Impact of LC-MS/MS on the laboratory diagnosis of catecholamine-producing tumors

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ABSTRACT

Applications of LC-MS/MS for the biochemical diagnosis of catecholamine-producing tumors have followed from parallel emergence of this technology with changes in emphasis of biochemical testing, in particular requirements that testing include measurements of plasma or urinary fractionated metanephrines. Since the turn of the century, when LC-MS/MS was first introduced into the routine laboratory, there have been numerous advances in analytical methodology that offer opportunities for breaking away from out-moded analytical procedures to methods that provide enhanced diagnostic information with dramatically improved analytical sensitivity, accuracy and precision as well as rapid sample throughput. LC-MS/MS also offers high analytical specificity, but is not infallible. Multiple reaction monitoring does not always guarantee selectivity and attention to sample purification and chromatographic separation remains important. Although other analytical methods persist, today LC-MS/MS represents the method of choice for high-throughput, low-cost, precise and accurate measurements of the catecholamine metabolites now routinely used for diagnosis of catecholamine-producing tumors.

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Contents

1. Introduction

1.1. Biochemical diagnosis of catecholamine-producing tumors

Catecholamine-producing tumors include pheochromocytomas, paragangliomas and neuroblastomas, all derived from cells of the neural crest. Pheochromocytomas and paragangliomas (PPGLs) are tumors of respective adrenal and extra-adrenal chromaffin cells that usually present in adulthood, whereas neuroblastomas are derived from immature embryonic neuroblast cells that also form tumors at adrenal and extra-adrenal locations, but present almost exclusively in childhood.

Both PPGLs and neuroblastomas are characterized by synthesis and metabolism of catecholamines within tumor cells. Intra-tumoral metabolism of catecholamines in PPGLs was first described in the 1960s [1]. Nevertheless, because PPGLs are characterized by hypertension and symptoms of catecholamine excess, those early findings were largely ignored and diagnosis continued to focus on measurements of catecholamines. It was not until the turn of the century that emphasis started moving from catecholamines to their O-methylated metabolites, the metanephrines [2].

Shift in emphasis from catecholamines to metanephrines for diagnosis of PPGLs followed development of a liquid chromatographic-electrochemical detection (LC-ECD) method for measuring the metabolites in plasma [3]. Developed to investigate extra-neuronal O-methylation pathways of catecholamine metabolism, that method established that these pathways were minor routes of metabolism compared to neuronal deamination pathways [4] (Fig. 1). Over 90% of all circulating metanephrine, the metabolite of epinephrine, and at least a quarter of all normetanephrine, the metabolite of norepinephrine, were formed within adrenal chromaffin cells, not after release of catecholamines by the sympatho-adrenal system [5]. Tumors derived from chromaffin cells were found to produce these metabolites by the same processes [6]. Since many PPGLs secrete catecholamines intermittently or in low amounts, the continuous intra-tumoral metabolism of catecholamines to metanephrines provides a diagnostic advantage for measurements of the metabolites over the catecholamines; this is now confirmed by numerous studies [7]. Consequently, todays recommendations mandate measurements of plasma free or urinary fractionated metanephrines as initial screening tests for PPGLs [7].

Intra-tumoral metabolism of catecholamines is more strongly established for neuroblastomas than PPGLs [8]. Neuroblastomas display a relative lack of catecholamine storage vesicles characteristic of mature chromaffin cells and their PPGL derivatives. Thus, these tumors usually do not present with hypertension or increased urinary outputs of catecholamines, so that biochemical diagnosis has always depended on measurements of catecholamine metabolites.

The two metabolites that have remained the mainstay for diagnosis of neuroblastoma are homovanillic acid (HVA) and vanillylmandelic acid (VMA). HVA represents the end-product of dopamine metabolism, whereas VMA is the end-product of norepinephrine and epinephrine metabolism [4] (Fig. 1). HVA is produced by deamination of methoxytyramine, the O-methylated metabolite, or O-methylation of 3,4-dihydroxyphenylacetic acid, the deaminated metabolite of dopamine. Both enzymes responsible for these steps are present in neuroblastoma cells so that HVA can be directly formed within tumors. In contrast VMA is almost exclusively formed within the liver by the actions of alcohol dehydrogenase on 3-methoxy-4-hydroxyphenylglycol [4]. That metabolite in turn is formed from deamination of normetanephrine or O-methylation of 3,4-dihydroxyphenylglycol, the latter largely formed within sympathetic neurons. Synthesis of norepinephrine requires translocation of dopamine into storage vesicles where the presence of dopamine-ß-hydroxylase facilitates conversion to norepinephrine. Production of HVA by neuroblastomas thus reflects
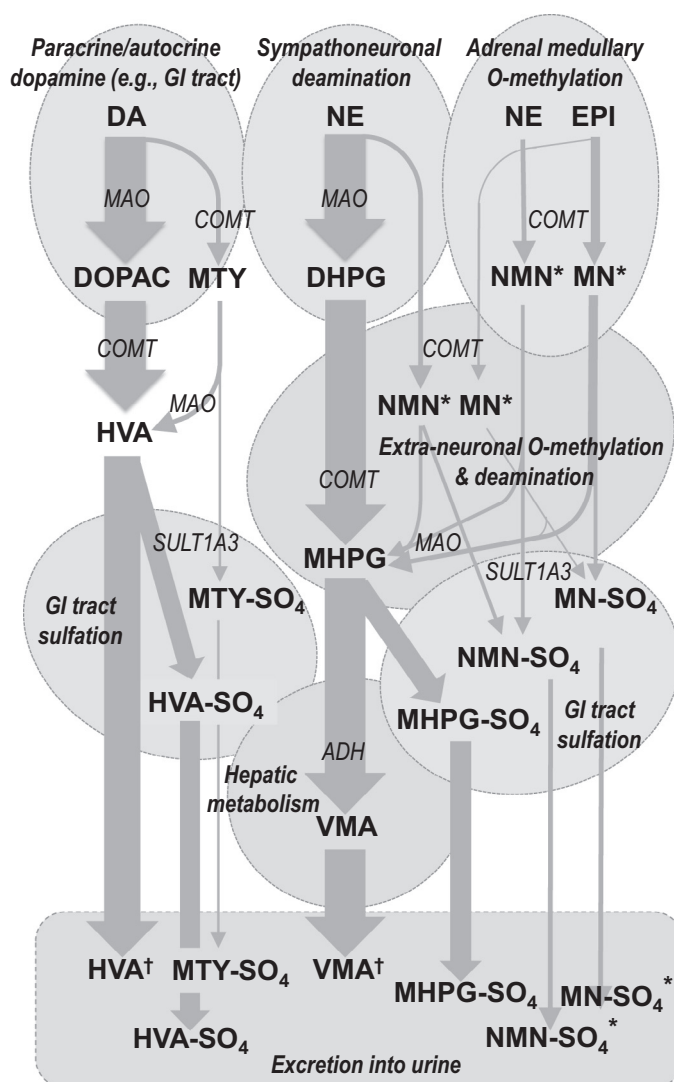

