-p35	25 (10-60)	paragangliomas malignant
SDH-D	11q23	30 (5-65)	paragangliomas

MEN-2 – Multiple Endocrine Neoplasia Type 2
NF1 – Neurofibromatosis Type 1
VHL – Von Hippel-Lindau
SDH – Succinate dehydrogenase

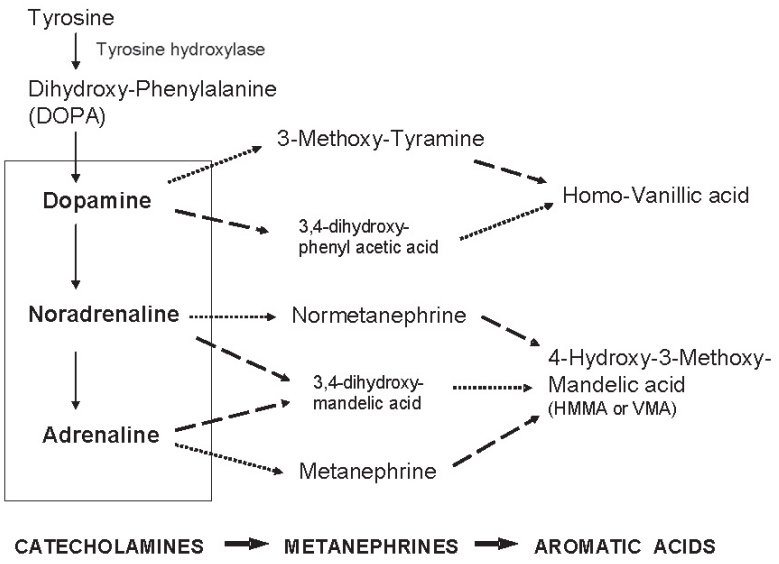

Figure 1. Biochemical pathways for the metabolism of catecholamines. The dotted line shows reactions catalysed by catechol-O-methyl-transferase, while the dashed line indicates reactions catalysed by monoamine oxidase.

Catecholamines taken up into cells by transporters can be metabolised by O-methylation to metanephrines (also called metadrenalines) by the enzyme catechol-O-methyl-transferase (COMT), or deaminated by monamine oxidase (MAO) to intermediates such as dihydroxy-phenylacetic acid (DOPAC), dihydroxy-phenylglycol (DHPG) and dihydroxy mandelic acid (Figure 1). These latter intermediates can then be further metabolised by COMT, leading to homovanillic acid (HVA) from dopamine and hydroxy-methoxy-mandelic acid [HMMA, also called VMA (vanillylmandelic acid)] from noradrenaline and adrenaline. As a result of differential expression of the enzymes MAO and COMT, catecholamines produced at neuronal and adrenal medullary locations follow different pathways of metabolism.[44] In adrenal chromaffin cells, catecholamines are metabolised by COMT, accounting for more than 90% of circulating metanephrine (also called metadrenaline) and 24% to 40% of circulating normetanephrine (also called normetadrenaline).[45] In contrast, most noradrenaline produced by the nervous system is metabolised initially to DHPG in sympathetic nerves and released into the plasma, where it is taken up by the liver and converted to HMMA.[39] Except for HMMA, all the catecholamines and their metabolites can be conjugated with sulfate by the sulfotransferase enzyme (SULT1A3).[46] The conjugated compounds are water soluble and eliminated by urinary excretion. A more detailed description of catecholamine metabolism is given by Eisenhofer et al. [39]

Chromatographic Methods for Catecholamines and Metabolites

The most widely used method in the routine clinical laboratory for the measurement of urinary catecholamines and their metabolites is HPLC. All large pathology laboratories would be expected to have at least one instrument, which can be purchased from a number of commercial suppliers. Original methods relied on fluorescence detection to gain analytical sensitivity,[47,48] but the availability of electrochemical detection provided superior specificity and this mode of detection is the most common today.[49] However, the use of tandem mass spectrometry is becoming more popular as laboratories realise its advantages, particularly in detecting the low concentrations of metanephrines present in plasma.

