Advances in Catecholamine and Metabolite Measurements for Diagnosis of Pheochromocytoma

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Assessment of catecholamine production and excretion is important in the laboratory detection of pheochromocytoma, a rare but curable cause of hypertension. Advances in catecholamine and metabolite methodologies have enhanced the diagnostic acumen by increasing analytical sensitivity and eliminating many of the interferences observed with earlier methods. Estimation of urinary catecholamines, metanephrine and vanillylmandelic acid is routinely used in the biochemical detection of pheochromocytoma and in monitoring the completeness of tumor excision as well as the possibility of recurrence. Traditional spectrophotometric and fluorometric methods for urinary catecholamines and their metabolites are being replaced by highly sensitive and selective chromatographic methods. The ability to quantify individual catecholamines and metanephrines by high-performance liquid chromatography is of particular value for detecting familial forms of the tumor that may secrete epinephrine. Plasma norepinephrine and epinephrine measurements are of additional diagnostic value in determining recent catecholamine release and response to clonidine suppression. For either urine or plasma measurements, appropriate patient preparation, sample collection, and method validation along with an understanding of the variable pattern of catecholamine secretion and metabolism in pheochromocytoma are essential. Advances in laboratory methodology and reference intervals for catecholamines for clinical interpretation are reviewed.

Pheochromocytoma is a rare but important cause of hypertension, and physicians investigating hypertension are advised to be guided by the dictum, "Think of it, confirm it, find it, and remove it" (1). The laboratory role in confirming the diagnosis is challenging because of the low incidence (0.1–0.5%) of pheochromocytoma in the hypertensive population and the tumor's protean clinical and biochemical manifestations (2–4). Although these tumors are capable of expressing and secreting several neurochemical and peptide products, overproduction of catecholamines is primarily responsible for the major clinical findings and forms the basis for biochemical detection and monitoring.

Dopamine (DA), norepinephrine (NE), and epinephrine (E) are the three naturally occurring catecholamines; their in vivo pathway of synthesis has been known since 1939 (5). Early estimates of catecholamine concentrations in biological fluid and tissue were performed by colorimetric and bioassays that lacked either sensitivity, specificity, or both (6, 7). Not until 1950 were adequate fluorometric methods for measuring NE and E in plasma and urine used in the detection and management of patients with pheochromocytoma (8, 9). Because unaltered catecholamines ordinarily represent <2% of the urinary excretion products (10), elucidation of the metabolic pathway (11–13) has led the way to additional diagnostic measurements for vanillylmandelic acid (VMA), total metanephrines (MN) and, to a more limited extent, homovanillic acid, methoxyhydroxyphenylglycol (MHPG), and other metabolic intermediates (14–17). Recent years have seen a significant increase in the understanding of catecholamine metabolism (10), advances due, in part, to a progressive change toward more sensitive and specific laboratory methods. Many of these newer methods are now widely applied in the clinical laboratory, and Figure 1 illustrates the increasing use of HPLC methods over the past 10 years. According to proficiency survey data obtained from the College of American Pathologists, the majority of participating laboratories currently use HPLC to measure catecholamines, and more than one-third of the laboratories use HPLC methods for metabolite testing. Given this change in clinical laboratory practice, it is appropriate to review the advances in methodology and their current interpretation. The reader is also referred to a recent review of the broader biomedical applications of catecholamine measurements (18) and a selective review of plasma catecholamine analysis (19).

**Plasma Catecholamines**

Current and traditional methods for catecholamine measurement are based on the oxidative, amphoteric, or chemical derivitization properties of the catecholamines. Although based on similar principles, plasma and urine methodologies involve different strategies to deal with sample matrix and catecholamine concentration differences. Highly sensitive, specific, and reliable methods are essential for measuring the low concentrations of NE, E, and DA in plasma. Because the catecholamines may oxidize spontaneously, anti-oxidants are often added as a plasma preservative, but stability studies (20–22) indicate that this practice may not be necessary. Catecholamines circulate in plasma as free or sulfon conjugated forms, the biological activity residing in the free fraction (23). Although both forms are increased in plasma from patients with pheochromocytoma, they are often used concurrently in a composite assay to improve diagnostic sensitivity. A cut-off value is used to determine the usefulness of this approach. Conventional HPLC or high performance anion exchange chromatography is widely used in the plasma measurement of catecholamines. The method is sensitive and specific for plasma catecholamines with detection limits under 2 pg/ml. Ratios of NE to E are also used as a determinant of possible tumor presence. Ratios of greater than 3:1 are suggestive of a tumor, whereas ratios of less than 0.5:1 are more suggestive of a non-tumor cause of hypertension.