Fig. 1. Pathways of catecholamine metabolism. *Denotes catecholamine metabolites recommended for routine biochemical diagnosis of pheochromocytomas and paragangliomas. †Denotes catecholamine metabolites in routine use for biochemical diagnosis of neuroblastoma. Abbreviations: DA, dopamine; NE, norepinephrine; EPI, epinephrine; DOPAC, 3,4-dihydroxyphenylacetic acid; MTY, methoxytyramine; DHPG, 3,4-dihydroxyglycol; NMN, normetanephrine; MN, metanephrine; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; MTY-SO4, methoxytyramine-sulfate; MN-SO4, metanephrine sulfate; NMN-SO4, normetanephrine sulfate; HVA-SO4, homovanillic acid sulfate; MHPG-SO4, methoxy-4-hydroxyphenylglycol; MAO, monoamine oxidase; COMT, catechol-O-methyltransferase; SULT1A3, sulfotransferase type 1A3; ADH, alcohol dehydrogenase; GI, gastrointestinal.

impaired conversion of dopamine to norepinephrine and the immaturity of catecholamine storage and secretory pathways.

Since the HVA and VMA derived from neuroblastomas are diluted by considerable amounts of the same metabolites produced from other sources, these metabolites are relatively poor diagnostic markers for these tumors. In a prospective trial involving 1.5 million neonates only 73% of all infants detected at follow-up with neuroblastoma had elevated urinary excretion of HVA or VMA at screening [9]. HVA and VMA nevertheless remain the principal catecholamine metabolites used for biochemical diagnosis of neuroblastoma. The metabolites are excreted in large quantities and are relatively easy to measure so that in contrast to PPGLs there has been relatively little effort in LC-MS/MS method development directed to neuroblastoma.

Table 1
Advantages and disadvantages of LC-MS/MS versus LC-ECD and immunoassays for measurements of catecholamine metabolites

LC-MS/MS	LC-ECD	Immunoassay
Advantages	**Advantages**	**Advantages**
Minimal consumable costs	Minimal consumable costs for in-house methods	Minimal expense of instrumentation
High sample throughput	Relative simplicity of operation	Kit methods simple to set up
Sample preparation relatively simple	Some kit methods available, but high consumable costs	Minimal operator expertise required
High analytical sensitivity	Moderate analytical sensitivity	
High analytical specificity and relative freedom from interferences	Chromatographic interferences can be identified	
Methoxytyramine can be measured with precision	Methoxytyramine measurements possible	**Disadvantages**
High versatility of LC-MS/MS instruments		High costs of kit method consumables
	Disadvantages	Lengthy sample preparation/analysis time
Disadvantages	Moderate capital cost of instrumentation	Each metabolite must be measured separately
High capital cost of instrumentation	Cumbersome sample preparation	Difficult to identify presence of interferences
High level of operator expertise required	Prone to analytical interferences	Poor accuracy – negative bias
Need to develop in-house methods	Low sample throughput	Poor analytical sensitivity
		Not possible to measure methoxytyramine

1.2. Plasma versus urine measurements

The utility of measurements of metanephrines in plasma instead of urine for diagnosis of PPGLs was first indicated in a series of studies culminating in a report with cumulative experience in over 800 patients [10]. Although urine fractionated metanephrines provided the one test for which diagnostic sensitivity approached that for plasma metanephrines (97% vs 99%), diagnostic specificity for measurements in urine was nevertheless lower than for plasma (69% vs 89%). Higher accuracy of plasma over urinary metanephrines for diagnosis of PPGLs has been indicated by others [11]. Nevertheless, the differences are small and it remains a matter of debate whether one test is superior to the other. Until resolved, measurements of plasma free and urinary fractionated metanephrines both remain recommended as initial screening tests for PPGLs [7].

For metanephrines in urine there are other considerations concerning measurements of the free versus deconjugated metabolites, the latter reflecting the sum of free plus conjugated metabolites [12]. Metanephrines in urine are usually measured after an acid hydrolysis step, in which conjugated metanephines are converted to free metabolites. The sulfate-conjugates are metabolites produced by a sulfotransferase enzyme localized mainly to gastro-intestinal tissues [13] (Fig. 1). Use of an acid-hydrolysis deconjugation step is based by convention on early methods for which analytical sensitivity was insufficient for quantification of the relatively low concentrations of free metabolites. With LC-MS/MS, measurements of free metanephrines are no longer a problem. Although it is not clear whether urinary free metanephrines offer improved diagnostic performance compared to the deconjugated metabolites, there are other advantages, such as lack of need for acid-hydrolysis deconjugation. Since efficiency of this step depends on pH, temperature and incubation time, variations in these conditions can negatively impact precision and accuracy of measurements [14].

