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Review

Liquid chromatography tandem mass spectrometry for plasma metadrenalines

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ABSTRACT

Evidence is accumulating which may result in plasma free metadrenalines (PMets) becoming the preferred test for diagnosing phaeochromocytoma and paraganglioma. Moreover, increased availability and benefits over other analytical methods like liquid chromatography with electrochemical detection and immunoassay are causing liquid chromatography tandem mass spectrometry (LC-MS/MS) to become the method of choice for PMet measurement. This review explores the evidence-base supporting this, and summarises published LC-MS/MS analytical methods for PMet analysis. Key aspects of methods (including SPE extraction, HILIC chromatography, MRM MS-detection and standardisation) are discussed. Common causes of analytical interference (e.g. ion suppression/enhancement, ionic cross talk, in source transformation and isobaric interferences) are outlined to illustrate the importance of sample purification and chromatographic resolution. The importance of supine, fasting sampling and Bayesian interpretation against supine, fasting reference intervals are explained, as well as the importance of age-specific reference intervals for normetadrenaline. Confounding factors like diet, drugs, renal function and acute illness are explored, along with potential strategies to address these (e.g. CKD-specific reference intervals).

1. Introduction

Clinical practice guidelines [1] currently advocate with high-level evidence that total fractionated urine metadrenalines (UMets) or plasma free metadrenalines (PMets) should be used in the initial biochemical screening for phaeochromocytomas and paragangliomas (PPGL), with no preference expressed for either test. In addition, UMets and PMets are both suggested for biochemical monitoring of recurrent or metastatic disease [1]. Metadrenaline (MA, synonym metanephrine), normetadrenaline (NMA, synonym normetanephrine) and 3-methoxytyramine (3MT) are, respectively, *O*-methylated metabolites of the catecholamines adrenaline (synonym epinephrine), noradrenaline (synonym norepinephrine) and dopamine. Total urine metadrenalines refers to measurement of both free and conjugated metadrenalines (synonym metanephrines), with acid hydrolysis or enzymatic deconjugation being used to liberate free metadrenalines from their sulphate-conjugated metabolites. The term metadrenalines often relates to MA and NMA only. Separate measurement of MA and NMA is termed "fractionated". Only the unconjugated (free) metadrenalines are measured in plasma [2]. Evidence is accumulating that urinary free metabolites (without deconjugation by acid hydrolysis) may confer advantages over UMets and that PMets offers superior diagnostic performance over urinary metabolites in high-risk patients with appropriate pre-analytical precautions [3]. Differences in diagnostic

Abbreviations: ACN, acetonitrile; AmF, ammonium formate; APCI, atmospheric pressure chemical ionisation; CE-IVD, Conformité Européenne In Vitro Diagnostic; CKD, chronic kidney disease; ESI, electrospray ionisation; FA, formic acid; FN, false negative; FP, false-positive; HILIC, hydrophilic interaction liquid chromatography; HLB, hydrophilic–lipophilic balance; HMMA, 4-hydroxy-3-methoxymethamphetamine; HPLC, high performance liquid chromatography; IA, immunoassay; LC, liquid chromatography; LC-ECD, liquid chromatography with electrochemical detection; LC- MS/MS, liquid chromatography tandem mass spectrometry; LLMI, lower limit of measuring interval; MA, metadrenaline; MAO, monoamine oxidase; MCX, mixed mode cation exchange; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MRM, multiple reaction monitoring; MRM^3, multistage fragmentation; NMA, normetadrenaline; PFP, pentafluorophenyl; PGC, porous graphite column; PMets, plasma free metadrenalines; PPGL, phaeochromocytomas and paragangliomas; Sen, sensitivity; SPE, solid-phase extraction; Spec, specificity; U, unknown; ULRI, upper limit of the reference interval; UMets, total fractionated urine metadrenalines; WCX, weak cation exchange; 3-MT, 3-methoxytyramine

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Table 1
Summary of the advantages and disadvantages of PMets compared with UMets. Adapted from [5].

Advantages PMets vs UMets	Reflect the free metadrenalines produced directly by tumours.
	Samples are more convenient and reliable for patients to collect than 24 h urines.
	Dietary and drug confounders are easier to control than in urine.
	More useful in chronic kidney disease (CKD).
Disadvantages PMets vs UMets	Requirement for supine/fasted samples.
	Greater analytical challenges (picomolar concentrations in a complex matrix necessitating extraction).
	Sample instability (collected on ice, with prompt centrifugation and freezing).
	Increased cost of clinic sampling.

Abbreviations: PMets - plasma free metadrenalines; UMets - total fractionated urine metadrenalines.

performance between plasma and urine were not evident in patients with low pretest prevalence [3]. Superior performance of PMets (sampled in the supine position) over UMets which disappears in low-risk patients is corroborated [4]. Further advantages and disadvantages of PMets compared with UMets are listed in Table 1 (adapted from [5]). PMets may become the preferred test in future guidelines with preference shifting to urinary tests when accurate plasma methods, appropriate reference intervals, or adherence to pre-analytical blood sampling precautions cannot be achieved [3]. The diagnostic performance (sensitivity and specificity) of published PMet and UMet methods have been reviewed elsewhere [4,5]. Clinical practice guidelines [1] and a recent systematic review [4] both recommend measuring PMets by liquid chromatography tandem mass spectrometry (LC-MS/MS) or liquid chromatography with electrochemical detection (LC-ECD) in supine samples to provide biochemical evidence of PPGL. Immunoassay (IA) methods have been published but suffer from lower diagnostic sensitivity, imprecision, negative bias for MA and NMA and false-negative (FN) results compared with other methods [1]. Moreover, 3MT measurement is generally not possible by IA and stereospecificity is an issue [6,7]. Measurement of PMets is challenging due to low physiological concentrations, hydrophilic properties, complexity of the matrix and lack of unique chemical characteristics. This review aims at summarising published LC-MS/MS methods for measurement of PMets. Historical methods have previously been reviewed [8], as have ECD and urine methods [9].