**References**

1. Nonstandard abbreviations: GLC, gas-liquid chromatography; NE, norepinephrine; E, epinephrine; DA, dopamine; DOPA, 3,4-dihydroxyphenylalanine; NM, 3-methoxynorepinephrine (normetanephrine); VMA, 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid); MN, 3-methoxyepinephrine (metanephrine); and MHPG, methoxyhydroxyphenylglycol.

Received February 21, 1991; accepted April 4, 1991.
require diamine condensation are, etc., plasma and concentration undergoing plasma ganglionic influence. Methods Comparison metaboltfe FIg. NE 40 60 80 Ut, permission in in the more metabolic pathway studies of Axelrod (11). This indirect technique is based on enzymatic O-methylation of the catechol ring with a tritium-labeled methyl donor, S-adenosylmethionine. The most commonly used commercial kit application (Upjohn Diagnostics, Kalamazoo, MI) is based on the method of Peuler and Johnson (30). The reaction is catalyzed by catechol-O-methyltransferase isolated from the soluble fraction of liver homogenates. After extraction and back-extraction of the tritiated O-methylcatecholamines, the derivatives are resolved by thin-layer chromatography, eluted, and further purified, and the radioactivity of the label is counted. Advantages of the method include small sample volume (50 μL), no sample pretreatment, and the ability to simultaneously detect low amounts of NE (1 pg), E (1 pg), and DA (6 pg). Modifications of the method have incorporated plasma pretreatment and alternative organic extraction steps (31, 32). The procedure may also be abbreviated to measure just the total concentration of NE and E. Study of the kinetics of the enzymatic O-methylation has revealed that optimized reaction conditions as well as purity of the catechol-O-methyltransferase are critical for accurate measurement (33). Plasma inhibitors of the methylation reaction have been identified in uremic and hypertensive patients (34, 35), thus reinforcing the need for internal standardization techniques. Technical disadvantages of radioenzymatic methods include the complexity and length of the procedure as well as the higher intra- and interlaboratory variability as compared with chromatographic methods (36, 37).

GLC methods with flame ionization (38), electron capture (39), or mass fragmentographic (40–43) detection have been reported for plasma catecholamines. Detection of 0.1–2 pg with these methods often exceeds the limits for the most sensitive radioenzymatic methods. Plasma deproteinization and alumina adsorption are generally used for sample pretreatment, followed by derivatization with trifluoroacetic or pentafluoropropionic anhydride. Because of the potential for multiple derivatization products and limited routine availability of equipment, especially the more specific mass-fragmentography detector, GLC methods are not widely used in the routine clinical setting.

HPLC is the most promising technique for simultaneous quantification of the plasma catecholamines and has gained increasing use in the clinical laboratory. Several methods involving either electrochemical detection (37, 44–51) or fluorescent derivatization by pre- (52) or post-column (53–55) techniques have been developed and validated. Method modifications have focused primarily on extraction techniques and chromatographic conditions. To measure the low concentration of cate-
cholamines in plasma requires preanalytical extraction and concentration. The common alumina pretreatment involves a batch extraction technique with well-defined conditions and alumina preparation (56). Others have shown, however, that readily available alumina sources are usable and that the amount of alumina and the adsorption pH are not as critical as originally defined (49). Alumina columns have also been used to allow more automated extraction (57). Because alumina extraction of plasma-based standards may not result in a valid analysis, standardization with aqueous calibrators has been proposed (58). Additional problems with selectivity of alumina extraction methods have led to even lengthier two-stage extraction procedures with alumina and ion-exchange steps (47, 48). However, formation of a borate gel complex with catecholamines provides a simple alternative to alumina; more recent methods have validated this approach, demonstrating higher absolute recovery than with alumina (59, 60). For chromatographic separation of the extracted catecholamines, several column conditions and stationary phases have been tried. Except for a few procedures involving cation-exchange columns (44, 53), reversed-phase chromatography with ion-pairing reagent is used. To quantify the eluted catecholamines, electrochemical detection is widely used, and several commercial detectors are available for either amperometric or coulometric measurement. Clinical evaluation of reversed-phase HPLC methods with electrochemical detection have shown detection limits similar to those of the radioenzymatic methods, and the lower absolute values measured by HPLC point to a greater specificity (37, 53, 61). HPLC methods are not, however, without potential interferences. Dihydrocaffeic acid, for example, may co-elute with either NE or E, depending on the extraction and chromatographic conditions (62). Nonetheless, HPLC is a versatile technique and, unlike the radioenzymatic methods, its analytical (chromatographic) conditions can often be altered to identify and eliminate potential endogenous or exogenous interferences.