Sample Preparation

Prior to chromatography, some pre-analytical clean-up step is required to extract and sometimes concentrate the catecholamines or metanephrines from the biological urine or plasma matrix. For catecholamines, urine is processed directly to isolate only free noradrenaline, adrenaline and dopamine, while for urine metanephrines an acid hydrolysis step is nearly always included to release the conjugated forms, which are the major molecular species present. In an acidified urine, the biogenic amines are protonated as cations, and can be isolated on alumina columns or commercial cartridges packed with ion-exchange supports.[47,49-51] To increase processing speed, vacuum manifolds are used to facilitate conditioning, loading, washing and elution steps. Alternatively, fully automated solid-phase extraction (SPE) systems can be purchased to handle larger numbers of samples.[49]

To extract catecholamines from urine, advantage can be taken of the unique affinity of boronate compounds for the cis-diol structural moiety, to which a covalent linkage is formed at

alkaline pH.[52] The complexed catecholamines can then be subjected to liquid-liquid extraction[53] or direct immobilisation is possible with solid-phase extraction on PBA (phenylboronic acid) cartridges[54] followed by elution of the catecholamines under acidic conditions. Talwar et al. found it more convenient to extract the complexed catecholamines from solution with C18-bonded silica cartridges at pH 8.5, and then elute them with acid, as this avoided solvent extraction steps while stabilising the catecholamines from oxidation.[55] This approach has been extended to SPE on Bond Elut Plexa™, a new hydrophobic-hydrophilic synthetic support with improved binding properties for small molecule extractions from biological fluids, and recovery of biogenic amines in formic acid which is compatible with mass spectrometry.[56]

Acid-hydrolysed urines are used routinely for total metanephrine measurement, but are often heavily pigmented and require clean-up prior to HPLC separation of normetanephrine and metanephrine. Isolation procedures used in the past involve solvent extraction, or SPE on cation-exchange resins.[49] A popular option, which is available commercially, uses dual cation-exchange and anion-exchange columns to separate metanephrines from catecholamines, non-phenolic amines and other interfering hydrophobic compounds.[49,57] Recently, an SPE method developed for free catecholamine extraction from urine was found to also recover metanephrines with high yield. This allowed the simultaneous measurement of urinary metanephrines with catecholamines following HPLC separation and detection by tandem mass spectrometry.[56]

For plasma free metanephrines, direct extraction is possible by SPE on polymer-based supports after the addition of internal standard to correct for extraction efficiency.[58] To fully automate the analytical process, an on-line cation-exchange cartridge was used recently in combination with tandem mass spectrometry to produce a run time of 11 minutes per sample.[59,60] This allows high throughput of plasma samples but is restricted to large laboratories with sufficient analytical resources to purchase and operate the sample processor as well as the tandem mass spectrometric instrumentation. A recent simple alternative to SPE techniques used isopropanol to precipitate and remove proteins prior to normal phase liquid chromatography with tandem mass spectrometry.[61]

Chromatography

In the past few years, manufacturers of HPLC columns have released new column technology with smaller particle sizes (sub-2 micron particle diameter) to increase sample throughput in chromatography laboratories.[62] These columns have increased resolving power such that the same separations of mixtures of compounds can be achieved in much shorter run times. However, for laboratories to utilise these new columns some upgrade of older HPLC systems may be required to withstand the higher operating pressures. Since analytical methods can be transposed to the new faster HPLC columns without major modifications, this advance is of potential benefit to laboratories that process high workloads.

Another recent advance in HPLC is the development of new column phases based on reversed-phase chromatography with modifications to allow retention and separation of polar compounds, such as the biogenic amines, without using ion-pairing reagents. Examples of this type of column packing are the Atlantis dC18 or T3 columns (Waters) and Synergi Hydro or Polar-RP (Phenomenex). Simple acidic mobile phases with pH as low as 2 can be used for separation of catecholamines and metanephrines, as well as other biogenic amines such as HVA, serotonin and 5HIAA. An example of the chromatographic separation of catecholamines and metanephrines obtained on an Atlantis T3 column is shown in Figure 2, with detection by tandem mass spectrometry.

Chromatographic Detection

The most widely used method for the detection of catecholamines and metanephrines that have been extracted from urine or plasma and separated by HPLC is electro-chemical detection (ECD). This is illustrated by enrolments in the biogenic amines quality assurance program of RCPA Pty Ltd where over 90% of laboratories state their method of analysis as HPLC with ECD. While this detector is very sensitive, it requires a high level of maintenance with frequent cleaning of the electrode and analytical interference from drugs may occur, in particular, paracetamol metabolites and the sympathomimetics.[63,64] Issues relating to drug interference are discussed below.