2. LC-MS/MS analytical methods

LC-MS/MS is regarded as the "gold-standard" method [1] and is the method of choice, as reflected by its predominance in international quality assurance programs [3]. Table 2 summarises the relative advantages and disadvantages of LC-MS/MS methods for PMets compared to LC-ECD and IA methods (adapted from [10]). Another benefit of LC-MS/MS methods is the ability to accurately and precisely measure 3MT. The majority of PPGL give rise to increased NMA (either alone or in combination with increases in other PMets) [10]. However, there are some dopaminergic tumours that can only be detected through solitary increased 3MT [11]. These are associated with gene mutations encoding succinate dehydrogenase subunits or metastatic disease [12–14]. 3MT measurement is useful for detecting these tumours and has the greatest clinical utility in the evaluation of metastatic disease. High analytical sensitivity is required for 3MT measurement owing to upper limits of reference intervals (ULRI) < 100 pmol/L [10]. Studies have shown that combined plasma free NMA, MA and 3MT detected more biochemically active head and neck paragangliomas than 24 h urinary deconjugated NMA, MA and 3MT [15]. Moreover, that urinary 3MT has limited utility for screening dopamine-producing PPGLs [16] and plasma free 3MT is superior to urinary free and deconjugated 3MT for assessing tumoural dopamine production [3].

LC-MS/MS methods comprise extraction of the metadrenalines from the complex plasma matrix, followed by chromatography, and mass spectrometric detection. Table 3 summarises the key analytical and performance characteristics of in-house LC-MS/MS methods for PMets. It is important to note that performance characteristics e.g. precision and lower limit of the measuring intervals (LLMI) were variably defined and may not be equivalent but have been presented as stated in the publication. Recent guidance on clinical method validation [17] should reduce this variability in future. An earlier method which required preconcentration has not been included [18], and neither have gas chromatography mass spectrometry methods which are not practicable or high enough throughput for routine analysis.

2.1. Extraction

The polar and hydrophilic nature of metadrenalines (Fig. 1) complicates their extraction. Solid-phase extraction (SPE) is the most popular sample preparation technique to minimise matrix effects from endogenous plasma constituents like phospholipids, salts, and proteins that can cause ion suppression. The majority of methods employ weak cation exchange (WCX) [19–25]. The basic nature of metadrenalines (pKa values in Fig. 1) permits isolation by cation-exchange. The quaternary amine group makes strong cation exchange unsuitable. WCX binds strong bases using a carbonyl ion-exchanger at pH > 5, allowing washing with water and acetonitrile (ACN) without elution of metadrenalines. An acidic mobile phase is then used for elution [19]. A minority of methods use mixed mode cation exchange (MCX) [26–28], which exploit reverse-phase interactions and a sulphonic acid strong cation exchange for biogenic amine retention [26]. Ion-pairing reagents may be required for this [27]. Other extraction techniques involve mixed mode (hydrophilic–lipophilic balance, HLB) [29] or isopropanol protein precipitation [30]. Sample volume requirement is highly variable (100–1000 μL). The majority of methods involved off-line extraction, with some on-line extractions described [19,25,27] (not all of these are still supported). On-line extraction requires capital investment, but gives simpler and less labour-intensive sample preparation so is preferable to complex manual SPE if available.

2.2. Chromatography

Hydrophilic interaction liquid chromatography (HILIC) is the most common chromatographic approach used for measurement of PMets. HILIC allows polar analytes to be separated on polar stationary phases (like silica or cyano) using highly organic mobile phase (often ACN) with relatively low back pressures. It is thought that separation occurs through partitioning of the analyte of interest between the bulk organic mobile phase and a partially immobilised layer on that stationary phase surface that is enriched with water. Additional mechanisms are possible too [31]. Buffers containing ammonium formate (AmF) help to stabilise charge on both the analytes and the stationary phase. HILIC also offers enhanced sensitivity in electrospray ionisation (ESI) methods owing to efficient desolvation in the highly organic mobile phase at which elution occurs [31]. However, HILIC columns are unable to chromatographically resolve metadrenalines [19,20,22,24–26,30], with the potential for analytical interference (see Section 3.2). Amide HILIC columns retain the advantages of HILIC with greater chromatographic separation [10]. Reverse phase columns have also been utilised for enhanced chromatographic resolution including Hypercarb porous graphite column (PGC) with ion-pairing reagents [27], Acquity UPLC

Table 2
Summary of the advantages and disadvantages of LC-MS/MS, LC-ECD and IA methods for PMets. Adapted from [10].

	LC-MS/MS	LC-ECD	IA
Advantages	Minimal consumable costs.	Minimal consumable costs (in-house methods).	Minimal instrumentation costs.
		More moderate capital cost of instrumentation.	
	High sample throughput (facilitated by less stringent requirements for chromatographic separation).	Relatively simple operation and troubleshooting (expertise implications).	Easy to set up kit methods.
	Simplified sample preparation.	Some kit methods available with high consumable costs.	Minimal operator expertise required.
	High analytical sensitivity.	Moderate analytical sensitivity.	
	High analytical specificity with fewer interferences.	Chromatographic interferences can be identified.	
	Precise measurement of 3MT.	3MT measurement possible.	
	High versatility of LC-MS/MS instruments.		
	Kit methods and commercial calibrators / quality control materials available. Some of these are on-line.		
Disadvantages	High capital cost of instrumentation.	Labour-intensive sample preparation.	High costs of consumables for kit methods.
	Operator expertise required.	Susceptible to analytical interferences.	Lengthy sample preparation / analysis times.
	Some laboratories need to develop in-house methods.	Low sample throughput (long sample run times).	Requires separate measurement of each metabolite.
	High cost of kit methods.		Interferences difficult to identify.
			Poor accuracy with negative bias and potential for false negatives.
			Poor analytical sensitivity.
			3-MT measurement not possible (with implications for dopamine producing tumours, hereditary and metastatic disease).

Abbreviations: IA - immunoassay; LC-ECD - liquid chromatography with electrochemical detection; LC- MS/MS - liquid chromatography tandem mass spectrometry; PMets - plasma free metadrenalines; 3MT- 3-methoxytyramine.

HSS T3 [28] and C18 [23]. Other analytical columns include LUNA Cyano which exploit dipole interactions [29] and pentafluorophenyl (PFP) which can utilise dipole, π-π, and shape interactions facilitated by the halogenated stationary phase [21].