1.3. Emergence of mass spectrometry

From bioassays, colorimetric and fluorometric methods to radioenzymatic assays, analytical techniques for diagnosis of catecholamine-producing tumors have also emerged that employ gas or liquid chromatography, coupled to electrochemical, fluorometric or mass spectrometric detection. At the same time there has been additional emergence of immunoassays for measurements of metanephrines. Although many early methods for measuring catecholamine metabolites were described that combined gas or liquid chromatography with a single mass filter [15–20], these methods involved numerous shortcomings and it was not until emergence of the triple quadrupole design that mass spectrometry began to take hold in the routine laboratory environment. As reviewed elsewhere

[21], LC-MS/MS has since risen to the forefront, increasingly taking center stage as the analytical tool of choice for diagnosis of PPGLs.

Relative freedom from analytical interferences, simpler sample preparation, as well as high sample throughput, offer significant advantages of LC-MS/MS over other methods (Table 1). Sample throughput considerations are particularly important for high volume commercial laboratories where the capital costs of LC-MS/MS instrumentation may be easily recouped. For smaller laboratories, instrument costs, requirements for skilled laboratory personnel and the need to develop in-house analytics can be a disincentive for LC-MS/MS, providing advantages for immunoassays supplied as kit methods. Nevertheless, as outlined in the Endocrine Society Guidelines on PPGLs, immunoassays are not recommended for measurements of plasma free metanephrines [7]. This is because immunoassays suffer not only from imprecision, but also from inaccuracy involving underestimation of plasma concentrations of metanephrines, a consequence of calibration problems [22].

LC-MS/MS as the analytical technique of choice for laboratory evaluation of catecholamine-producing tumors has risen to particular prominence in the United States and Australasia; in the former economically competitive, high-throughput considerations have been instrumental to this emergence, whereas in the latter there is value placed on high level expertise and quality assurance as integral components of the chemical pathology profession. Despite the advantages of LC-MS/MS, over immunoassays, the latter have remained prominent in places where interrelated economic, health insurance and regulatory issues impede adoption of more advanced laboratory technologies.

Adoption of technologies requiring advanced expertise can be particularly difficult in small hospital-based laboratories. Such environments favor the expense of immunoassay kit methods easily utilized on available automated immunoassay analyzers. For such kits and analyzers there are minimal requirements for advanced technical expertise; there is also no need for a large capital outlay for instrumentation not easily recouped in a low throughput environment. For routine use, kit methods must also be certified by regulatory agencies and are immediately ready for use, whereas for LC-MS/MS there can be barriers associated with instrument certification and the need to develop in-house methods requiring additional time and expertise for validation to meet regulatory compliance.

Despite the above limitations to emergence of LC-MS/MS, there remain over-riding advantages that will ensure impact of the technology even among regions and centers resistant to change (Table 1). Once expertise is in place and methods are developed, the costs of consumables are slight compared to kit methods. Modern LC-MS/MS instruments are also quite robust and flexible allowing multiple applications to be run seamlessly on one instrument, thereby minimizing problems associated with recouping capital costs of

instrumentation for low sample throughput laboratories. In-house methods can also be easily adapted and improved upon, such as with inclusion of additional analytes to a panel (e.g., methoxytyramine). In contrast, for kit methods there is considerable expense required by manufacturers in meeting certification standards, so that even if deficiencies become recognized they may not be acted upon [22]. As covered below there are many continuing advances in LC-MS/MS technology. These developments offer further opportunities for breaking away from out-moded analytical procedures to mass spectrometric-based methods that offer dramatically improved analytical sensitivity, accuracy and precision with attendant potential impact for improved patient diagnostics, management and care.

2. LC-MS/MS method development

2.1. Analytes and ionization strategies

The analytes used in LC-MS/MS applications directed to diagnosis of catecholamine producing tumors include most commonly normetanephrine, metanephrine and methoxytyramine and less commonly the parent catecholamines, norepinephrine, epinephrine and dopamine measured in plasma or urine (Fig. 2). Urinary HVA and VMA are also used for diagnosis of neuroblastoma. All are polar compounds existing as positively (amines) or negatively (acids) charged ions in solution, favouring electrospray ionization (ESI) as the method of choice for optimal signal strength. ESI in the positive mode is standardly used for the amines, whereas negative electrospray is employed for VMA and HVA.

2.2. Specimen processing

The high analytical specificity offered by multiple reaction monitoring (MRM) in LC-MS/MS applications enables considerably simplified sample preparative procedures compared to the laborious procedures demanded by other techniques. Nevertheless, ion suppression or enhancement associated with matrix impurities still mandates sample clean up before analysis. For analytes, such as HVA and VMA, present in urine at high concentrations, this can simply amount to removal of particulate matter followed by a dilution before injection [23]. One method for measurements of urine deconjugated metanephrines has similarly detailed direct injection after acid hydrolysis and centrifugation [24]. Usually for such analytes present in sample matrices at relatively low abundance, a more involved sample purification procedure is necessary; for plasma free metanephrines, and particularly methoxytyramine, it can be useful for purification to also include analyte enrichment.

One LC-MS/MS method for plasma metanephrines has been described in which purification and concentration was achieved by isopropanol protein precipitation followed by dry down and mobile phase reconstitution [25]. Virtually all other published methods for plasma and urinary metanephrines have incorporated a solid phase extraction (SPE) step [26–39]. Similarly, for the few LC-MS/MS methods developed for measuring plasma or urine catecholamines, either alone [40–42] or in combination with metanephrines [29,37], all have involved an SPE step.