Clinical interpretation of plasma catecholamine concentrations determined by previously described methods is normally based on comparison with reference intervals determined with normotensive subjects. For standardization of physiological conditions, blood should be collected from a fasting subject who has been resting in the supine position for 20–30 min after insertion of a venous catheter. Figure 2 summarizes a literature review of adult reference intervals for NE and E in plasma from healthy normotensive subjects in the supine position. Although not shown in the Figure, plasma DA reference intervals are lower than the intervals for E. The mean concentration of plasma NE in these studies ranged from 165 to 311 ng/L with mean E concentrations ranging from 16 to 59 ng/L. Variability in the statistical upper limit (mean + 2 SD) for NE (250–620 ng/L) and E (38–129 ng/L) may be explained, in part, by the number of subjects, the difference in adherence to collection conditions, and normal circadian variations (70). The differences do not, however, appear to be related to the laboratory methodology used. Most clinical laboratories report plasma NE concentrations >500 ng/L and E concentrations >100 ng/L as above-normal in comparison with values from normotensive adults. Pediatric reference intervals for plasma catecholamines have been reported (71), but application to the diagnosis of pheochromocytoma in childhood is rare (72).

**Urinary Catecholamines**

Urinary catecholamines represent a quantitatively small but diagnostically important component of the excretion products. As with plasma measurements, the unconjugated fraction is least affected by diet (73); therefore, the methods do not include a hydrolysis step. Cells of the adrenergic system are a direct source of urinary unconjugated NE and E, whereas DA originates primarily from peripheral metabolism of 3,4-dihydroxyphenylalanine (DOPA) in the kidney (74–76). Unlike the case in plasma, DA is the major unconjugated catecholamine in urine, and its measurement, along with NE and E, is especially useful in the diagnosis of malignant forms of the tumor.

Numerous approaches have been reported for the purification of urine samples before quantifying the catecholamines. An alumina extraction procedure developed initially by Anton and Sayre (56) may offer sufficient purification when used alone (77), but is typically coupled with other techniques to ensure adequate removal of interfering compounds (78–80). Although generally used in batch form, alumina columns allow automation through on-line column-switching techniques (81). Catecholamines may be efficiently isolated by solvent extraction by using ion-pair formation with diphenylborate under alkaline conditions (82). Cation-exchange resins have been used extensively in sample pretreatment. Primarily, weak ion-exchange resins such as Bio-Rex 70 are used, although methods for
simultaneous analysis of catecholic amino acids require strong ion-exchangers (83). Borate can be used to selectively elute catecholamines from ion-exchange resins (84) rather than less-specific elution by pH adjustment. Finally, boric acid affinity gels provide for selective adsorption of catecholamines (78, 85, 86). Commercially prepared columns are available and make use of either immobilized boric acid (Affigel 601) or phenylboric acid (Amicon 30). The linkage of boric acid to the support matrix influences the adsorptive properties of the resin and may ultimately affect the selectivity of the extraction (87).

Traditional methods for the quantification of urinary E and NE are similar to the early plasma catecholamine methods, which relied on the production of detectable fluorophores. Although not sensitive enough for plasma, the trihydroxyindole method (88–90) is adequate for urine testing. Drugs such as α-methyldopa, isoprotenerol, quinidine, propranolol, labetalol, and tricyclic antidepressants, which also show fluorescent properties, may interfere. Modifying the original methods to include iodine as the oxidizing agent allows for the additional measurement of DA (85). Although they are still used in some clinical laboratories, fluorescent methods are being used less frequently in routine practice (Figure 1) and are being replaced by newer chromatographic methods.