2.3. Detection

Positive ionisation mode is used for PMet analysis since these polar compounds exist as positively charged amines in solution. The majority of methods [19–28,30] utilise ESI for optimal signal strength [10] with some examples of atmospheric pressure chemical ionisation (APCI) [29]. NMA, MA, and 3MT (molecular masses 183, 197, and 167, respectively) are protonated in positive ion mode to give molecular ions with *m/z* 184 (NMA), 198 (MA) and 168 (3MT). As illustrated in Fig. 2, the protonated molecular ions for NMA and MA undergo spontaneous loss of water to yield precursor ion fragments with *m/z* 166 (NMA) and 180 (MA). Characteristic product ions are formed by collision-induced dissociation with further loss of a protonated methoxy group in the collision cell to fragments of *m/z* 134 (NMA) and 148 (MA). These transitions are more specific than water loss transitions, and the use of multiple reaction monitoring (MRM) pairs increases specificity [29]. In MRM mode, the majority of methods use 166 → 134 (NMA) and 180 → 148 (MA) transitions for quantification [19–22,24–27,29,30]. As shown in Fig. 3, facile loss of ammonia from the protonated molecular ion of 3MT yields a precursor ion of *m/z* 151 [10,20,28], with a characteristic product ion of 119. Thus, the majority of methods use the 151 → 119 transition for quantification of 3MT [19,25,28]. This transition should be investigated for ionic cross talk [32]. Qualifier ions are used for enhanced specificity in detection. Qualifier transitions for NMA included 166 → 106 [24,28] and 166 → 121 [22]. Qualifier transitions for MA included 180 → 165 [24], 180 → 148 [28] and 180 → 120 [22], with some methods using the 180 → 165 transition for quantification [23,28]. Qualifier transitions for 3MT were 151 → 91 [19,28], with some methods using this as the transition for quantification [20].

2.4. Standardisation

Cerilliant certified reference material in solvent is available for NMA and MA (Catecholamine Mix 2) and 3MT. However, higher-order reference material in human plasma or reference measurement methods are not available for PMets [33]. These would be required for standardisation and full comparability between methods. However, commercial suppliers have started to produce PMet kits. One is the Recipe ClinMass® Complete Kit for Free Metanephrines in Plasma (Recipe Chemicals + Instruments GmbH, Munich, Germany; MS11000, March 2019). This meets the regulatory requirements to be certified a Conformité Européenne In Vitro Diagnostic (CE-IVD) medical device. It comprises simple sample preparation (protein precipitation), on-line SPE and isotope-labelled internal standardisation. An alternative Recipe ClinMass® complete kit with manual sample preparation for standard LC-MS/MS systems is also available (MS11100, March 2019). NMA, MA and 3MT exhibit similar retention times in this method which has potential interference implications (as discussed in Section 3.2). Chromsystems also have multiple options for MassChrom® Free Metanephrines in Plasma. One uses manual sample preparation on sample clean up columns (Chromsystems Instruments & Chemicals GmbH, Munich, Germany; 81000/C, March 2019) and another uses 96 SPE Well Plates (81000, March 2019) with either manual or automated sample preparation. Centrifugation is used to pass solvents through the SPE. These are CE-IVD validated kits which promise straightforward sample preparation, isotopically labelled internal standardisation and importantly from an interference perspective, chromatographic resolution is achieved for all PMets. Cost is one of the disadvantages of these kits but advantages include traceability and decreased staff requirements for verification and sample preparation. Moreover, there are two commercial sources of calibrators (Chromsystems 6PLUS1® Multilevel Plasma Calibrator Set Free Metanephrines, 81039; Recipe ClinCal® lyophilised Serum Calibrator Set for Metanephrines (Level 0–4), MS11013; both March 2019) and quality control materials (Chromsystems MassCheck® Free Metanephrines Plasma Controls, 0384-0386; Recipe ClinChek® lyophilised Serum Controls for Metanephrine, MS11083; both March 2019). This decreases reliance on in-house

Table 3
Summary of key analytical and performance characteristics of in-house LC-MS/MS methods for measurement of PMets.