Analytical interference problems are minimised by the higher analytical specificity of tandem mass spectrometry. A summary of published tandem mass spectrometry methods for catecholamines, metanephrines and other metabolites is shown in Table 2.[56,58,60,65-68] All methods make use of internal standards labelled with heavy isotopes such as deuterium, as these are essential to correct for ion suppression effects and any losses that occur in the sample preparation procedure. Although the use of HPLC coupled to tandem mass spectrometry still has some way to go for widespread adoption in clinical chemistry laboratories,[59,69] for those who have the equipment and operating resources, the technique does improve workflows and the reliability of analytical results for small molecules that are not easily quantified by automated immunoassays. An ELISA kit has been developed commercially and used to measure total urine metanephrines, after their conversion to acyl derivatives,[70] but the accuracy of

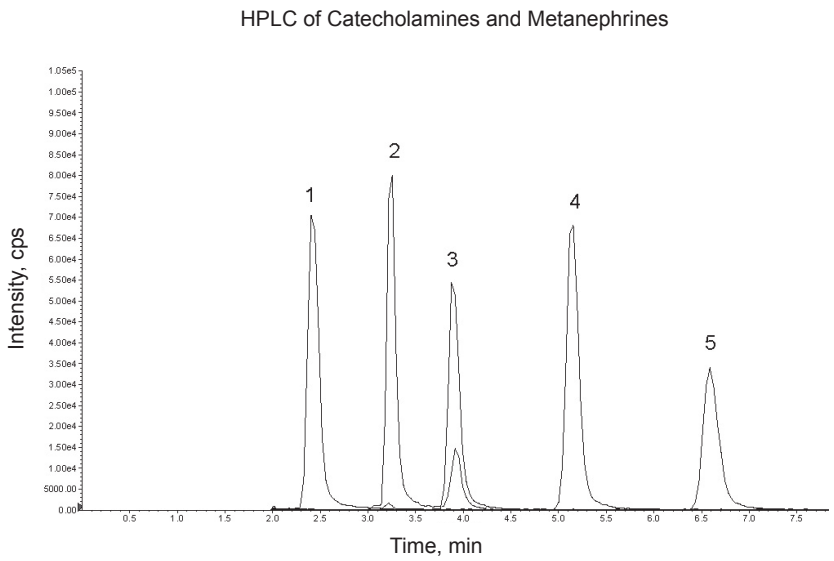

Figure 2. Separation of five biogenic amines (50 pmol each on-column) on an Atlantis T3 column (150 x 2.1 mm, 3 µm particle size) at 25°C with a flow rate of 200 µL/min of 0.2% formic acid-methanol (98/2, v/v). Peaks were detected by tandem mass spectrometry with multiple reaction monitoring of the ion transition pairs as follows: (1) noradrenaline 170→152, (2) adrenaline 184→166, (3) normetanephrine 166→134, (4) dopamine 154→137 and (5) metanephrine 180→148. The smaller normetanephrine peak is due to the 184→166 ion pair which is common with adrenaline.

immunoassays with respect to plasma free metanephrines has not been well established.

Diagnostic Efficacy of Biochemical Tests

Requests for biochemical tests for phaeochromocytoma are most often in the context of a low pre-test probability. These are requested as part of the work-up of patients with spells, difficult to control hypertension, or incidentally identified adrenal masses. Because of the potentially serious consequences of missing a phaeochromocytoma, test thresholds are set for high sensitivity and consequently have only moderate specificity. The combination of low pre-test probability and moderate specificity results in many false positive tests and dealing with these efficiently is a challenge for both clinicians and laboratory scientists.