Since metanephrines contain the same functionally charged amino group, the most commonly employed SPE process involves cation exchange [26–30,32,34,36–38]. For catecholamines, methods

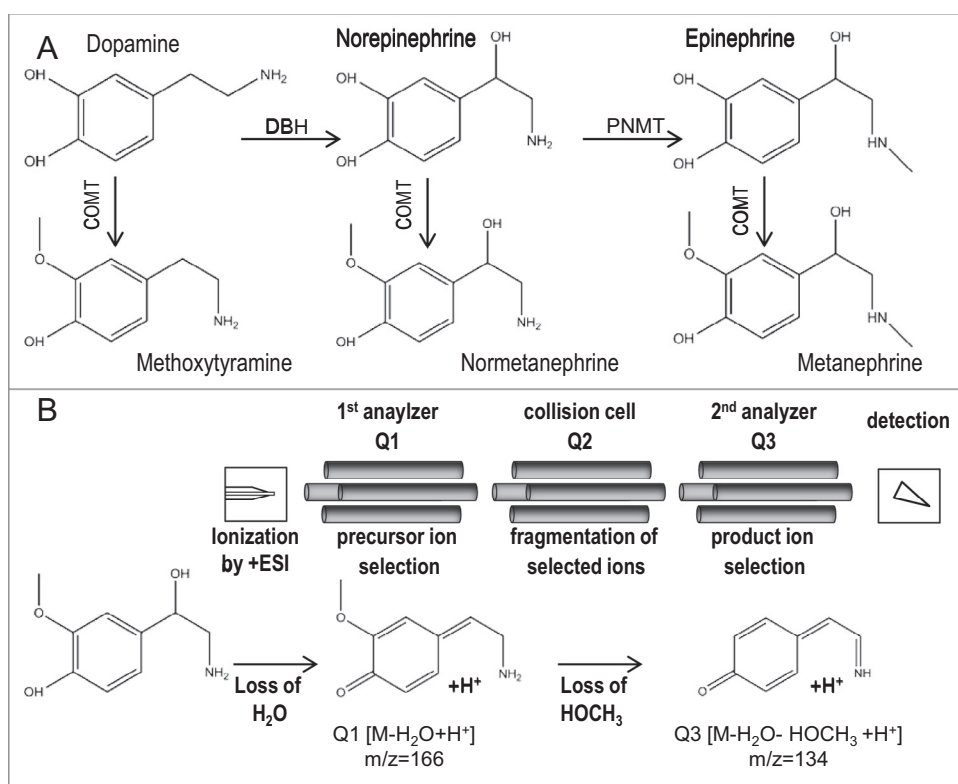**Fig. 2.** Chemical structures of catecholamines and O-methylated metabolites (including pathways of metabolism) employed for diagnosis of catecholamine producing tumors (A) and MRM transitions for normetanephrine employed for mass spectrometric analysis of this metabolite (B). Abbreviations: DBH, dopamine beta hydroxylase; PNMT, phenylethanolamine-N-methyltransferase; COMT, catechol-O-methyltransferase.
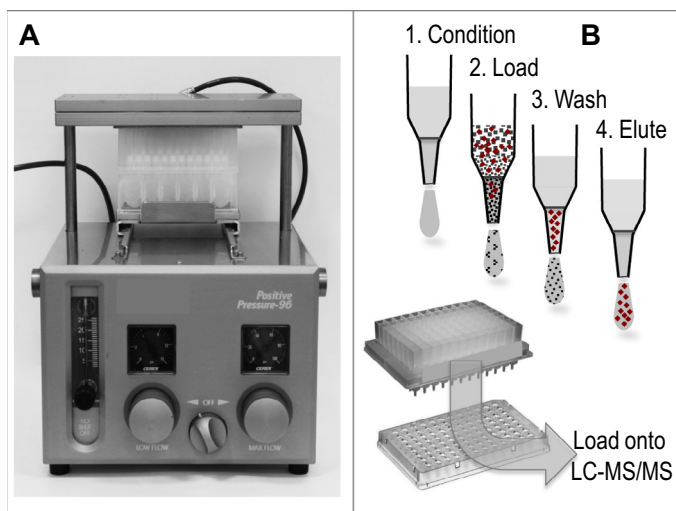
Fig. 3. Experimental set-up for 96-well solid phase extraction using positive pressure (A) and diagrammatic representation of the scheme for cation-exchange SPE procedures typically used for purification of metanephrines prior to loading of 96-well plates onto the sample manager and solvent delivery system (B). SPE extraction cartridges are first conditioned (1) before samples with internal standards are loaded (2). There follows a wash step (3) followed by elution of the analytes (4) into 96 well plates for loading onto the LC-MS/MS.

cipal is the same, requiring loading of the sample followed by washing and elution steps (Fig. 3). To minimize ion suppression it is important that the final extract be prepared in a solution identical to or closely resembling the mobile phase into which the extract is injected, this most readily achieved by a dry-down and reconstitution step or elution from SPE cartridges using mobile phase.

SPE sample preparation to date has involved mainly offline applications [26,27,29,30,32,34,36,37], with online applications increasingly possible due to advances in robotic instrumentation [28,38], which in different adaptations is also applicable to offline applications. Initial SPE procedures employed single unit cartridges [26,27,29], which have now been superseded by 96 well SPE plates [30,32,34,36,37]. Traditionally a vacuum (negative pressure) is applied to SPE columns to draw through the solvents and sample; however, positive pressure units have been introduced for the 96 well SPE format that more effectively push rather than pull the solvents and sample through the SPE material (Fig. 3). The 96 well sample processing format provides substantially increased efficiency, including loading of extracts in 96 well plates directly into sample and solvent delivery systems. A dry-down and mobile phase reconstitution step remains possible for measurements in which sample enrichment can aid analytical sensitivity [36].