Sample volume (μL)	Extraction	On-line	LC Column	Guard column	Mobile Phases	Instrumentation	Run time (mins)	Linearity (pmol/L)	LLMI (pmol/L)	Accuracy (%)	Precision (%)	Diagnostic Performance (%)	Reference interval (pmol/L)	Reference
1000	Oasis HLB cartridges (Waters)	No	LUNA Cyano 4.6 × 150 mm, 5 μm (Phenomenex)	Yes	Isocratic: ACN/water (40:60 by volume) containing 1.5 mM ammonium acetate and 0.6 g/L FA	API 3000 Atmospheric pressure chemical ionisation source (Sciex)	6	10,000 (NMA, MA)	200 (NMA, MA)	U	Intra: ≤ 11 Inter: ≤ 13	U	120 to 1100 pmol/L (NMA) 50 to 470 (MA) No sex differences. NMA increased with age.	[29]
500	Oasis WCX cartridges (Waters)	Yes	Atlantis HILIC Silica 2.1 × 50 mm, 3 μm (Waters)	No	A: 100 mM AmF in water adjusted to pH 3.0 with FA B: ACN	Quattro Premier with Z Spray ion source (Waters)	8	18,210 (NMA, MA) 19,930 (3MT)	50 (NMA) 30 (MA) 60 (3MT)	U	Intra: ≤ 8.0 Inter: ≤ 11.1	U	230 to 1070 (NMA) 70 to 330 (MA) < 170 (3MT)	[19]
200	Isopropanol protein precipitation	No	Atlantis HILIC Silica 2.1 × 30 mm, 3 μm (Waters)	No	A: 95% ACN, 3% methanol, 2 mM AmF in water (pH 3) B: 80% ACN, 20 mM AmF in water (pH 3)	Quattro Micro (Waters)	4.6	20,000 (NMA, MA)	170 (NMA) 90 (MA)	U	Intra ≤7.0 Inter ≤17.3	U	U	[30]
100	Oasis WCX μElution (Waters)	No	Atlantis HILIC HPLC 2.1 × 50 mm, 3 μm (Waters)	No	A: 100 mM AmF in water (adjusted to pH 3.0 with FA) B: ACN	Quattro Premier (Waters)	3.5	23,000 (NMA, MA, 3MT)	50 (NMA) 40 (MA) 60 (3MT)	88 to 99	Intra: ≤ 11.5 Inter: ≤12.9	Sen:100 Spec: 96	120 to 1180 (NMA) 80 to 510 (MA) < 180 (3MT)	[20]
500	MCX cartridges (Waters)	No	Atlantis 2.1 × 100 mm, 3 μm (Waters)	No	A: 20 mM AmF in water containing 0.2% FA (pH 3.2) B: ACN	Quattro Premier XE (Waters)	10	U	100 (NMA) 60 (MA)	U	Intra ≤11 Inter ≤7.5	U	U	[26]
500	Oasis WCX cartridges (Waters)	No	Ultra PFP Propyl 2.1 × 100 mm, 3 μm (Restek)	Yes	A: 1 mM AmF in water with 0.1% FA B: 1 mM AmF in methanol with 0.1% FA	QTRAP 5500 (AB Sciex)	5.75	22,200 (NMA) 8200 (MA)	10.80 (NMA) 30 (MA)	81 to 107	Intra and total: ≤ 6.8	U	U	[21]
500	TurboFlow MCX-2 0.5 × 50 mm (Thermo Scientific) Perfluoroheptanoic acid for ion-pairing	Yes	Hypercarb PGC 3 × 50 mm, 5 μm (Thermo Scientific)	No	Assorted (see reference for details)	TSQ Vantage (Thermo Scientific)	12	4345 (NMA)* 1949 (MA)*	57 (NMA)* 27 (MA)*	80.6 to 101.7	Intra: ≤ 10.0 Inter: ≤ 8.9	U	U	[27]
200	Oasis WCX μElution (Waters)	No	Atlantis HILIC Silica 2.1 × 50 mm, 3.0 μm (Waters)	Yes	A: ACN B: 100 mM AmF (pH 3.0).	Xevo TQS (Waters)	3.5 to 4	100,000	100 (NMA, MA)	102.8 to 104.0	Intra: ≤ 11.5 Inter: ≤ 9.7 Total: ≤ 15.1	U	0 to 890 (NMA) 0 to 490 (MA)	[22]
900	Oasis MCX (Waters)	No	Acquity UPLC HSS T3 2.1 × 100 mm, 1.8 μm (Waters)	Yes	A: 0.2% FA in water B: 0.2% FA in ACN	QTRAP 5500 (AB Sciex)	4	55,000 (NMA) 56,000 (MA) 63,000 (3MT)	24 (NMA) 20 (MA) 24 (3MT)	97.5 to 104	Intra: ≤ 13.5 Inter: ≤ 11.7	Sen: 100	170 to 730 (NMA) 80 to 310 (MA) 40 to 130 (3MT) Age-related differences for NMA and 3MT	[28]
150	Oasis WCX cartridge (Waters)	Yes	Atlantis HILIC Silica 2.1 × 50 mm, 3 μm (Waters)	Yes	A: 100 mM AmF in water (adjusted to pH 3.2 with FA) B: ACN	Xevo TQS (Waters)	7.15	32,300 (NMA) 30,000 (MA, 3MT)	75 (NMA, 3MT) 37.5 (MA)	80 to 109	Intra: ≤ 9.5 Inter: ≤ 9.3	Sen: 94.4 Spec: 96.5	U	[25]
500		No		No	A: Water containing 0.1%	Agilent 6490 (Agilent)	5	100,000 (NMA)	80 (NMA) 40 (MA)	92.5 to 102.0	Intra: ≤ 3.55 Inter: ≤ 7.50	Sen:100 Spec: 97.7	U	[23]

(*continued on next page*)

Table 3 (*continued*)

Sample volume (μL)	Extraction	On-line	LC Column	Guard column	Mobile Phases	Instrumentation	Run time (mins)	Linearity (pmol/L)	LLMI (pmol/L)	Accuracy (%)	Precision (%)	Diagnostic Performance (%)	Reference interval (pmol/L)	Reference
	Strata CW-X cartridges (Phenomenex)		Unison UK C18 2.0 × 100 mm, 3.0 μm (Imtackt)		FA B: ACN containing 0.1% FA			50,000 (MA)						
100	Oasis WCX μElution (Waters)	No	Atlantis HILIC Silica 50 × 2.1-mm, 3 μm (Waters)	No	A: 25 mM AmF (pH 3.0) B: ACN	API QTRAP 5500 (AB Sciex)	7	9100 (NMA) 4600 (MA)	100 to 200 (NMA) 50 to 160 (MA)	U	Inter: ≤ 9.9	U	< 900 (NMA) < 400 (MA) (Data not shown)	[24]

Abbreviations: ACN - acetonitrile; AmF - ammonium formate; FA - formic acid; HILIC - hydrophilic interaction liquid chromatography; HLB - hydrophilic–lipophilic balance; HPLC - high performance liquid chromatography; LC - liquid chromatography; LC- MS/MS - liquid chromatography tandem mass spectrometry; LLMI - lower limit of measuring interval; MA - metadrenaline; MCX - mixed mode cation exchange; NMA - normetadrenaline; PFP - pentafluorophenyl; PGC - porous graphite column; PMets - plasma free metadrenalines; Sen - sensitivity; Spec - specificity; U - unknown; WCX – weak cation exchange; 3-MT - 3-methoxytyramine.
* Indicates that concentrations were calculated in pmol/L assuming the monoisotopic mass of NMA, MA and 3MT were 219.67, 233.69 and 203.67, respectively.

material and in combination with inter-laboratory quality assurance programmes facilitates inter-laboratory harmonisation [10,34]. This is important due to difficulties of making in-house matched calibrators owing to the ubiquitous presence of metadrenalines in spiked plasma.