Choice of initial test

Plasma free metanephrines or urine total metanephrines are the currently recommended biochemical tests for phaeochromocytoma screening.[71] Until recently, the recommended initial test was 24-hour urine free catecholamines (adrenaline and noradrenaline).[14,72-75] This recommendation was predicated on three principles. Firstly, catecholamines produced by chromaffin cells have a very short plasma half-life, approximately 80 and 150 seconds for adrenaline and noradrenaline respectively.[41-43] Thus, plasma sampling lacks reliability. Secondly, urine free catecholamines represent the 24-hour integral of catecholamine production as the total filtered at the glomerulus.[76,77] This second premise is weakened by the effect of renal tubular reabsorption of catecholamine.[78,79] Thirdly, the concentrations in the urine could be accurately measured by the available laboratory techniques.[80] Most importantly, the empirical data from clinical studies had shown 24-hour urine free catecholamines to better predict phaeochromocytoma than other measures available at the time.[14,72]

In the early 1990s, specific assays for metanephrine and normetanephrine became more available and some of these assays were sufficiently sensitive to accurately quantitate free metanephrine in plasma.[81-84] There are two reasons to hypothesise metanephrines as the preferred analyte in assessing chromaffin cell catecholamine production. Firstly, as outlined previously, metanephrines are the catecholamine metabolites of COMT, thus providing a way of preferentially testing chromaffin cell production of catecholamines with reduced interference from catecholamines produced in the sympathetic nervous system. Secondly, metanephrines have a longer half-life than catecholamines improving the reliability of plasma as a substrate.[85] The validity of metanephrines as the preferred analyte has been demonstrated in several large case control studies and a meta-analysis using appropriate statistical methods including ROC curve evaluation.[84-95] The first International Symposium of Phaeochromocytoma considered this issue in detail and recommended plasma or urine metanephrine testing as the initial test for investi-

Table 2. Features of published methods for the determination of biogenic amines by tandem mass spectrometry.

Analyte	Fluid/Volume	Clean-Up	LC Column	MRM Ion Pairs m/z	Imprecision Inter-assay CV	Internal Standards	Reference
Free catecholamines	Urine/0.3 mL	Liquid-liquid extraction (LLE)	Allure Basix 50 x 2 mm RT 3.5 min	9 ion pairs	4 to 7%	Deuterated	Kushnir et al. [65]
Total metanephrines	Urine/1 mL	SPE Oasis HLB	RP Amide C16 50 x 4.6 mm RT 3 min	180 to 148 183 to 151 166 to 134 169 to 137	6 to 9%	Tri-Deuterated	Taylor and Singh [66]
Free metanephrines	Plasma/1 mL	SPE Oasis HLB	Luna CN 150 x 4.6 mm RT 6 min	180 to 148 183 to 151 166 to 134 169 to 137	6 to 13%	Tri-deuterated	Lagerstedt et al. [58]
Free metanephrines	Plasma/0.5 mL	On-line SPE	Atlantis HILIC 50 x 2.1 mm RT 8 min	9 ion pairs	2 to 14%	Deuterated	De Jong et al. [60]
Combined catecholamines and metanephrines	Urine/0.5 mL	SPE Bond-Elut Plexa	Atlantis T3 150 x 2.1 mm RT 5 min	15 ion pairs	5 to 7%	Four deuterated, one 13C, 15N	Whiting [56]
HMMA (VMA)	Urine/0.6 mL	SPE Oasis HLB Automated	RP Amide C16 50 x 4.6 mm RT 3 min	197 to 137 200 to 140	2.5%	D3-VMA	Magera et al.[67]
HVA	Urine/1.2 mL	SPE Accu-Bond C18 Automated	RP Amide C16 50 x 4.6 mm RT 3 min	181 to 137 189 to 145	3 to 5%	13C-18O-HVA	Magera et al.[68]

gation of suspected phaeochromoctytoma.[71] Catecholamine testing is no longer recommended as first line testing for phaeochromocytoma due to lower sensitivity and consequent false negatives.

Many laboratories in the UK have yet to make a switch from catecholamine to metanephrine testing.[96] This is also true in Australia as shown by enrolments in the urine biogenic amine quality assurance programme of the Royal College of Pathologists of Australasia. In 2008, there were 45 laboratories enrolled for catecholamines compared with 15 laboratories for metanephrines.

Plasma or Urine

The choice of plasma or urine as test specimen is more controversial. Both have been demonstrated to have high sensitivity for metanephrines. Proponents of plasma point to its very high sensitivity and the convenience of sample collection.[86,91] Proponents of urine point to its greater specificity and consequent reduction in false positives.[92,94] The consensus guidelines accept either specimen.[71] Published sensitivities and specificities are dependent on choice of patient and control groups, choice of test threshold and analytical precision.[97] The sensitivity and specificity achieved in clinical practice will additionally depend on factors such as patient preparation and sampling environment.