Since sample preparation is the main determinant of operator time and represents a significant bottleneck in clinical mass spectrometry, automation of the extraction process with coupling to the chromatographic system provides significant gains in efficiency and minimizes sample handling and associated operator errors. Online SPE also enables increased analytical sensitivity since the sample can be concentrated before elution, with all of the concentrated extract injected onto the column. Online SPE systems are designed to automatically process through a series of programmable functions during which the SPE cartridge is equilibrated, loaded and washed (Fig. 4).
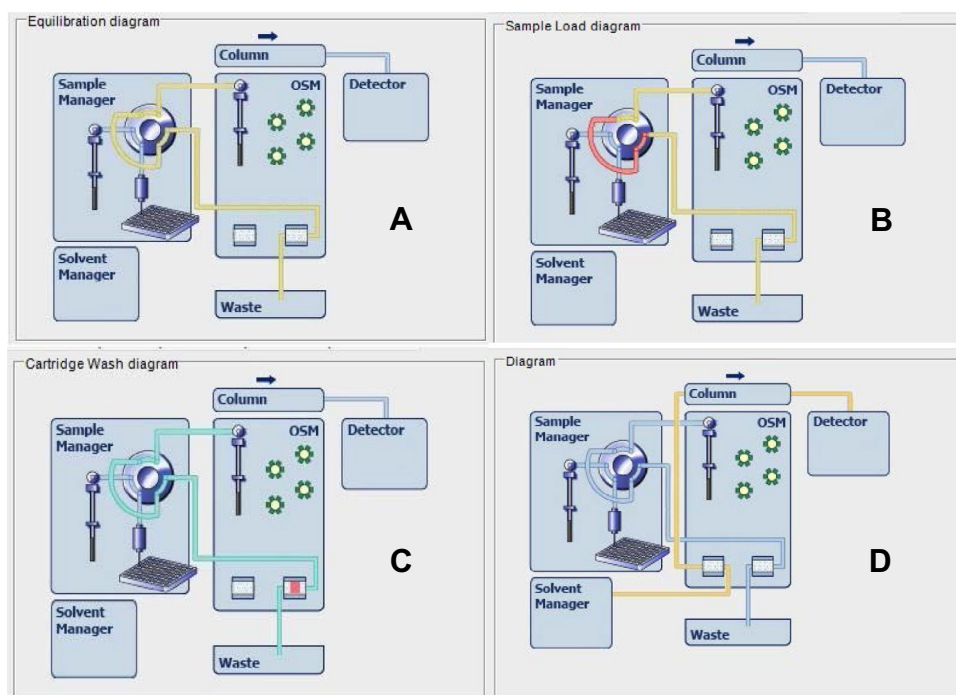
employing alumina or phenylboronic acid for complexing catechols have been described [41,42], but cation exchange remains preferable since it also enables combined extraction and measurements of urine free catecholamines and metanephrines [29,37]. SPE methods employing weak, strong, or mixed mode reversed phase strong cation exchange have been described, but in all cases the principal

Fig. 4. Diagrammatic representation of an automated on-line sample purification system used for LC-MS/MS measurements of plasma free metanephrines. There are 4 steps to the process: A equilibration, in which gripper places cartridge in right clamp and high pressure dispenser (HPD) applies conditioning and equilibration solvents to cartridge; B sample loading in which the sample manager draws sample during conditioning and equilibration steps, the loop is brought in line with loading solvent from the HPD and the sample is pushed through the cartridge with the analyte trapped in the cartridge and the stream diverted to waste; C sample washing in which the HPD supplies wash solvents across the cartridge; and D sample elution in which the gripper moves the cartridge to the left clamp for the elution step. The elution time is defined within the software, providing selectivity of extraction.

2.3. Liquid chromatography

Initial methods for measurements of plasma free metanephrines utilised LC-ECD, a labor-intense method that also requires extended sample run times (up to 40 minutes) for chromatographic separation of metabolites and interferences [3]. The selectivity offered by MRM not only enables simplified sample purification, but also less stringent requirements for chromatographic separation, thereby reducing sample run times and maximizing sample throughput. In some applications, there is virtually no chromatographic separation of metabolites and the purpose of chromatography is to simply separate polar metabolites from the solvent front and non-polar interferences [28,30,34,38,43]. For these applications, analytical specificity depends principally on the selectivity of ion monitoring.

As discussed later and in detail by others [44], over reliance on the specificity of the mass spectrometer can be problematic and for this reason the additional specificity offered by chromatography should be balanced against the needs for high sample throughput. Advances in liquid solvent delivery systems and column technology provide opportunities for such optimization. In particular, solvent delivery systems, such as ultra high performance liquid chromatography (UHPLC) that operate at much higher pressures than conventional systems enable chromatographers to utilize sub 2-micron particle size columns. The result is increased peak resolving power and analytical sensitivity, along with a significant reduction in column re-equilibration time following gradients. The net gain is faster and superior chromatography facilitated by availability of a host of analytical columns specially suited for this purpose.

Analytical columns employing hydrophilic interaction liquid chromatography (HILIC) are especially suitable for separation of polar compounds from matrix interferences and enable enhanced analytical sensitivity with electro spray ionization. For the desolvation process, an organic solvent is more efficient and the higher concentrations of organic solvent (>80%) used with HILIC provide superior ionization efficiencies than possible with the primarily aqueous mobile phases required for traditional reverse phase analytical columns. Nevertheless, almost all published applications with HILIC, although demonstrating rapid chromatography, have displayed poor separation of O-methylated metabolites [28,30,34,38,43]. Introduction of amide HILIC columns has overcome these issues, allowing separation of O-methylated metabolites while still retaining the benefits of HILIC (Fig. 5A). Continued use of traditional reverse phase columns by others reflects the more easily attained separation possible with these columns [24,29,32,35–37], which combined with UHPLC also enables rapid chromatography at high analytical sensitivity and specificity [36,37] (Fig. 5B). Where necessary, improvement in separation of amines on reverse phase columns is possible using ion-pairing reagents [45], but these must be volatile to minimize ion suppression.

