



Mass Spectrometry Technology Leads to Improved Clinical Utility of Laboratory Tests

LC/MS/MS and GC/MS Technology Guide

In an effort to simplify and reduce the cost of immunoassays, manufacturers have developed products that have reduced sample preparation (eg, extraction and/or purification), eliminated the use of radioactive isotopes, and minimized manual labor. Unfortunately, for some analytes, this has led to highly variable and inaccurate immunoassays.¹ Such assays may be subject to cross-reactivity with similar analytes, matrix effects that are not eliminated by the calibrators and their matrix, interferences due to binding proteins, and other unknown interferences. Consequently, liquid chromatography tandem mass spectrometry (LC/MS/MS) and gas chromatography mass spectrometry (GC/MS) methods have been implemented to eliminate interferences and improve the accuracy and precision of measurement.

The high analytical specificity of MS detection results from the molecular mass of the analyte coupled with its specific disintegration pattern. Following chromatographic separation, the sample is ionized and separated by the MS according to the mass-to-charge ratio of the ions created. For LC/MS/MS, parent ions generated in the first MS step are collected and fragmented into daughter ions and then separated by mass-to-charge ratio a second time. The resultant ion count, once corrected for manipulation losses using the internal standard, is compared to a calibration curve to quantitate the analyte(s). Thus, extraction, purification, and 1 or 2 MS separations account for the lack of interference from related compounds, drugs, and other substances providing excellent specificity. Each of these steps also contributes to elimination of background noise, increasing the signal-to-noise ratio; thus, sensitivity is also improved. The correction for manipulation losses provides accuracy and precision suitable for reference methods.

Both LC/MS/MS and GC/MS provide multi-analyte profiling from a single analysis and use minimal sample volume, both of which are especially important for pediatric patients. The excellent accuracy and precision of LC/MS/MS enable the laboratory standardization necessary for use of universal cutpoints recommended by expert panel consensus. These analytical advantages also translate into improved clinical correlation to support diagnosis and patient management as described in the remainder of this document.

Evaluation of Adrenal Function

Primary Aldosteronism

The recommended screening test for primary aldosteronism (PA) is the aldosterone-to-renin ratio (ARR); several confirmatory tests including captopril suppression of aldosterone are available to confirm the diagnosis.² The Endocrine Society suggests using LC/MS/MS for more consistent and accurate aldosterone measurement and correct estimation of ARR.² Secondly, the reliability of aldosterone measurements during suppression testing has come into question.³ When a nonextraction RIA was compared to a LC/MS/MS method, RIA results were on the average 33% higher and showed an unacceptably high scatter at levels <8 ng/dL.⁴ Levels <8 ng/dL are clinically significant as they are typically observed in cases of normal aldosterone suppression. The clinical advantages of specific, accurate, and reproducible measurement of aldosterone are presented in Table 1.

Adrenal Insufficiency

A lack of specificity of immunoassays for plasma cortisol has complicated the diagnosis of adrenal insufficiency.^{6,7} Adrenal insufficiency is best diagnosed with serum cortisol determination before and after a 250 µg dose of cosyntropin.⁷ The most commonly used criteria to rule out adrenal insufficiency is achieving a level of post-cosyntropin cortisol of >20 µg/dL. In critically ill adults, the American College of Critical Care Medicine recommends the diagnosis be made when the basal cortisol level is <10 µg/dL or the post-cosyntropin increase is <9 µg/dL.⁷ Using these criteria, the concordance of distinguishing responders from non-responders was 44% when 3 immunoassays were compared to a chromatographic reference method.⁶ The differences were attributed to differing degrees of antibody cross-reactivity to steroids that would be increased by cosyntropin. Furthermore, in patients with sepsis, cross-reactivity of immunoassays with cortisol precursors and metabolites that accumulate in sepsis complicates test interpretation.⁷ Thus, highly specific measurement by LC/MS/MS can improve the clinical utility of cortisol measurement (Table 1).

Assessing adrenal function in critically ill patients using baseline or cosyntropin-stimulated serum total cortisol may be misleading.⁸ During critical illness, albumin and cortisol

binding globulin (CBG) are frequently decreased resulting in misleadingly lower total cortisol concentration. Measurement of serum free cortisol levels, rather than total cortisol, can overcome the illusion of adrenal insufficiency and suboptimal response to cosyntropin (Table 1).⁸

Cushing's Syndrome

Symptomatic patients who are not taking exogenous glucocorticoid should initially be tested for 1 of the following: urine free cortisol, which is increased in Cushing's syndrome; day-time and late-night free salivary cortisol to document loss of diurnal variation; overnight dexamethasone suppression; or longer low-dose dexamethasone suppression.⁹ Immunoassays not employing chromatography overestimate levels of urinary free cortisol by at least 2-fold, presumably due to cross-reactivity with common urinary cortisol metabolites, such as dihydro- and tetrahydrocortisol compounds.^{10,11} Similarly, bedtime salivary cortisol measurement, which has been proposed as an alternative screening test, is overestimated by RIA compared to LC/MS/MS.¹² Table 1 details the analytical and clinical advantages of LC/MS/MS technology for these assays.