Free or Total Metanephrines

Metanephrines are metabolised by sulfation and excreted in the urine predominantly as sulfated metabolites.[39] Total metanephrines, in this context, refers to the total of free metanephrine and normetanephrine, and their sulfated metabolites. In urine, total metanephrines have been the preferred analyte for two reasons. Firstly, due to the higher concentrations, earlier less-sensitive assays were able to quantitate total metanephrines. Secondly, as the 24-hour urine test is a measure of 24-hour production by measurement of all metanephrines filtered at the glomerulus, total metanephrine should more closely approximate catecholamine production. Free urine metanephrine excretion has not yet been robustly evaluated in clinical studies.

In contrast, metanephrine concentration in plasma is a function of production and clearance. Measurement of free metanephrine reflects this, whereas total metanephrine concentration also reflects a third variable, clearance of the conjugated metanephrine and thus signal is likely to be lost. The validity of this premise has been demonstrated by empirical data from clinical studies.[87] It is for these reasons that plasma free metanephrines or urine total metanephrines are the currently recommended biochemical tests for phaeochromocytoma screening.[71]

Total or Fractionated Metanephrines

These terms have been widely used and cause confusion. Fractionated metanephrines refers to the separate quantitation and reporting of metanephrine and normetanephrine. Total, in this context, refers to the sum of the two analytes. We believe this term should no longer be used as it is confused with "total" meaning metanephrines plus sulfate metabolites. This is further confused as "total" concentration of a hormone or drug usually refers to the total of free and protein bound concentrations excluding metabolites and other related moieties. Although catecholamines and metanephrines do bind with low affinity to albumin and haemoglobin the clinical relevance of this is unknown and this will not be considered further.[98,99] In this review, metanephrines refers to separate measurement and reporting of metanephrine and normetanephrine and total refers to the sum of free metanephrine and conjugated metabolite.[100]

Interpreting Clinical Studies, Pre-test Probability and False Positives

The most useful studies in evaluating a diagnostic test include large numbers of both cases and controls. For rare conditions such populations are only found at major referral centres which see a selected group of patients, and, by definition, relatively fewer patients without phaeochromocytoma. For biochemical investigation of phaeochromocytoma, the US National Institute of Health (NIH) and the Mayo Clinic have reported their data extensively.[86-89,91,94] Large European series have been reported from Vienna, Oxford, Essen and Lille.[85,92,93,95] Sawka et al. summarised the published data prior to 2004 in a metanalysis.[90] Not being major referral centres, Australasian hospitals are likely to have lower case rates than the large US referral centres and thus a greater proportion of false positives can be expected.

False positive tests cause anxiety, inappropriate use of resources and in some cases patient harm. As phaeochromocytoma is very rare, the pre-test probability of phaeochromocytoma is nearly always low. Thus, most patients with a positive initial test will not have a phaeochromocytoma. The best way of reducing false positives is to reduce inappropriate testing. For example, testing all patients with hypertension is not recommended.[71] Appropriate testing is on the basis of pre-test probability and patients will usually fall into one of four groups. These are difficult hypertension, unexplained spells, adrenal masses identified by abdominal imaging and patients with a personal or family history of a genetic syndrome associated with phaeochromocytoma.

Patients with Spells

Due to the lack of discriminating clinical features, it is hard to offer simple rules to further define pre-test probability in these

patients. With the frequency of late diagnosis and "missed" phaeochromocytoma, any clinical suspicion should prompt the clinician to "actively exclude phaeochromocytoma". Clinical clues include: sweating, headache, tremor, pallor or episodes of non-exertional palpitations. Although the presence of an alternative explanation for these symptoms is often sufficient to exclude phaeochromocytoma, biochemical testing can be useful in the investigation of spells.

Hypertensive Patients

The prevalence of phaeochromocytoma in a general hypertensive population has been estimated at about 0.3%.[23-25, 101-103] By selecting patients with a history of paroxysms or of "treatment-resistant hypertension", this can increase to about 2.5%. In this scenario, for a test with a sensitivity of 99% and a specificity of 87%, 40 patients would be tested with five false positives to identify one true case.