Significant improvements in the specificity of catecholamine measurements have resulted from a coupling of HPLC separation and fluorescent detection. HPLC with detection of native fluorescence provides sufficient sensitivity (5 μg/L) after catecholamine isolation by cation-exchange (91, 92) and concentration with alumina (93). Many of the drug interferences in the traditional fluorescence procedure are eliminated by the selectivity of the chromatographic separation, but some compounds such as the α-methyldopa metabolite, α-methylnorepinephrine, may elute close to MN and NM, depending on the chromatographic conditions (91). Derivatizations may also be used to further enhance sensitivity and specificity. Post-column adaptations include ethylenediamine condensation (94), trihydroxyindole (95), glycyglycine (96), and 2-cyanoacetamide (97) methods. These methodologies, although reported to have picomole limits of detection, have not yet been extensively investigated for possible interferences. Pre-column methods include 1,2-diphenylethyleneamine treatment (98) or dansylation of E, NE, and DA (99) and derivatization of NE and DA by O-phthalaldehyde and mercaptoethanol (100). Nohta et al. (101) have combined electrochemical oxidation and fluorescence derivatization to co-analyze catecholamines, metanephrines, and other related compounds. This approach involves sequential chromatographic separation, coulometric oxidation, and final chemical derivatization with 1,2-diphenylethyleneamine to fluorescent products. These derivatization methods have not, however, gained widespread use in the clinical laboratory.

Electrochemical detection with ion-pairing adap-

indentions of reversed-phase chromatography are the most
common methodologies, and many adaptations for ur-
inary catecholamine measurement have been reported
(77–81, 87, 102–114). Ion-pairing with alkyl sulfonates
or sulfates is generally used to enhance retention of
 catecholamine moieties on lipophilic stationary phases.
Alteration of chromatographic retentions through mod-
ification of the ion-pairing agents, ionic strength, or
organic components has been previously reviewed (115).
The method of Moyer et al. (78), in which samples are
initially purified by alumina and boric acid gel, is
commonly used. With this method, each of the catechol-
amines and the 3,4-dihydroxybenzylamine internal
standard display similar recoveries (~60%), except in
urine samples with abnormally high amounts of gluco-
ose. Extensive review of possible drug interferences
shows only α-methyldopa to cause problems, by eluting
close to NE. Abstention from this medication is, there-
fore, required for accurate quantification of the catechol-
amines. Acetaminophen (116), labetalol, and captopril
(117) have since been reported as possible interferences
under similar conditions. Some have attempted to de-
velop chromatographic techniques that provide for the
coa-analysis of catecholamines and their metabolites.
After varying the sample preparation procedures, met-
anephrines and vanillylmandelic acid (77, 110) may be
coa-analyzed without instrument changeovers to offer
significant savings of analysis time. Chan and Siu (114)
have since reported that catecholamines and their
O-methylated metabolites may be simultaneously
quantified with a single sample injection after a one-
step sample preparation on Bio-Rex 70 cation-exchange
resin.

Ion-exchange chromatography coupled with electro-
chemical detection offers unique separatory and sensi-
tivity advantages over ion-pair reversed-phase applica-
tions. Early applications of ion-exchange were ham-
pered by deficiencies in column reproducibility, which
resulted in loss of catecholamine retention and separa-
tory properties (118); however, subsequent applications
suggest that ion-exchange may be superior to reversed-
phase chromatography when analyzing samples pre-
treated by an immobilized phenylboronic acid affinity-
column technique (80). When using cation-exchange
pretreatment, XAD-4 resin must also be used to remove
late-eluting metabolites of labetalol (119). Mefford (120)
recently reported that semi-irreversible loading of C18
columns with lauroyl sarcosine results in separations
that are dependent on ion-exchange mechanisms.
Subsequent work with N-methyl oleoyl taurate resulted in
chromatographic separations during which epinephrine
was eluted before NE, allowing enhanced sensitivity for
the normally lower concentration of E (121). Further
sensitivity for E was achieved by using a mobile phase
at neutral pH, which enhanced the overall electrochem-
ical responses by increasing the fraction of unproto-
nated amine and quinone formed. Compared with ion-
pairing techniques, these sensitivity enhancements of
the cation-exchange method imparted a threefold rela-
tive increase in the analytical signal for E vs NE. This enhanced sensitivity may be of particular value in detecting small tumors that preferentially secrete E.