3. Analytical considerations for interpreting results

3.1. Reference intervals

False-positive (FP) results are a significant issue for PMet testing, and sampling conditions like sampling from a seated position, medication and dietary factors are a notable contribution to these [35]. Clinical practice guidelines advocate collecting PMet samples after 30 min of supine rest and establishing reference intervals under the same conditions to avoid FPs in samples from a seated position interpreted against reference intervals from a supine position (lower diagnostic specificity) as well as the FNs associated with samples from a seated position interpreted against reference intervals from a seated position (lower diagnostic sensitivity) [1]. Upright posture is a powerful stimulus for the sympathetic nervous system activation and, thus, increases plasma noradrenaline and NMA concentrations [1,10]. Consequently, seated reference intervals are higher than the equivalent supine reference intervals [1]. Significant posture-associated changes are not observed in patients with PPGL [1]. Sampling from the seated position decreases diagnostic accuracy of LC-MS/MS methods, negates the benefits compared with urinary fractionated metadrenalines and may necessitate alternative urine testing [10]. When samples are collected from a seated position for ease, repeat sampling from a supine position is recommended to exclude FP results [1]. There benefits of supine over seated PMet sampling on diagnostic accuracy have been demonstrated in more recent studies [4,35–39]. Other studies have concluded acceptable diagnostic performance for PMets collected from a seated position interpreted against reference intervals from a seated position [40] or against supine, fasted reference intervals [41]. Although not extensively studied, diurnal variability does not appear to be a significant factor, thereby allowing sampling at any time of the day [42].

Owing to the clinical implications of a missed diagnosis, the ULRI for PMets must be optimised for diagnostic sensitivity and negative predictive value. Laboratories should also establish or verify their own reference values before routine use [43]. Negative results reliably exclude PPGL in the majority of cases, but all positive results require follow-up. Follow-up should be guided by the clinical presentation, pre-test probability, as well as the magnitude and pattern of the elevation above the ULRI [1]. FPs are less likely if NMA and MA are elevated, and results are highly suggestive of PPGL if either NMA or MA are elevated at least 3-fold relative to the ULRI [1]. ULRI thresholds of 2-fold [43] and 3.5- to 4-fold [44] have also been used in other publications. This Bayesian approach differs from the conventional binary interpretation against clinical chemistry reference intervals. Further assurance can be provided by demonstrating similar metadrenaline patterns in paired plasma and urine samples [5]. In these cases imaging must be used to locate the tumour. Plasma NMA and MA can also help to predict tumour size (strong positive relationship with tumour diameter) and location (higher concentrations of MA to NMA in adrenal tumours) [44]. Increased 3MT has also been associated with dopamine-secreting extra-adrenal tumours [14].

Borderline results (1–3 x ULRI) are more difficult to interpret in patient populations with a low pre-test prevalence, particularly owing to sub-optimal diagnostic specificity [1,5]. These results are not always followed-up appropriately and carefully selected interpretive comments may be helpful [45]. In these cases, pre-analytical factors that contribute to FP results should be considered since FP cases exceed true-positive cases [46]. It may be useful for confirmatory testing after exclusion of sampling position, pharmacological, dietary, and other causes of FPs.

When plasma NMA is elevated and clinical suspicion is moderate/

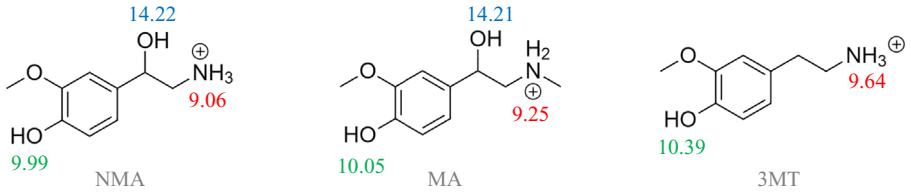

Fig. 1. Structure of NMA, MA and 3MT at pH 3.0, including all pKa values. Adapted from [79]. *Abbreviations:* MA - metadrenaline; NMA - normetadrenaline; 3MT - 3-methoxytyramine.

high, the clonidine suppression test measuring plasma NMA can help to distinguish true-positive results from FPs [1,43]. Others studies suggest follow-up of positive PMet results by measurement of chromogranin and urinary fractionated metadrenalines [47]. Where there is a low clinical probability of PPGL and borderline positive results, biochemical monitoring after at least 6 months for sustained or more marked elevations in PMets may suggest increased likelihood of a small/enlarging tumour [1].

Plasma NMA concentrations significantly increased with age, whereas weaker positive relationships were observed for plasma MA and 3MT. In the absence of age-specific reference intervals FP NMA results were significantly greater in patients over 60 years old compared with patients under 45 years old. Age was found to be an important contributor to FP PMet results and diagnostic test performance was improved through use of age-specific reference intervals for NMA and optimised slightly above 99.5 percentiles for MA and 3MT [48]. The applicability of these reference intervals to different LC-MS/MS methods in other laboratories can, in part, be determined from bias observed in inter-laboratory quality assurance programs [7]. Combined with a common certified reference material harmonisation of reference intervals may then be possible [48]. Plasma MA and 3MT concentrations were significantly higher in males than in females but sex-specific reference intervals are not required in plasma [48]. This corroborates earlier studies that advocated age-adjusted reference intervals for plasma NMA [49].

3.2. LC-MS/MS interference

Sample purification and chromatographic separation are important for selectivity and specificity, even with LC-MS/MS methods for PMets [10]. Ion suppression or enhancement, ionic cross talk, in source transformation and isobaric interferences can all cause analytical interference in LC-MS/MS measurement of PMets [10].

When alternative matrices are used (e.g. for in-house calibrators) differential ion suppression / enhancement between the analyte and its deuterated internal standard should be investigated to ensure quantification is not affected [50]. Ion suppression of the analyte and/or internal standard may occur but have negligible impact on quantification e.g. cimetidine, ephedrine, labetalol, and pseudoephedrine [22].