Patients with Incidental Adrenal Masses

In these cases pathologies other than phaeochromocytoma also need to be considered. Large series estimate a prevalence of phaeochromocytoma of about 4%.[17-20,27,104,105] The imaging characteristics of these lesions can help to discriminate further, for example, low attenuation lesions on non-contrast CT (<10 Hounsefield units) are unlikely to be phaeochromocytomas.[19] If there is any doubt, consultation with a clinical endocrinologist is recommended in addition to the evaluation of the possibility of other hyper-secreting adrenal lesions.

Drugs and Other Test Confounders

Noradrenaline from the sympathetic nervous system is the source of ~66% of circulating normetanephrine.[39] Thus, anything that increases noradrenaline production or decreases noradrenaline uptake will increase normetanephrine concentrations and may result in false positive tests.[106] Physiological stress increases noradrenaline production whereas supine posture and sleep decrease metanephrine production.

The drugs that most commonly give false positive results are those that inhibit noradrenaline uptake or block sufficient adrenoreceptors to cause increased noradrenaline production. These include tricyclic antidepressants, atypical antipsychotics, alpha-2 receptor blockers and beta blockers, as shown in Table 3.[106-110] It is the responsibility of the clinician interpreting the test to carefully consider the potential impact of medications. The magnitude of interference is patient- and dose-dependent. Thus, the most practical method of accounting for interferences is to repeat testing under controlled conditions. Although not widely

Table 3. Medications with potential to cause false positive results.

Mechanism	Class	Drugs*
Noradrenaline re-uptake inhibition	Tricyclic antidepressants	Amitriptyline, clomipramine, dothiepin, doxepin, imipramine, maprotiline, mianserin, nortriptyline, trimipramine.
	Antipsychotics	Clozapine, quetiapine, amisulpride, aripiprazole olanzapine, risperidone, ziprasidone, chlorpromazine, fluphenazine, pericyazine, trifluoperazine.
	Noradrenaline re-uptake inhibitors	Bupropion, reboxetine, viloxazine.
	Other	Prochlorperazine, reserpine.
Adrenergic receptor blockers	Beta blockers	Carvedilol, labetolol, acebutolol, atenolol, bisolprolol, celiprolol, metoprolol, pindolol, propranolol, sotalol.
	Alpha-2 blockers	Phenoxybenzamine, phentolamine.
Monoamine oxidase inhibition	Monoamine oxidase inhibitors	Moclobamide, phenelzine, tranylcypromine.
Recreational drugs	Amphetamines, xanthines	Cocaine, methamphetamine, caffeine.
Structurally related[#]	Sympathomimetics	Pseudoephedrine, phenylephrine, dobutamine, isoprenaline, salbutamol, terbutaline.
	Others	Paracetamol, levodopa.

*As insufficient data are available to reliably catalogue interfering drugs, this list is extrapolated from known interfering agents. Drugs were selected from the Australian Medicines Handbook 2008 and the NZ Pharmaceutical Schedule November 2008
[#]Applicable to electrochemical but not mass spectrometry detectors.

used, monoamine oxidase inhibitors may increase false positive metanephrines by 'diverting' peripheral metabolism of noradrenaline to COMT. Paradoxically, amphetamines and xanthines also increase catecholamine concentrations by induction of tyrosine hydroxylase and competitive antagonism of catecholamine transport. A second group of medications can cause false positive results with electrochemical, but not mass spectrometry detection. These are the sympathomimetics and other similar molecules that cause assay interference.[111,112] Importantly, this list includes paracetamol, which is metabolised to catechol compounds with a high incidence of chromatographic interference.[64]

Whilst drugs have not been reported to cause false negative results, alpha-2 receptor agonists and dopamine agonists have been reported to lower catecholamine and metanephrine concentrations.[113,114] The clinical significance of this effect is currently unknown, although failure of suppression by clonidine, an alpha-2 agonist, is used as a diagnostic test with high specificity for phaeochromocytoma.[106]

There is limited data on the effect of renal impairment on metanephrines.[107] From first principles one would expect plasma free metanephrines to be only modestly affected, whereas plasma total metanephrines would increase due to decreased clearance of the conjugated metabolites. Similarly, one would expect only modest changes in urine total metanephrines, whereas there might be a significant decrease in urine free metanephrines.

Other Analytes

Chromaffin cells also produce molecules other than biogenic amines and these can be used as markers of chromaffin cell mass in the diagnosis of phaeochromocytoma. The best validated of these is chromogranin A. Granins are a family of acid proteins present in the secretory granules of a wide variety of endocrine and neuroendocrine cells, with chromogranin A being specific to chromaffin cells.