Reference intervals for 24-h urinary excretion of unconjugated catecholamines have been studied in normotensive adults. Figure 3 summarizes NE and E excretion in studies involving either the traditional fluorometric method or HPLC. Mean excretion rates for NE (31–59 μg/24 h) and E (7–14 μg/24 h) were reported in these studies, and the statistical upper limit (mean + 2 SD) varied for both NE (41–135 μg/24 h) and E (9–37 μg/24 h). Several of these studies also included DA data, with means ranging from 120 to 278 μg/24 h and upper statistical limits ranging from 308 to 681 μg/24 h. Other studies of normotensive adults have reported the range of concentration and point out the non-Gaussian distribution of catecholamine excretion data. The upper limit of the range, based on the 95th percentile by Moyer et al. (78), was NE, 80 μg/24 h; E, 20 μg/24 h; and DA, 400 μg/24 h. Diagnostic testing based on overnight rather than on 24-h urine collection has been suggested as a sensitive diagnostic index of autonomous catecholamine secretion by a pheochromocytoma (124). However, reference intervals must be established for these studies because excretion of catecholamines varies during the sleep–wake cycle. Figure 4 depicts a representative example of excretion of NE, E, and DA during wake and sleep intervals in a healthy normotensive subject as determined in our laboratory. For the entire 10-day study period, amounts of NE and E excreted were significantly lower during sleep, whether expressed as hourly output or per milligram of creatinine. DA did not, however, show significant diurnal variation. Diurnal variations in the excretion of NE and E have been demonstrated by others but DA was not measured in this early study (125). We have also studied reference intervals for pediatric populations based on creatinine output and have shown excretions to be significantly higher and more variable at birth, with a progressive decrease in concentrations with increasing pediatric age (126).

Urinary Vanillylmandelic Acid

VMA is a major catecholamine metabolite formed by the action of catechol−O-methyltransferase and monoamine oxidase. It is excreted by the kidney and represents an average of 40–50% of the urinary excretion production of NE and E. NE is the major source of VMA with metabolism through MHPG as the major pathway (10, 127). VMA is not significantly conjugated and, therefore, is measured without a hydrolysis step. VMA was first isolated and identified in the urine of a patient with pheochromocytoma (13), and its analysis is commonly performed to detect the presence of the tumor (2, 128–131).

For urine measurement, a 24-h collection is recommended to assess daily metabolite excretion and to ensure detection of tumors with sporadic secretion patterns. Urine should be collected under acidic conditions at 4 °C to ensure stability. The preferred methods of
quantification require an initial extraction or ion-exchange step to remove potential interferences (132–134). Extraction procedures involve acidification of urine with concentrated HCl to a pH less than 2, which converts the VMA to the less-soluble protonated form of the acid, followed by saturation with NaCl and extraction with ethyl acetate. Alternatively, anion-exchange column chromatography is an efficient purification step that is gaining widespread use. Extraction efficiencies are high with either the solvent or ion-exchange technique (80–95%).

Quantification is generally accomplished by spectrophotometric methods based on the direct vanillin method of Pisano et al. (132). VMA is converted to vanillin by oxidation with sodium periodate, followed by spectrophotometric quantification at 360 nm (134). This wavelength is used instead of the vanillin absorption maximum of 347–350 nm, because of the presence of an interfering absorption peak from p-hydroxybenzaldehyde, an oxidation product of p-hydroxymandelic acid. α-Methyldopa and p-hydroxymandelic acid interfere with the spectrophotometric method, but dietary vanillin and aromatic phenols do not when anion exchange is used (133). Other methodologic interferences have been reported and include labetalol metabolites, clofibrate, and nalidixic acid (135). VMA concentration in urine may also be affected by the pharmacologic action of certain drugs. Monoamine oxidase inhibitors and α-methyldopa may decrease VMA output; L-dopa, phenothiazines, and tricyclic antidepressants may have a variable effect on VMA production. Another method (136) involves treatment of the urine sample with magnesium silicate to remove interfering compounds, oxidation of VMA to vanillin with ferricyanide, extraction, and formation of the pink carbonium complex of vanillin and indole. The indole condensation method has also been used after an anion-exchange purification step (133).

To enhance the specificity of the measurement, researchers have developed chromatographic and electrophoretic methods for isolating VMA. Earlier methods include paper chromatography (13, 137), thin-layer chromatography (138), and high-voltage electrophoresis on cellulose acetate (139). Detection in these methods often involved reaction with diazotized p-nitroaniline. Judged against current standards, these early methods are imprecise, time-consuming, and impractical for current use. Several GLC methods with flame ionization (140–143) or mass fragmentography (144, 145) detection have been developed. GLC methods are highly specific and can selectively measure other catecholamine metabolites. These methods are not, however, routinely used in clinical laboratories because of the need for expensive equipment and the complexity of the procedure. HPLC is the most frequently used chromatographic method, featuring isocratic reversed-phase separation with electrochemical, spectrophotometric, fluorometric, or post-column detection (146–152). HPLC methods are relatively free of interference and may provide simultaneous measurement of VMA and other metabolites, including MN, NM, homovanillic acid, and MHPG (55, 150, 153–156). Comparable accuracy for spectrophotometric and HPLC methods has been reported, with HPLC demonstrating better sensitivity and precision (148, 157).