2.4. Selectivity, interferences and other analytical issues

Analytical interferences with LC-MS/MS measurements of catecholamines and their metabolites are less troublesome than for other methods, but as detailed elsewhere [44], may occur in several different forms: 1. ion suppression or enhancement; 2. ionic cross talk; 3. in source transformation and; 4 isobaric interferences. Optimization of sample purification and chromatography as part of method validation should avoid most problems with ion suppression. Variable specimen matrix composition can nevertheless result in signal loss for isolated samples. For example, as described for HILIC methods some dugs (e.g., cimetine, ephedrine, labetolol and pseudoephedrine) result in ion suppression-associated signal loss, though with negligible impact on quantification [34]. Quantification can, however, be impacted by matrix effects leading to differential ion
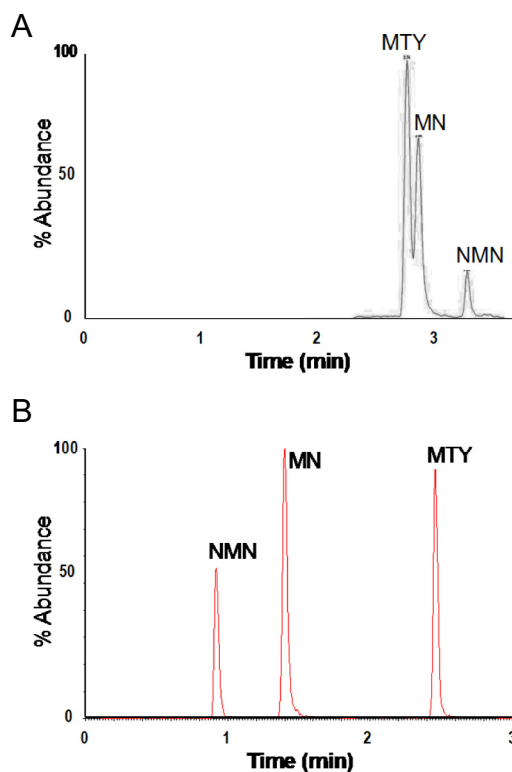

Fig. 5. Ultra-high performace liquid chromatographic separation of normetanephrine (NMN), metanephrine (MN) and methoxytyramine (MTY) achieved on an amide HILIC analytical column (A) compared to a reverse phase analytical column (B).

suppression and loss of signal for the analyte and its deuterated internal standard [46].

Ionic cross talk has been described for methods in which O-methylated metabolites are not chromatographically resolved and where in-source fragmentation can result in formation of ions mimicking methoxytyramine, leading to over-estimated concentrations of this analyte. A larger problem for methoxytyramine has been identified to result from co-chromatography of this metabolite with 3-O-methyldopa, which is present in plasma at concentrations more than 3 orders of magnitude higher than methoxytyramine itself [47]. In source decarboxylation of 3-O-methyldopa leads to a product ion identical to that for methoxytyramine (Fig. 6); this results in substantial overestimation of methoxytyramine, a problem only avoided by chromatographic separation.

Others have described interferences with measured concentrations of normetanephrine by 4-hydroxy-3-methoxymethamphetamine [48], a metabolite of the recreational drug 3,4-methylenedioxymethamphetamine, which also has been reported by others to cause similar interference [34]. The beta-adrenergic agonists, isoproterenol and isoetharine, are other drugs reported to interfere respectively with measurements of normetanephrine and metanephrine [34]. In all above cases the interfering compounds bear structural similarities to measured O-methylated metabolites and likely interfere by either ionic cross talk or in source transformation. Isobaric interference can occur between epinephrine and normetanephrine, which share identical molecular masses, but is less problematic for catecholamines and their metabolites than for other analytes (e.g., steroids). In all above cases interference can be avoided by optimization of chromatography.

Interferences from co-chromatographing substances with MRM transitions of target analytes can be assessed from ratios of quantifier to qualifier ions. Using these ratios and chromatographic interferences that precluded baseline resolution, Wright and
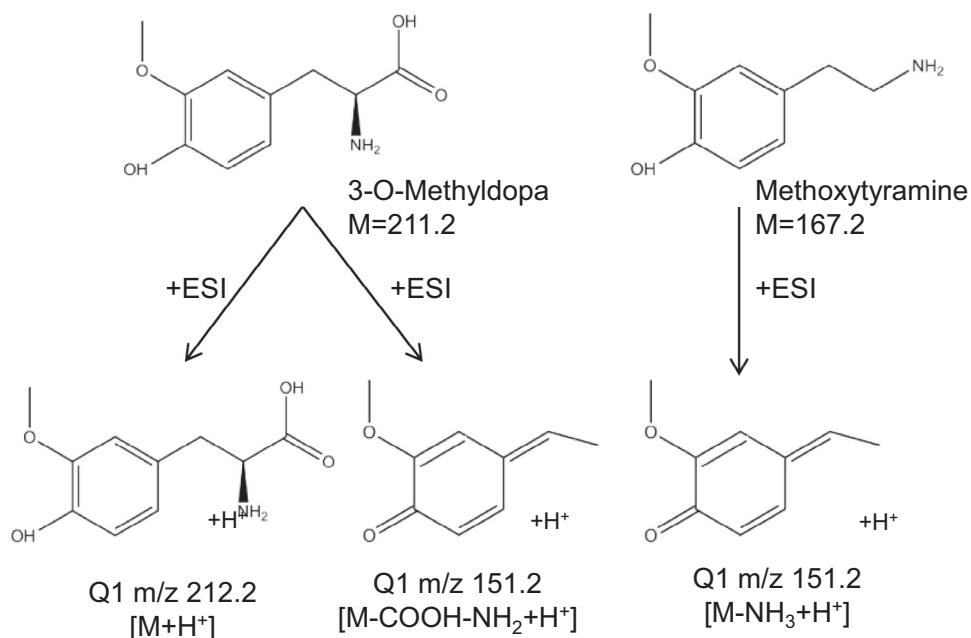
Fig. 6. Basis of interference with LC-MS/MS measurements of plasma methoxytyramine due to in-source fragmentation 3-O-methyldopa. Most 3-O-methyldopa remains insource as the positively charged precursor ion, but a small proportion undergoes insource decarboxylation to a positively-charged ion identical to that produced from methoxytyramine.

colleagues reported interferences with 1% of all patient samples analyzed by LC-MS/MS for plasma metanephrines [43]. These investigators further established how these interferences could be eliminated by MRM with multistage fragmentation, whereby the conventional product ion produced by collision induced fragmentation of the precursor ion is further fragmented in a linear ion trap to produce a "second generation product ion". Although a significant advance that takes advantage of hybrid triple quadrupole ion trap instruments, MRM with multistage fragmentation cannot be used to resolve interferences resulting from in-source transformation and in which a product is produced identical to that being measured. For such interferences chromatographic resolution remains essential [47].