Chromogranin A is highly sensitive but less specific than metanephrines in the diagnosis of phaeochromocytoma.[115-120] Importantly, it is biochemically unrelated to catecholamines and thus cleared by different metabolic pathways, which makes it a relatively independent test analyte. Chromogranin A is used in two ways: as a second line test in the diagnostic work-up of patients with raised metanephrines (Figure 3) and as a tumour marker in the follow-up of patients after treatment for chromaffin cell tumours. An important confounder of this test is treatment with proton pump inhibitors, which are widely used and cause marked elevation of chromogranin A.[121] Chromogranin A testing is discussed in more detail in recent articles by Grossrubatscher et al.[119] and Bilek et al.[120]

An Approach to Biochemical Testing for Suspected Phaeochromocytoma

A suggested algorithm for biochemical testing for phaeochromocytoma is outlined in Figure 3. This is adapted for Australasian practice from an approach published by Eisenhofer et al. in 2003.[106] Subsequent published experience, particularly by the Mayo Group, has influenced this adaptation.[87-96,106]

Before requesting any test, the pre-test probability should be considered. How likely is phaeochromocytoma? Is there an alternative explanation for the symptoms? In most cases, tests for rare conditions should not be part of first line investigations. If testing is clinically indicated, the initial test should be plasma free metanephrines or urine total metanephrines. The choice is dependent on pragmatic issues and local circumstances. However, when the plasma test is available, the convenience is hard to resist. A negative test reliably excludes phaeochromocytoma and alternative explanations for the presenting symptoms should be considered. If the test does not exclude phaeochromocytoma, the options are endocrinology referral or to do a second test.

A (nor)metanephrine concentration above the test threshold is most likely a false positive result, unless it is very high (>4x threshold).[94,117] Thus a second test is required and this should seek to minimise false positive results. For this reason, we favour 24-hour urine total metanephrines after stopping medications known or likely to be associated with false positive results (Table 3). The optimal time after stopping medications varies substantially and is predominantly determined by the pharmacokinetics of the individual drug. In most cases five half lives is likely to be sufficient. However, pharmacodynamics may also need to be considered, for example, the effect of phenoxybenzamine, a non-competitive alpha antagonist, will persist until there has been sufficient regeneration of alpha receptors, which may take over a week.[122]

If the second test is negative, phaeochromocytoma can be excluded. If the test is also positive, one needs to consider both the clinical suspicion (pre-test probability) and the concentration observed (is the result just above the threshold or several times the threshold?). At this stage we recommend referral to a clinical endocrinologist with expertise in this area. Expensive imaging tests should only be undertaken after biochemical diagnosis.

Subsequent Tests

Endocrine investigation of hypersecretory conditions utilises biochemical suppression of production. Most false positive tests are due to production of noradrenaline by the sympathetic nervous system. Clonidine, an alpha-1 receptor agonist,