Reference interval studies, based on 24-h urinary excretion of VMA in normotensive adults, are listed in Table 1. The mean excretion reported varies from 3.9 to 6.0 mg/24 h with statistical upper limits (mean + 2 SD) ranging from 5.3 to 9.4 mg/24 h (55, 136, 158–161). Reports of the upper limit of the range for normotensive individuals vary from 4.9 to 8.0 mg/24 h (18, 134, 136, 146, 157, 161, 163). The differences in mean or range for these studies do not appear to be method dependent. VMA excretion in hypertensive patients has also been studied and mean values, although higher, do not differ significantly from those of normotensive subjects (136, 162). VMA excretion >8 mg/24 h is reported as abnormal by most clinical laboratories. Pediatric reference ranges for 24-h urine VMA excretion are lower than for adults (55, 164, 164). Reported reference values for pediatric age groups increase from <1 mg/24 h at age one year to near adult values by age 15–16 years (165, 166). However, when expressed on the basis of creatinine excretion, the relative excretion of VMA in the pediatric group decreases with age and shows more variability than adults values (154, 164, 167).

**Urinary Metanephrines**

Normetanephrine (NM) and metanephrine (MN) are metabolic products of NE and E, respectively, and are formed by the action of catechol-O-methyltransferase without deamination. As a result of active neuronal re-uptake and deamination of NE, NM normally represents <5% of the total NE excretion products in urine. MN, however, even with its lower urinary concentration relative to NM, represents a major excretion product of E (10). The metanephrines are excreted in both conjugated and unconjugated forms (168). Unlike the catecholamines, total metanephrine excretion is not significantly influenced by diet (73). As a result, the metanephrines are routinely measured after acid hydrolysis or sulfatase pretreatment.

Isolation of metanephrines from the sample matrix is usually accomplished with ion-exchange chromatography.

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phy. Metanephrines are adsorbed onto cation-exchange resins under weakly acidic conditions. Column treatment with stronger acid or alkaline buffers is then used to elute the metanephrines as a single fraction or individual components. Weak cation-exchange resins are predominantly used, but occasionally suffer from recovery problems because of retention inefficiencies encountered with urine samples containing high salt concentrations. Sample desalting by electro dialysis (169), dilution (170, 171), and dual column usage (172) has been used to minimize variability in metanephrine recovery. Some investigators have also used a combination of strong and weak cation-exchange to enhance recovery (173–176). Catecholamines that may interfere in the analysis are removed in sample pretreatment steps through adsorption onto alumina (169–171, 177), destruction in alkaline ammonia elution buffers (173–176), or differential elution with borate (178, 179). Metanephrines may also be isolated from interferences in the sample matrix through solvent extractions. Under alkaline conditions, metanephrines are extracted into ethyl acetate (180, 181) or cyclohexanone (182), with repeated extractions often being necessary to ensure adequate recoveries. Alternatively, Gupta et al. (183) have described a solvent clean-up procedure with ethyl acetate under acidic conditions. Compounds such as VMA and HMPG are extracted into the organic phase, while metanephrines remain in the original aqueous sample.

Early methods for analysis include electrophoresis (180, 184) and paper (11, 182, 185, 186) and thin-layer chromatography (181). Spraying with dichloroquinone chlorimide or diazotized p-nitroaniline yielded chromogen products that could be measured spectrophotometrically. These assays have been replaced by less technically demanding methods, which possess greater sensitivity.

Spectrophotometric methods continue to be widely used for the measurement of total urinary metanephrines (15, 187). In this method the hydroxyl- and amino-containing side chains are oxidatively cleaved by periodate after sample hydrolysis and isolation of the metanephrines by ion-exchange chromatography. Oxidation of both MN and NM results in the formation of a common end product, vanillin, which is measured spectrophotometrically. Similar to the VMA method, vanillin absorption is monitored at 360 nm to minimize potential interferences such as labetalol (188, 189). Increased analytical specificity can be accomplished by extracting the vanillin before differential spectrophotometry (190). Spectrophotometric measurement of the metanephrines is also subject to negative interferences. Johnson et al. (191) reported that methylglycine, a component of some radiopaque dyes, preferentially consumes the periodate needed for the oxidation of the metanephrines to vanillin, resulting in an underestimation of the metanephrines. Inaccurate blank absorbances may result from a 4-hydroxylated metabolite of propranolol (192).