Product ion cross talk is an important cause of 3MT over-estimation that occurs when two different precursor ions give rise to a product ion with the same *m/z* that are not chromatographically resolved. 3-O-Methyldopa is endogenously produced by catechol-*O*-methyltransferase action on the dopamine precursor L-dopa and is present at much higher concentrations than 3MT. Interference in 3MT measurement is particularly significant in patients with higher 3-O-Methyldopa concentrations e.g. renal insufficiency or L-dopa treatment for Parkinson's disease. It is attributable to in-source decarboxylation of 3-O-Methyldopa (relative molecular mass 211.1) to produce a co-eluting fragment with *m/z* 151.1 i.e. the same as the precursor ion for 3MT [51] (Fig. 4). Another example is ionic cross talk between MA and 3MT and is caused by in-source fragmentation of MA to produce ions mimicking 3MT, possibly by loss of methyl or hydroxyl groups to form 3MT precursor ions. The higher concentrations of MA relative to 3MT results in 3MT over-estimation in any method where NMA, MA and 3MT are not chromatographically resolved e.g. HILIC methods [32]. Interference of both 3-O-Methyldopa and MA in 3MT measurement can be eliminated by chromatographic separation [32,51].

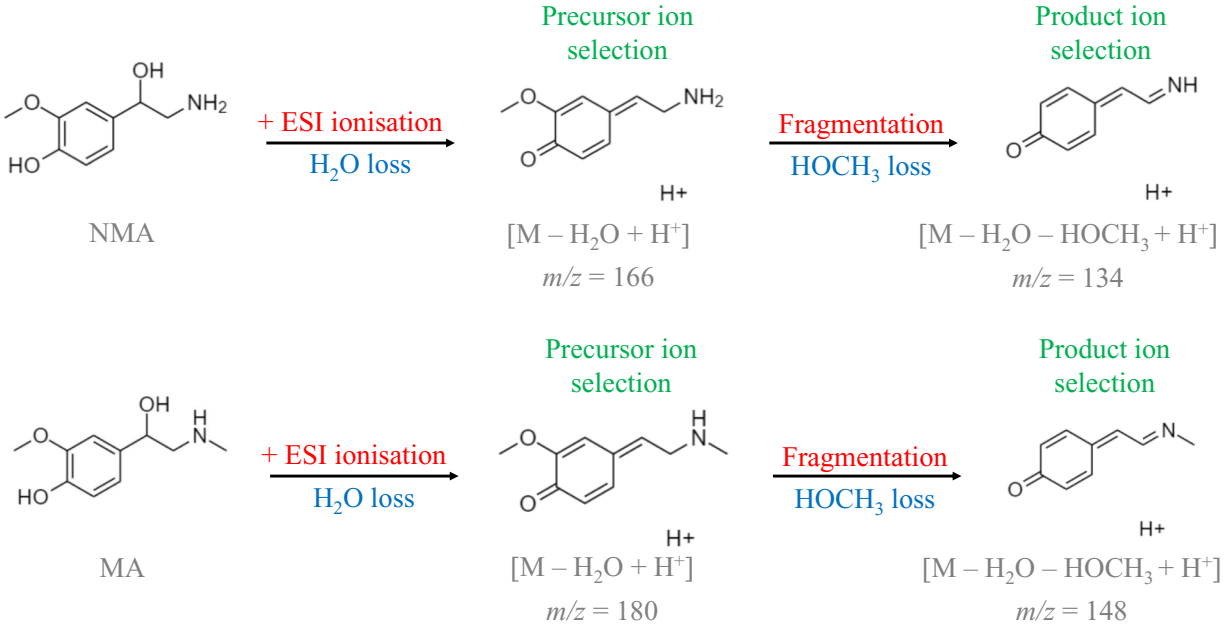

Fig. 2. Characteristic ionisation and fragmentation of NMA and MA used for quantification in LC-MS/MS methods for PMets. Adapted from [10]. *Abbreviations:* ESI - electrospray ionisation; LC- MS/MS - liquid chromatography tandem mass spectrometry; MA - metadrenaline; NMA - normetadrenaline; PMets - plasma free metadrenalines.

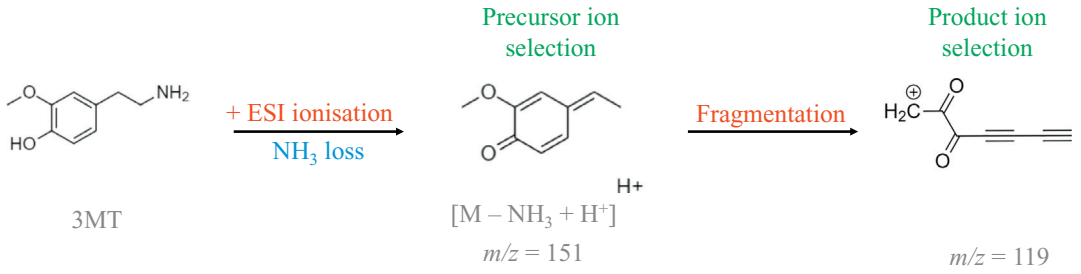

Fig. 3. Characteristic ionisation and fragmentation of 3MT used for quantification in LC-MS/MS methods for PMets. Adapted from [10]. 3MT product ions inferred from [80]. *Abbreviations:* ESI - electrospray ionisation; LC- MS/MS - liquid chromatography tandem mass spectrometry; PMets - plasma free metadrenalines; 3MT - 3-methoxytyramine.

Adrenaline and NMA are isomers and, thus, are subject to isobaric interference in LC-MS/MS methods unless chromatographic separation is achieved [10]. Owing to different fragmentation mechanisms, different product ions are observed for adrenaline (*m/z* 107) and NMA (*m/z* 134) which can help distinguish between them [10,29].

For enhanced analytical selectivity for PMets, one study advocates using multistage fragmentation (MRM^3), which can be achieved on quadrupole-ion trap tandem mass spectrometers. Collision-induced dissociation produces primary fragment ions which are isolated and fragmented further to produce secondary fragment ions for quantification. MRM^3 for NMA and MA on a HILIC column allowed for removal of co-eluting interferences present in MRM which prevented quantitation by MRM. In MRM mode the transitions used were *m/z* 166 → 134 (NMA quantifier), 166 → 106 (NMA qualifier), 169 → 137 (D3-NMA), 180 → 148 (MA quantifier), 180 → 165 (MA qualifier) and 183 → 151 (D3-MA). MRM^3 transitions were *m/z* 166 → 134 → 79 (NMA quantifier), 166 → 134 → 116 (NMA qualifier), 180 → 149 → 121 (MA quantifier) and 180 → 149 → 107 (MA qualifier). The MRM^3 method was less sensitive and more imprecise than MRM but still deemed sufficient for clinical use [24]. Quantifier:qualifier ratios allow co-chromatographing interferences to be identified. However, MRM^3 cannot remove interference from in-source transformation yielding identical products. Chromatographic resolution is required to remove this interference [10,51].