2.5. Quality assurance

As outlined earlier, one of the disadvantages of LC-MS/MS applications in the routine clinical chemistry laboratory is the need for in-house development, which also requires considerable time for method validation and integration with procedures and programs to ensure quality assurance (QA). Requirements for method development and validation have been reviewed elsewhere [49–51]. Reliance on in-house produced calibrators and quality control material has been or is being addressed by commercial suppliers. These efforts are assisting with harmonization of the many varied assay methods, with this being further facilitated by other practices and initiatives, in particular those involving inter-laboratory QA programs [52].

One particularly relevant international QA program for laboratory measurements of catecholamines and their metabolites is that run by the Royal College of Pathologists of Australasia (RCPA). The RCPA QA program (http://www.rcpaqap.com.au) directed to catecholamines and their metabolites was established by the Australasian Association of Clinical Biochemists working party on biogenic amines. The biogenic amines QA program, established in 1988 [53], was extended in 2008 to cover plasma free metanephrines [54]. Over the ensuing 7 years there has been a clear shift from

mainly LC-ECD methods to near exclusively LC-MS/MS methods for measuring plasma metanephrines. Among the 36 laboratories participating in the first cycle of 2015, 32 (89%) were using LC-MS/MS, only 1 was using LC-ECD and 3 immunoassays. Apart from enzyme immunoassays, which continue to show considerable negative bias and imprecision, there has been a steady improvement over the 7 years in agreement of results between laboratories.

3. Diagnostics

3.1. Diagnostic performance

As with any laboratory test, accuracy and precision of measurements of catecholamine-related biomarkers are key prerequisites to ensure best possible diagnostic performance. LC-MS/MS provides the best currently available analytical tool for such assurance, but even when performed with high accuracy under the strictest conditions of quality assurance there can be over-riding more important factors that impact the performance of any test for diagnosis of catecholamine-producing tumors [55]. As outlined in the introductory sections, choice of the best available biomarkers according to their selectivity for diagnosis is crucial. For biomarkers with poor diagnostic accuracy, such as urinary HVA and VMA used for detection of neuroblastoma, even when the analytes are measured by state of the art LC-MS/MS methods, impact of the technology on diagnostic performance is unlikely to be significant. As discussed below, high diagnostic performance also remains only possible when testing for PPGLs is carried out under appropriate preanalytical conditions and with appropriately determined reference intervals.

3.2. Preanalytical considerations

In contrast to analytical interferences, which are less of a problem with LC-MS/MS than other methods, pharmacophysiological interferences involving effects on production or clearance of measured analytes are method-independent and equally important to consider for all methods. Such method-independent interferences can

derive from medications, diet or environmental sources, including conditions of sampling.

For measurements of normetanephrine, tricyclic and other antidepressants that block neuronal uptake of norepinephrine are one of the most common causes of false-positive results [56]. There are numerous other drugs that can either increase norepinephrine release by sympathetic nerves or impact clearance mechanisms and thereby cause false positive increases of plasma or urine metanephrines. As in the case of tricyclics, most such drugs only increase the likelihood of false positive results so that withdrawal is only necessary when positive results are encountered. Monoamine oxidase inhibitors, which block metabolism of all O-methylated metabolites and cause large increases in metanephrines, are one exception for which withdrawal is necessary before testing. Although L-dopa used to treat Parkinson's disease elevates plasma and urinary outputs methoxytyramine, it has only minor influences on normetanephrine and metanephrine measured by LC-MS/MS [57].

Apart from medications, dietary influences can also impact concentrations of circulating and urinary catecholamine-related biomarkers. L-dopa, dopamine and other amines, common to many foods, are established to increase plasma levels of L-dopa, dopamine and their sulfate-conjugated metabolites [58,59]. Such dietary compounds can lead to substantial increases of not only urine outputs of deconjugated normetanephrine and methoxytyramine, but also plasma free methoxytyramine [60]. Blood samples for measurements of the latter metabolite should therefore be collected after an overnight fast [61]. For urine, procedures to avoid dietary influences are problematic and not commonly employed.

For plasma free metanephrines, inappropriate sampling conditions, in particular seated rather than supine blood sampling, represents the most common and easily correctible cause of false positive results [55]. Since upright posture is a powerful stimulus for activating the sympathetic nervous system, sampling in the seated position results not only in elevated plasma concentrations of norepinephrine, but also its metabolite normetanephrine. False-positive results may thereby be increased by as much as 7-fold [61]. As indicated by areas under receiver operating characteristic curves, diagnostic accuracy of plasma free metanephrines measured by LC-MS/MS for samples taken in seated positions is significantly reduced compared to supine sampling and offers no better if not worse diagnostic accuracy compared to urinary fractionated metanephrines. Thus, when blood cannot be collected in the supine position, any diagnostic advantage of the plasma over the urine test is negated; under these circumstances and for ease of measurement the urine test may be preferable.

3.3. Reference intervals

Because of the potentially deadly consequences of missing the diagnosis for catecholamine-producing tumors, upper cut-offs of reference intervals for plasma and urinary metanephrines should primarily be optimized to ensure high diagnostic sensitivity. Such optimization provides confidence that patients with the tumors will not be missed, also meaning that disease can be reliably excluded by negative results so that no further testing is necessary.

As outlined in published recommendations [7,62], to minimize false negative results, reference intervals for plasma free metanephrines must be established for blood sampled in the supine position. When measurements include plasma methoxytyramine, reference intervals should also exclude dietary influences. Dangers of inappropriately established reference intervals have been highlighted by those used in immunoassay kit methods [22]. Upper cut-offs of reference intervals in package inserts were far too high resulting in up to a quarter of all patients with PPGLs failing to be diagnosed. Thus, reference intervals should not be set blindly according to kit package inserts or information in textbooks, but should be validated at every laboratory where the measurements are established for routine use (Fig. 7).