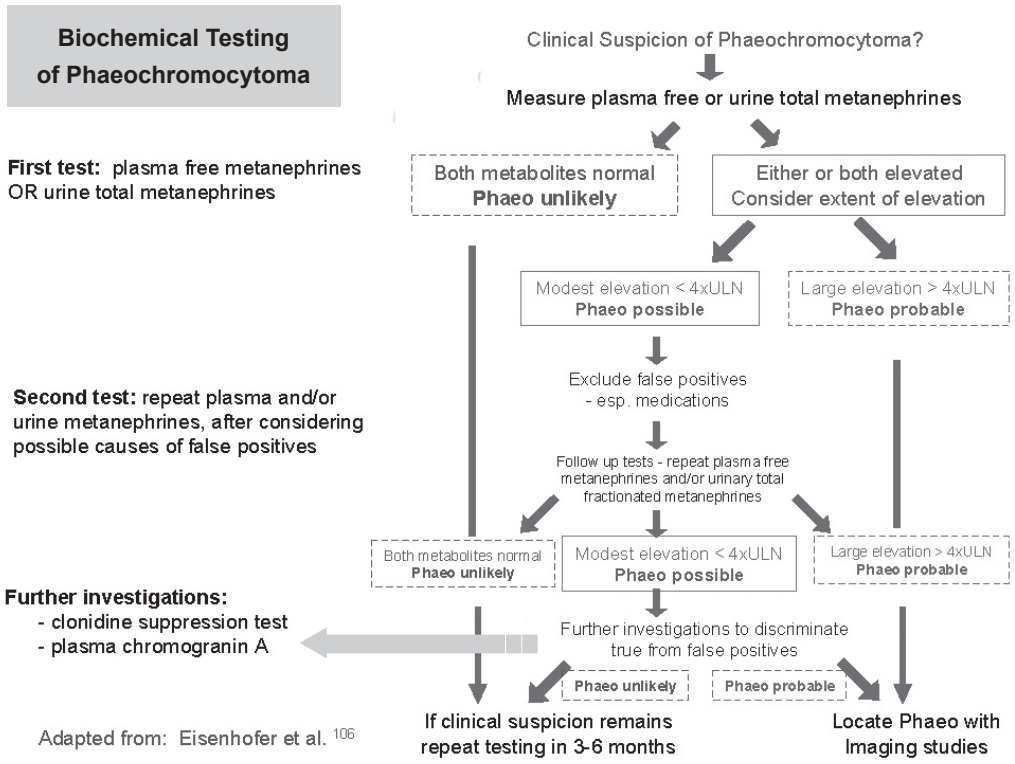

Figure 3. Suggested algorithm of biogenic amine testing for phaeochromocytoma screening. ULN = upper limit of normal.

suppresses noradrenaline production by sympathetic nerves, which is the basis of the highly specific clonidine suppression test. The limitation of this test is that it only has about 66% sensitivity, thus suppression of plasma noradrenaline or normetanephrine three hours after clonidine excludes the diagnosis of phaeochromocytoma, but failure to suppress is supportive rather than confirmatory.[106] This test should be limited to patients with moderate to high pre-test probability of phaeochromocytoma on the basis of clinical suspicion and metanephrine testing.

Specimen Collection Requirements

Proper sample collection is important for the reliable measurement of catecholamines or metanephrines, whether it be in a 24-hour urine collection or in plasma. Laboratories analysing urine specimens should provide instructions for adult patients on how to collect an accurate 24-hour collection, since interpretative reference ranges are expressed in amount excreted per day. Because of the difficulty in obtaining complete 24-hour urine collections in children, excretions of catecholamines and metanephrines are expressed per mmol creatinine, and age-dependent reference ranges used for biochemical testing.[123] With adults the effect of age and gender are small and considered not important for general testing.

Other factors such as drugs and diet can affect assay results. This can be due to direct analytical interferences, such as paracetamol interference in the determination of urinary metanephrines by HPLC with electrochemical detection,[64] or, by altering urinary and plasma concentrations of catecholamines or metanephrines. The effects of medications have already been discussed. Diet and recreational stimulants such as caffeine and nicotine are known to influence urinary catecholamines, but are thought to have minimal effect on metanephrines.[107] Dietary restrictions are not considered necessary for urine metanephrine testing in the majority of patients.[49,107]

The collection of plasma is more convenient than a 24-hour urine for most patients and other biochemical tests can be ordered at the same time in one clinic visit. For plasma free metanephrines, it has been recommended that patients should fast for at least four hours and then rest in the supine position for 30 minutes prior to the collection of EDTA-plasma.[87] A change in posture from supine to seated has been shown to increase plasma metanephrines by an average of 30% in one study,[124] and to have no significant effect in another.[85] If the optimal posture for plasma metanephrine sampling is resting-supine after an overnight fast, this is not usually practical and we therefore suggest a fasting morning EDTA-plasma collected from a quietly seated patient, with interpretation in the context of the sampling conditions.

Catecholamines and metanephrines are known to have different chemical stabilities in urine specimens stored under

various conditions. A drawback with urine collections has been the requirement to add concentrated acid to the collection container in order to stabilise catecholamines from oxidation. This also introduces the possibility of deconjugation, artefactually elevating free catecholamine concentrations. Urine metanephrines are more stable than catecholamines, and do not require acid preservative for collection or storage for up to seven days at room temperature.[80]

In conclusion, metanephrines should be the first line test in the investigation of possible phaeochromocytoma. False positive results remain a common finding complicating the investigative pathway. Effective investigation and management require collaboration between an adequately resourced specialty laboratory and experienced endocrinologists.

Competing Interests: None declared.

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