Endocrine Hypertension

Causes of low-renin hypertension include primary aldosteronism, glucocorticoid-suppressible aldosteronism, apparent mineralocorticoid excess (AME) due to 11 β -hydroxysteroid dehydrogenase deficiency, and Liddle syndrome (hypertension with low renin and low aldosterone). Steroid profiling in urine can distinguish these often treatable causes of endocrine hypertension (Table 1).

Pheochromocytoma

An international consensus recommendation advises that initial testing for pheochromocytoma always include measurement of either plasma free or fractionated urinary metanephrines.¹⁵ This recommendation is based on the high diagnostic sensitivity of high-performance liquid chromatography (HPLC) methods used for fractionating metanephrines in either plasma or urine. Such measurements demonstrated improved clinical sensitivity over urinary total metanephrines, urinary vanillylmandelic acid, and fractionated catecholamines in plasma or urine.¹⁶ Furthermore, the diagnostic specificity of fractionated metanephrines improves when non-MS HPLC methods are replaced by the more specific LC/MS/MS technology (Table 1).¹⁷

Table 1. Analytical and Clinical Advantages of MS Technology for Evaluation of Adrenal Function

Test Code	Test Name	Analytical Advantage	Clinical Advantage
17181X	Aldosterone, LC/MS/MS	Chromatography overcomes the 33% overestimation by nonextraction RIA ⁴	Helps avoid false-positive primary aldosterone diagnosis and unnecessary aldosterone suppression testing ³
		Overcomes imprecision and lack of sensitivity of RIAs at <8 ng/dL ^{4,5}	Helps avoid false-negative aldosterone suppression testing ³
11183Z	Aldosterone (LC/MS/MS): Plasma Renin Activity Ratio ^a	Aldosterone chromatography overcomes the 33% overestimation by nonextraction RIA ⁴	Helps avoid falsely elevated ARR and unnecessary confirmatory testing ³
11281X	Cortisol, Total, LC/MS/MS	<1% cross-reactivity with dexamethasone, methylprednisolone, prednisolone, and prednisone ⁵	Permits evaluation of adrenal function during glucocorticoid therapy
38149X	Cortisol Response to ACTH Stimulation, Serum	No cross-reactivity with cortisol precursors and metabolites or related steroids ^{5,6}	Adrenal insufficiency reliably diagnosed
36423X	Cortisol, Free, LC/MS/MS	Provides best measurement of biologically active cortisol (ie. free, unbound cortisol) Avoids interference from protein binding abnormalities (eg, malnutrition, increased CBG)	Accurately reflects glucocorticoid clinical status
11280X	Cortisol, Free, 24-Hour Urine, LC/MS/MS <i>Includes creatinine.</i>	Reference ranges for adult and pediatric populations agree with "true" literature values ^{10,11}	Provides accurate diagnosis of hypercortisolism
		No cross-reactivity to cortisol precursors or metabolites ^{5,11}	Helps avoid false-positive elevations
		Eliminates drug interference (eg, carbamazepine, fenofibrate) ^{5,13,14}	Helps avoid false-positive elevations
		<1% cross-reactivity with dexamethasone, methylprednisolone, prednisolone, and prednisone ⁵	Permits evaluation of adrenal function during glucocorticoid therapy
		Includes correction for extraction and chromatography losses	Helps avoid false negatives

continued

Table 1. Analytical and Clinical Advantages of MS Technology for Evaluation of Adrenal Function – continued

Test Code	Test Name	Analytical Advantage	Clinical Advantage
19897X	Cortisol, Saliva, LC/MS/MS	Ease of sample collection, especially in children	Assess circadian rhythm using a stress-free screening test
15026X	Hypertension, Endocrine <i>Includes 31 urinary adrenal and gonadal steroid metabolites.</i>	GC/MS enables simultaneous multi-analyte steroid profiling and ratio calculation	Differential diagnosis of secondary hypertension conditions associated with low renin
19548X	Metanephrines, Fractionated, LC/MS/MS, Plasma <i>Includes metanephrine, normetanephrine, and total metanephrines.</i>	<0.1% interference from acetaminophen, epinephrine, and norepinephrine ^{5,16} Includes correction for extraction and chromatography losses	Helps avoid false-positive elevations Helps avoid false negatives
14962X	Metanephrines, Fractionated, LC/MS/MS, 24-Hour Urine <i>Includes metanephrine, normetanephrine, and total metanephrines.</i>	<1% interference from acetaminophen, chlorpromazine, desipramine, dopamine, ephedrine, epinephrine, and norepinephrine ^{5,18} Includes correction for extraction and chromatography losses ¹⁸	Helps avoid false-positive elevations Helps avoid false negatives

RIA, radioimmunoassay; ARR, aldosterone-to-renin ratio; CBG, cortisol binding globulin; VMA, vanillylmandelic acid.