Although emphasis of reference intervals used in screening for PPGLs should primarily be directed to ensuring high diagnostic sensitivity, this does not imply that specificity should be neglected. For measurements of plasma normetanephrine, an age-adjustment employing higher upper cut-offs with advancing age can be particularly useful for minimizing false-positive results while maintaining high diagnostic sensitivity [63] (Fig. 7).

For 24 hr urinary measurements, gender can also be important to consider, with higher upper cut-offs usual in males than females. Use of age-appropriate reference intervals is also particularly important for measurements in children [64]. A related consideration is the use of creatinine for normalizations in spot urines. In contrast to 24-hour urinary outputs, urine outputs of normetanephrine and metanephrine normalized to creatinine show age-associated decreases, related to increases in total muscle mass and related production of creatinine. Similar influences also impact requirements for gender-specific reference intervals for urinary outputs of catecholamine metabolites.

3.4. Methoxytyramine

Methoxytyramine is present in plasma at very low concentrations with upper limits of reference intervals not extending beyond 0.1 nmol/L (Fig. 7). This mandates analytically sensitive detection methods, currently only possible with any precision using the latest generation of LC-MS/MS instruments. Although most PPGLs are readily detected by increases of normetanephrine, either alone or in combination with increases in metanephrine or methoxytyramine, a small percentage are manifest by solitary increases of metanephrine and others of methoxytyramine [65]. The latter dopaminergic tumors display immature phenotypic features often associated with metastatic disease or with paragangliomas due to mutations of genes encoding sub-units of succinate dehydrogenase [66,67]. Measurements of methoxytyramine aid detection of these tumors and provide one of the only currently available circulating biomarkers for metastatic PPGLs. With technical advances in LC-MS/MS, these measurements should further impact improvements in diagnosis and management of patients with PPGLs. Extension to neuroblastoma is another avenue that may offer improvements over current approaches reliant on urinary HVA and VMA [68].

3.5. Energy pathway metabolites

Apart from applications involving measurements of catecholamine-related biomarkers, LC-MS/MS-based applications for aiding diagnosis of patients with PPGLs have also been directed to measurements of mitochondrial energy pathway metabolites in tumor tissue [69]. This application followed identification of mutations of genes encoding sub-units of succinate dehydrogenase as a cause of PPGLs [70,71]. The associated defect in conversion of succinate to fumarate leads to substantially increased ratios of the two metabolites in tumor tissue offering a highly sensitive and specific method for identifying underlying abnormalities of mitochondrial energy metabolism due to genetic defects impacting associated enzymes [69]. The method is simple, cheap and can be employed on the same LC-MS/MS instruments used for measurements of catecholamine metabolites, thereby extending associated diagnostic capabilities.
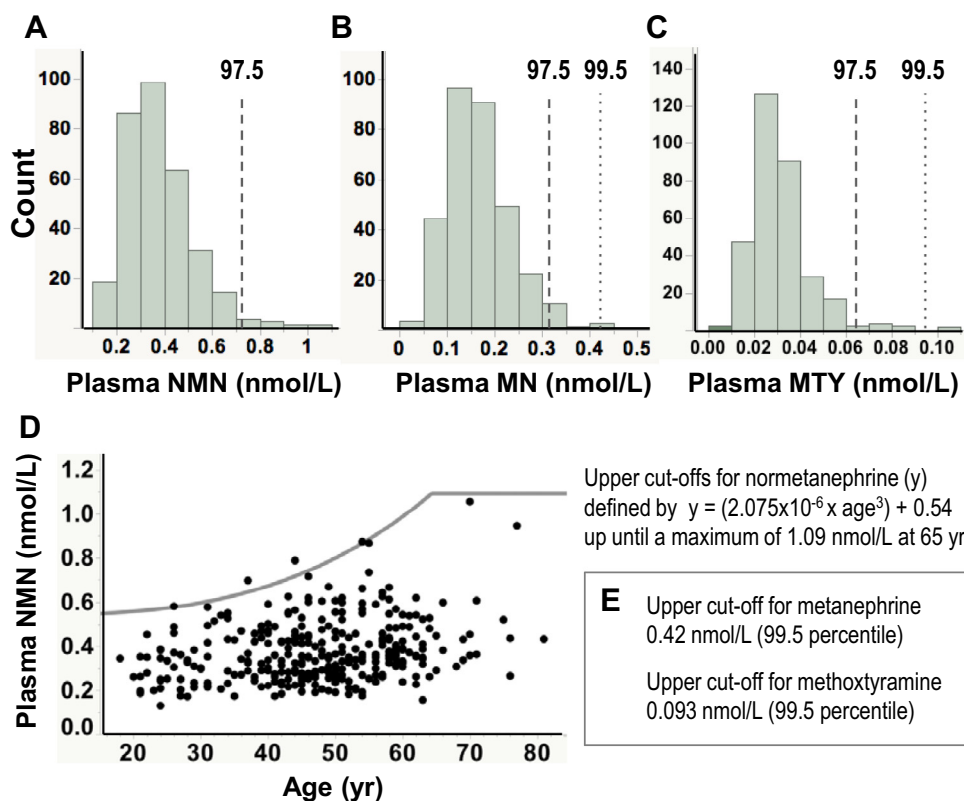
Acknowledgments

Fig. 7. Distributions of plasma concentrations of normetanephrine (A), metanephrine (B) and methoxytyramine (C) in a reference population used to establish reference intervals. To optimize diagnostic specificity, reference intervals for plasma normetanephrine are set up with an age-adjustment (D), whereas those for metanephrine and methoxytyramine utilize 99.5 percentiles rather than more commonly used 97.5 percentiles (E). Abbreviations: normetanephrine, NMN; metanephrine, MN; methoxytyramine, MTY.

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