The use of fluorescence methods has also been reported for metanephrine analysis. Fluorescent derivatization of the metanephrines by chemical oxidation is based on modification of the trihydroxyindole reaction used for catecholamines. As with the catecholamines, the individual metanephrines are estimated through chromatographic separation before the fluorescent derivatization (173, 174, 176), or through the use of differential pH conditions during oxidation (169–171, 175, 177). Ferricyanide (169–171, 177), iodine (169, 175–177), and periodates (173–175) have been used as oxidizing agents. After the oxidation, alkalinization in the presence of ascorbic acid results in tautomerization products, thought to be 3-O-methylated analogs of those produced by E and NE. Stability of the fluorescent products is variable, with some fluorescing as short as 10 min (173). This method appears to have limited application in current practice.

Radioenzymatic methods have also been used to quantify NM in plasma or urine (193–199). Phenylethanolamine-N-methyltransferase and [3H]S-adenosylmethionine convert NM to its [3H]-N-methylated derivative, [3H]MN. Common antihypertensive drugs, e.g., propranolol, guanethidine, reserpine, hydrochlorothiazide, labetalol, and methyldopa do not interfere with this method (197). An additional advantage of this method is the ability to detect picomole amounts of NM (194) within 4 h (195). However, because the assay allows quantification of only NM, it has gained only limited use.

The use of chromatographic methods involving GLC and HPLC for metanephrine analysis has also been reported. GLC may be used to quantify total metanephrines through production of vanillin, followed by its conversion to its volatile trimethylsilyl derivative (200). Alternatively, the metanephrines can be derivatized with perfluoro anhydride compounds and detected with mass-fragmentographic analysis (145, 201, 202). While offering advantages in sensitivity, specificity, and sample pretreatment, these techniques require the use of instrumentation that is not routinely available in many clinical laboratories. In contrast, HPLC measurement offers a practical alternative to other chromatographic or spectrophotometric methods. Typical applications include reversed-phase chromatography (203) with ion-pairing reagents (77, 168, 204–206) or the use of gradients (149) to provide adequate retention and separation of the metanephrines. Sufficient sensitivity for quantifying urinary concentrations may be obtained by using native fluorescence (204) or ultraviolet absorption (207), but most assays incorporate electrochemical methods, for which the limits of detection approach 0.2 ng. Analysis time must often be extended to 20 min to allow for late-eluting amines. Drug interferences with HPLC methods include viloxazine (207) and acetaminophen (116), which may falsely increase the estimation of NM. Labetalol may also interfere with metanephrine analysis (208), and treatment with α-methyldopa results in additional chromatographic peaks (204). As mentioned
in the urinary catecholamine section, chromatographic conditions have been developed for the co-analysis of metanephrines and catecholamines (77, 110, 114).

Progress is being made in the development of immunnoassays for the metanephrines. Initial metanephrine radioimmunoassays suffered from marginal sensitivity and specificity (209–212). More recently, however, Li-numa et al. (213) reported the use of antibodies that exhibited substantially reduced cross-reactivity toward the catecholamines and other related metabolites. Use of radiolabeled octopamine and synephrine as heterogeneous tracers (213, 214) provides specific activities and affinity constants capable of detecting 5 pg in both urine and plasma with no interference from hypertensive drugs. With this method, one can analyze many samples without the need for an extensive sample pretreatment step common to other methodologies. Although these recent reports are promising, more extensive testing is needed to evaluate their full potential.

Several studies have reported metanephrine excretion in adult populations. Table 2 summarizes 24-h excretion in normotensive subjects. In these studies, the upper limit for total metanephrine excretion is as great as 1300 µg/24 h. With selective measurement, NM concentrations consistently exceed those of MN, but absolute ranges vary between studies. Hypertensive populations typically exhibit higher NM or total metanephrine urinary excretions, but there is considerable overlap with values for the normotensive group (193, 199, 202, 205, 214). One-hour excretion of metanephrines has also been used in the diagnosis of pheochromocytoma (214), but diurnal variation in metanephrine excretion (169, 173) must be considered when short collection intervals are used.