^aThis test was developed and its performance characteristics have been determined by Quest Diagnostics Nichols Institute. It has not been cleared or approved by the U.S. Food and Drug Administration. The FDA has determined that such clearance or approval is not necessary. Performance characteristics refer to the analytical performance of the test.

Evaluation of Sex Steroid Status

Estradiol

Reliable and accurate measurement of low estradiol levels is required for the evaluation of prepubertal girls, postmenopausal women, breast cancer patients treated with aromatase inhibitor therapy, and men. In a study of postmenopausal women, non-extraction direct immunoassays overestimated estradiol levels by 68%, whereas extraction-based assays overestimated levels by 14% compared to a MS/MS method.¹⁹ With direct assays, overestimation may be due to serum matrix problems, binding of estradiol by sex hormone binding globulin, and the presence of water-soluble conjugated steroids with concentrations orders of magnitude higher than estradiol.²⁰ Such estradiol results may include over 70% artifact and compromise the clinical utility of estradiol measurement, especially when circulating concentrations are in the postmenopausal range (Table 2).²⁰

In addition, measurement of estradiol and estrone in breast cancer patients treated with aromatase inhibitor therapy is an important indicator of therapy response. Estrogen levels are usually very low and require ultrasensitive methods such as LC/MS/MS for adequate sensitivity.

Testosterone

When based on accurate measurement of total testosterone and sex hormone binding globulin, bioavailable testosterone calculation is recommended as the most useful, clinically sensitive test for hyperandrogenism in women and hypogonadism in men with equivocal total testosterone levels (200 to 320 ng/dL).²¹ However, testosterone assays that do not incorporate extraction and chromatography are unable to accurately quantitate the low serum total testosterone levels found in these cases.^{22,23} Thus, the Endocrine Society recommends testosterone methods that incorporate extraction and chromatography followed by RIA or mass spectrometry methods over direct immunoassays (Table 2).²¹

Free testosterone (FT) is a better reflection of the androgenic state in adults or post-pubertal children than total testosterone.²¹ FT is most commonly measured by analog-based immunoassays. Although these assays are relatively inexpensive, rapid, and convenient, they underestimate FT when compared to equilibrium dialysis and show high variability.²⁴ Thus, experts recommend FT by equilibrium dialysis or calculation of bioavailable testosterone over analog-based measurement (Table 2).²¹ Furthermore, both of these methods use calculations from total testosterone measurement thus requiring an accurate testosterone assay.

Table 2. Analytical and Clinical Advantages of LC/MS/MS for Evaluation of Sex Steroid Disorders

Test Code	Test Name	Analytical Advantage	Clinical Advantage
30289X	Estradiol, Ultrasensitive, LC/MS/MS	Overcomes immunoassay-dependent high bias ^{19,20}	Helps avoid false-positive elevations in children, postmenopausal women, women on aromatase therapy for breast cancer, and men
36169X	Estradiol, Free, LC/MS/MS <i>Includes estradiol, % free estradiol by equilibrium dialysis, and free estradiol concentration (calculated).</i>	Overcomes immunoassay-dependent high bias of total estradiol ^{19,20}	Helps avoid false-positive elevations in children, postmenopausal women, and men

continued

Table 2. Analytical and Clinical Advantages of LC/MS/MS for Evaluation of Sex Steroid Disorders – continued

Test Code	Test Name	Analytical Advantage	Clinical Advantage
15983X	Testosterone, Total, LC/MS/MS	Overcomes immunoassay overestimation in women and children ²²	Helps avoid false-positive hyperandrogenemia in women and children
		Overcomes low immunoassay bias in men ^{22,23}	Provides accurate measurements to better diagnose hygonadism in men and evaluate need for testosterone replacement therapy ²¹
14966X	Testosterone, Free, Bioavailable, and Total, LC/MS/MS <i>Bioavailable and free testosterone are calculated.</i>	High total testosterone specificity ^{21,23}	Improved diagnosis and management of hyperandrogenemia in women ²¹
		Calculated bioavailable testosterone correlates well with equilibrium dialysis results ²¹	More accurate differentiation between hypogonadal and eugonadal men ²¹
36170X	Testosterone, Free and Total, LC/MS/MS <i>Includes testosterone, % free testosterone by equilibrium dialysis, and free testosterone concentration (calculated).</i>	Overcomes underestimation and random variability observed with direct free testosterone measurement ²⁴	Improved diagnosis and management of hyperandrogenemia in women ^{21,24}