3.3. Drugs

As summarised in Table 4, there are numerous drugs that can cause FP PMet results by LC-MS/MS, both by pharmaco-physiological or analytical mechanisms. As such, medication-induced FP results should be excluded when positive PMet results are obtained e.g. by modifying interfering medication and re-testing [5,52]. However, LC-MS/MS is less susceptible to analytical interference than high pressure liquid chromatography with electrochemical detection [43].

Phenoxybenzamine is a nonspecific α-adrenoceptor blocker used in phaeochromocytoma treatment. Mechanisms for increasing NMA include decreased α_2-adrenoceptor-mediated feedback inhibition of noradrenaline release, potentially with sympathetic activation in reflexive response. This is a significant cause of FP NMA results and should be avoided during biochemical testing if possible [52].

Tricyclic and other antidepressants can block sympathoneuronal uptake of noradrenaline, thereby causing a significant number of FP NMA results [52]. Other mechanisms by which drugs cause FP NMA results include altered clearance mechanisms or increased noradrenaline release by sympathetic nerves [10].

By blocking the metabolism of all *O*-methylated metabolites, monoamine oxidase inhibitors vastly increase metadrenalines and should, therefore, be withdrawn before PMet testing. For most drugs, withdrawal is only required when positive results are encountered. [10].

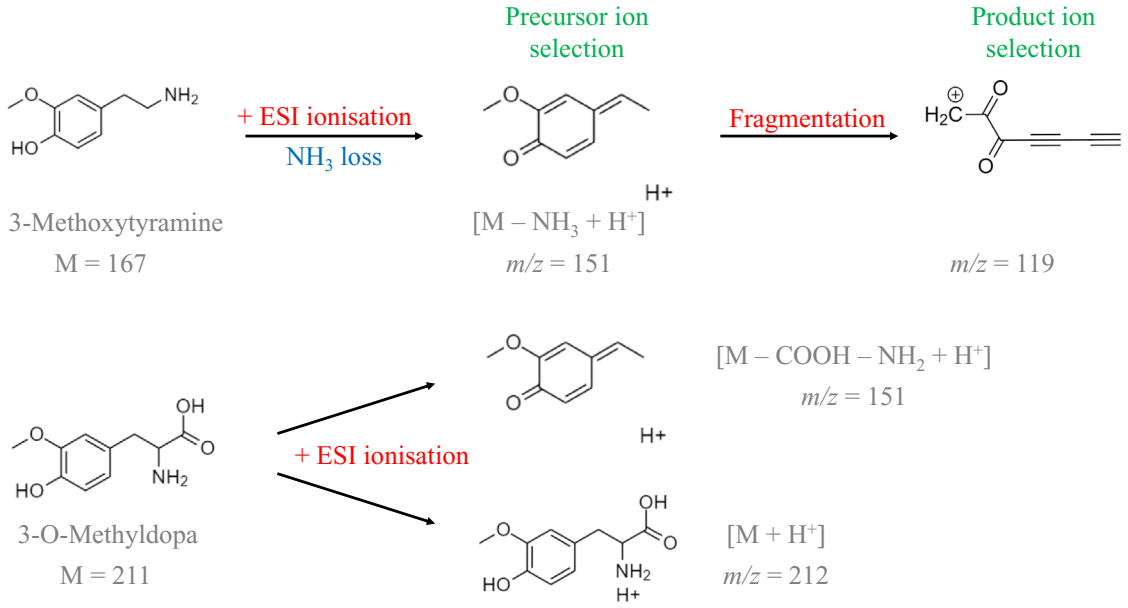

Fig. 4. Ionisation and fragmentation to explain the product ion cross talk which results in over-estimation of 3MT concentration by 3-O-Methyldopa interference when these are not chromatographically resolved. This is particularly significant in patients with renal insufficiency or those on L-dopa treatment for Parkinson's disease. Adapted from [10].

Table 4
Summary of drugs that can cause pharmaco-physiological or analytical interference in LC-MS/MS methods for PMets i.e. false positives. Adapted from [5].

Interferent	Cause of increase	NMA	MA	3MT	References
Phenoxybenzamine	Pharmaco-physiological	Y			[1,52]
Tricyclic (and other) antidepressants e.g. venlafaxine	Pharmaco-physiological	Y			[1,52,81]
Monoamine oxidase (MAO)-inhibitors	Pharmaco-physiological	Y	Y	Y	[1,10]
Sympathomimetics e.g. ephedrine, amphetamine	Pharmaco-physiological	Y	Y		[1,81]
Cocaine	Pharmaco-physiological	Y	Y		[1]
Midodrine	Pharmaco-physiological		Y		[1,53]
β-Adrenoceptor blockers	Pharmaco-physiological		Y		[52]
L-DOPA/Levodopa	Pharmaco-physiological			Y	[54]
L-DOPA/Levodopa	Analytical			Y	[51]
Isoetharine	Analytical		Y		[22]
MDA	Analytical		Y		[22]
Isoproterenol	Analytical	Y			[22]
MDMA	Analytical	Y			[22]
HMMA	Analytical	Y			[55]

Note: Figures to appear in colour in online version only.
Abbreviations: HMMA - 4-Hydroxy-3-methoxymethamphetamine; LC- MS/MS - liquid chromatography tandem mass spectrometry; MA – metadrenaline; MDA - 3,4-methylenedioxyamphetamine; MDMA - 3,4-methylenedioxymethamphetamine; NMA – normetadrenaline; PMets - plasma free metadrenalines; 3MT – 3-methoxytyramine.

The α-adrenoceptor agonist Midodrine is a pro-drug to desglymidodrine and used to treat orthostatic hypotension. It is hypothysesised that if either midodrine or desglymidodrine co-elute with MA they undergo in-source transformation to interfere in the MA transition [53].

β-Adrenoceptor blockers (e.g. atenolol, metoprolol, propranolol) as well as the α- / β-blocker labetolol may cause FP plasma MA but with a low frequency [52].