Urinary Measurements for Diagnosis of Pheochromocytoma

Clinical experience with plasma catecholamine measurements as well as the use of the clonidine suppression test as diagnostic aids in pheochromocytoma is discussed by Krakoff and Garbowit elsewhere in these Proceedings (215). Urinary measurements are more often used in the initial assessment of the patient, with the catecholamines, metanephrines, and VMA measurements commonly requested in various combinations to maximize clinical sensitivity and specificity. We have evaluated the clinical sensitivity and specificity of the HPLC method for urinary catecholamines (126) and the spectrophotometric methods for VMA (132) and total metanephrines (187) used in our laboratory. Figure 5 shows our test findings for 20 patients, in whom subsequent surgery has confirmed the presence of pheochromocytoma. These results have also been compared with test findings for 65 patients with essential hypertension. Based on the upper limit of our clinical laboratory reference interval for urinary NE (80 µg/24 h) and E (20 µg/24 h), a sensitivity of 75% was determined for either measurement. The use of an upper reference limit of 100 µg/24 h for the total NE plus E excretion increased clinical sensitivity to 85% but also decreased clinical specificity to 77%. Total metanephrine measurement was determined to be the best discriminator of essential hypertension and pheochromocytoma, having a sensitivity of 100% and a specificity of 84%. Measurement of VMA was less sensitive than measurement of metanephrines, with considerable overlap between essential hypertension and pheochromocytoma. These findings are consistent with several previous studies, which were recently reviewed by Mannelli (2). The poorer sensitivity of our urinary catecholamine measurements as compared with the findings of Moyer et al. (78) may be due to the relatively large percentage of multiple endocrine neoplasia cases in our group.

The high clinical sensitivity of the metanephrine measurement may have a metabolic basis. We expressed the increase in catecholamines and metabolite excretion as multiples of the upper limit of the reference range and, in accordance with the early work of Crout et al. (15), identified two populations of pheochromocytoma patients. One group showed a predominant increase in NE or E, the other group a predominant increase in

| Table 2. Reference Interval for Metanephrine Excretion in Urine |
|-----------------|-----------------|-----------------|-----------------|
|                 | Conc, µg/24 h   | MEAN (2 SD)     | RANGE           | MEAN (2 SD)     | RANGE           | TOTAL MN, mean (2 SD) | Ref.      |
| Method          | n               |                  |                 |                  |                 |                      |          |
| Spectrophotometry | 30              | 600 (600)        | 80              | 72 (50)          | 28–113          |                      | 187       |
|                 | 121             | 620 (560)        | 15              | 66 (52)          | 122–198         |                      | 15        |
|                 | 18              | 500 (280)        | 188             | 60 (18)          | 29–142          |                      | 188       |
| Fluorometry     | 19              | 158 (152)        | 80              | 149 (112)        | 79–258          |                      | 189       |
|                 | 16              | 245 (144)        | 173             | 174 (158)        | 74–297          |                      | 173       |
|                 | 12              | 231 (134)        | 202             | 154 (148)        | 74–297          |                      | 202       |
| GLC             | 18              | 238 (160)        | 203             | 172 (110)        | 96 (64)         |                      | 203       |
| HPLC            | 15              | 217 (218)        | 213             | 253 (180)        | 239 (202)       |                      | 213       |
| RIA             | 22              | 261 (146)        | 214             | 253 (180)        | 239 (202)       |                      | 214       |
| Thin-layer chromatography | 80     | 261 (146)        | 214             | 253 (180)        | 239 (202)       |                      | 214       |

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metabolites (Figure 6). The observed differences in these two groups have been attributed to the size of the tumor and its metabolic enzyme content (15). It is also noteworthy that in Crout's study, as well as our own, the increase in metanephrines relative to upper limit of the reference range was greater than the comparable increase in VMA for all patients with pheochromocytoma. This may be attributable to the lack of catecholamine re-uptake by the tumor and to a decreased chance for deamination by monoamine oxidase. Therefore, when tumor-secreted catecholamines are metabolized, MN, rather than VMA, becomes the preferred end product. This may account, in part, for the greater sensitivity of the measurement of MN in the diagnosis of pheochromocytoma.

**Future Directions**

Measurement of catecholamines and their metabolites remains an important laboratory aid in the diagnosis of pheochromocytoma. The recent development of chromatographic methods, which allow simultaneous analyses for catecholamines and their metabolites, may provide a simple and efficient approach for the future. Further displacement of the spectrophotometric, fluorometric, and radioenzymatic methods by chromatographic procedures should continue, with an emphasis on quality control and an expanded use of proficiency-testing programs to ensure test reliability. Looking to the future, development of immunologic methods holds promise for providing simple, more readily available techniques for measuring catecholamines and their metabolites. Advances in this area must await further development of antibodies of sufficient sensitivity and specificity. As methodological advances continue, clinical experience also continues to confirm the diverse metabolic potential of pheochromocytomas and to reinforce the need to use a combination of measurements for optimal clinical sensitivity in tumor detection.

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