In Parkinson's disease, L-dopa / Levodopa is used as a prodrug for dopamine which has potential for pharmaco-physiological and analytical interference in PMet measurement. It can be metabolised to dopamine and thereby its *O*-methylated metabolite 3MT [54]. Moreover, as previously discussed it can be metabolised to 3-O-Methyldopa with potential for 3MT analytical interference [51]. Methyldopa may co-elute with NMA in some methods [21].

Isoetharine and 3,4-methylenedioxyamphetamine (MDA) have been shown to positively interfere with MA quantification whereas isoproterenol and 3,4-methylenedioxymethamphetamine (MDMA) positively interfere with NMA quantification [22]. Isoetharine and isoproterenol are betaadrenergic agonists whereas both MDA and MDMA are recreational drugs. In all cases, structural similarity between the interferant and metadrenalines are likely to cause interference by in-source transformation or ionic cross talk [10].

4-Hydroxy-3-methoxymethamphetamine (HMMA), a metabolite of MDMA, has also been shown to interfere in NMA measurement. It is thought that in-source fragmentation (water loss from NMA and double demethylation of HMMA) give the same precursor ion (*m/z* 166) and product ion (*m/z* 134). Higher circulating levels of HMMA results in over-estimation of NMA unless chromatographic separation is employed [55].

3.4. Diet

Overnight fasting is recommended prior to PMet sampling when 3MT is being measured [1,56]. This is because many foods contain L-dopa, dopamine and other biogenic amines which can falsely increase plasma 3MT concentrations. Catecholamine-rich foods include fruit (e.g. bananas, pineapples), nuts (e.g. walnuts), potatoes, tomatoes, beans, etc. [56].

Caffeine [57,58] and nicotine can increase plasma catecholamines and, thus, some sources recommend avoiding them prior to testing [59]. Coffee contains the catechol caffeic acid. Caffeic acid and its derivative dihydrocaffeic acid can interfere in plasma catecholamine assays [58]. Moreover, these catechols can act as substrates for catechol *O*-methyltransferase, thereby providing a mechanism for catecholamine conversion to metadrenalines [59]. Coffee may also stimulate adrenomedullary catecholamine release [57]. However, this does not appear to have been studied using LC-MS/MS assays and one study using radioimmunoassay showed that although NMA concentration was increased significantly the changes were too small to confound interpretation [60].

3.5. Renal function

PMets are less affected by impaired renal elimination that urine metadrenalines [5]. The relatively mild effect of renal function makes PMets more suitable for the diagnosis of pheochromocytoma in patients with renal dysfunction [61], but renal function should still be considered when interpreting PMet results, particularly in patients on dialysis. Studies have shown that plasma NMA and MA concentrations are higher and increased above the ULRI in some patients with renal insufficiency [61,62]. Activation of the sympathetic nervous system (and, thus, increased plasma concentrations of catecholamines and their metabolites) or decreased renal clearance of circulating *O*-methylated metabolites are potential mechanisms for this increase [61,63,64]. Activation of the sympathetic nervous system is thought to be the most important factor since there is no strong inverse relationship between PMets and creatinine clearance [61] and circulatory clearance of PMets is relatively independent of renal function [61,65]. Some of these studies are based on electrochemical detection methods and chromatographic interference from matrix factors may be contributory, particularly in patients on dialysis [61].

Although renal dysfunction can lead to FP PMet results, the magnitude of the elevations (up to 2-fold) are not usually in a range more strongly suggestive of phaeochromocytoma [61]. This was not always the case for MA in a study based on enzyme-linked immunosorbent assays [62].

More recent studies based on LC-MS/MS methods have aimed at deriving CKD-specific reference intervals for NMA, MA and 3MT to account for the progressively higher PMet concentrations with more advanced CKD [66]. This could not be achieved for 3MT due to variability within the data set. CKD-stage specific reference intervals were more important for NMA, whereas similar adjustments could be made for all CKD stages for MA [66]. This strategy may help to decrease FP results but their applicability to different sites with different methods would need investigation.

3.6. Other factors

Other factors have been shown to affect PMet concentrations and, thus, warrant consideration when interpreting biochemical test results.

Other sympathetic or adrenal medullary stimuli can increase PMet concentrations. Physiological stress, as seen in severe illness in intensive care, can cause marked elevations [46,67,68]. To exclude FPs, repeat testing is advocated after recovery from the illness [1]. Imaging is required in an intensive care setting since the life-threatening, stressful conditions make biochemical screening unreliable [43]. Other co-morbidities causing PMet elevation include panic attacks and acute anxiety [69–72], severe pain (e.g. during cardiac ischemia or hypoglycaemia) [43], heart failure [68,73], and obstructive sleep apnea [74].

Two studies have found seasonal variation in PMets in patients without PPGL, with higher NMA concentrations in the winter than summer, with an associated increase in FP results in the winter [75,76]. Temperature and vasoconstriction/vasodilation effects on the sympathetic nervous system were thought to contribute [75]. Notably, at least one of these studies was based on an LC-ECD method [75]. Other studies have failed to find significant seasonal variation in more temperate climates [77].

Some reports advocate adjusted reference intervals following unilateral and bilateral adrenalectomy owing to lower MA and higher NMA concentrations in these patients [78].

4. Conclusion

Evidence is accumulating which may result in PMet becoming the preferred test for diagnosing PPGL. Moreover, increased availability and benefits over other analytical methods like LC-ECD and IA are causing LC-MS/MS to become the method of choice for PMet measurement. This review explores the evidence-base supporting this, and summarises published LC-MS/MS analytical methods for PMet analysis. Key aspects of methods (including SPE extraction, HILIC chromatography, MRM MS-detection and standardisation) are discussed. Common causes of analytical interference (e.g. ion suppression/enhancement, ionic cross talk, in source transformation and isobaric interferences) are outlined to illustrate the importance of sample purification and chromatographic resolution. The importance of supine, fasting sampling and Bayesian interpretation against supine, fasting reference intervals are explained, as well as the importance of age-specific reference intervals for NMA. Confounding factors like diet, drugs, renal function and acute illness are explored, along with potential strategies to address these (e.g. CKD-specific reference intervals).

Declarations of interest

None.

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