Evaluation of Metabolic Disorders

Acylcarnitine and Carnitine

Early diagnosis and treatment of fatty acid oxidation disorders and organic acid disorders can reduce associated morbidity and mortality. LC/MS/MS technology enables simultaneous, specific, and sensitive quantitation of free and total carnitine and the acylcarnitines (C2 through C18) that are associated with these disorders. Results can aid in the diagnosis, which, with rare exception, depends on such analyses (Table 3).²⁵

Steroid Testing for Congenital Adrenal Hyperplasia (CAH)

CAH caused by 21-hydroxylase deficiency is characterized by increased levels of 17-hydroxyprogesterone (17-OHP) and androstenedione and decreased levels of cortisol and 11-deoxycortisol. An increased level of 17-OHP may suggest the diagnosis; however, false-positive elevations in newborns can result from inaccurate immunoassays or from biological variation caused by prematurity, illness, or stress.²⁷ Therefore, some have suggested use of LC/MS/MS to measure 17-OHP along with other steroids (ie, steroid profiling with calculation of precursor-to-product ratios) (Table 3).^{27,28} In addition, GC/MS can be used for the differential diagnosis of multiple forms of CAH using a urine specimen collected from a diaper (Table 3).²⁹

Evaluation of patients with suspected androgen excess, which may be attributed to nonclassical or late-onset CAH, should include measurement of 17-OHP.³⁰ Accurate measurement of basal and cosyntropin-stimulated levels is required for accurate

diagnosis, but immunoassays are not reliable at the basal 17-OHP levels typically observed in these patients (Table 3).²⁸

Vitamin D

Serum 25-hydroxyvitamin D [25(OH)D] levels are used to determine vitamin D deficiency or intoxication and to monitor vitamin D therapy. Total circulating 25(OH)D should include accurate measurement of both 25(OH)D₂ (derived from ergocalciferol) and 25(OH)D₃ (from cholecalciferol).³³ However, immunoassays may underestimate 25(OH)D₂ and overestimate 25(OH)D₃ concentrations.^{33,34} LC/MS/MS methods accurately quantitate both forms of 25(OH)D (Table 3).

Serum 1,25-dihydroxyvitamin D [1,25(OH)₂D] measurement may be used to distinguish primary hyperparathyroidism from hypercalcemia of malignancy, to monitor vitamin D therapy in patients with chronic renal disease, and to help diagnose vitamin-D dependent rickets. Available methods include RIA, EIA, and the classical thymus radioreceptor assay (RRA) following chromatographic purification. Whereas the RRA method corrects for manipulation losses, commercial RIA and EIA methods that include extraction do not.³⁵ Furthermore, some immunoassays underestimate 1,25(OH)₂D₂³⁵ and overestimate the concentration of total 1,25(OH)₂D due to interference from 1 α -hydroxylated vitamin D metabolites.³⁶ Due to these limitations, correlation results between methods has been highly variable ($r^2 = 0.4$ to 0.9).³⁵⁻³⁷ LC/MS/MS overcomes many of these disadvantages (Table 3).

Table 3. Analytical and Clinical Advantages of MS Technology for Evaluation of Metabolic Disorders

Test Code	Test Name	Analytical Advantage	Clinical Advantage
14531X	Acylcarnitine, Plasma	LC/MS/MS enables simultaneous measurement of relevant acylcarnitines ranging from C2 through C18	Permits differential diagnosis of over 30 fatty acid oxidation disorders and certain organic acid disorders ²⁵
70107X	Carnitine, LC/MS/MS <i>Includes esters, free, and total carnitine.</i>	LC/MS/MS enables simultaneous measurement of free and total carnitine	Permits diagnosis and management of patients with primary or secondary carnitine deficiency ²⁶

continued

Table 3. Analytical and Clinical Advantages of MS Technology for Evaluation of Metabolic Disorders – continued

Test Code	Test Name	Analytical Advantage	Clinical Advantage
15269X	CAH Panel 1 (21-Hydroxylase vs 11 β -Hydroxylase Deficiency) <i>Includes androstenedione, cortisol, 11-deoxycortisol, 17-hydroxyprogesterone, testosterone, and precursor: product ratios.</i>	LC/MS/MS enables simultaneous multi-analyte steroid profiling using minimal sample volume ²⁷	Improves the differential diagnosis in low-birth weight or stressed newborns ^{27,31}
10046X	CAH Panel 11, Neonatal Random Urine	GC/MS simultaneously measures 34 steroids and calculates 11 precursor: product ratios using urine from a diaper	Distinguishes 3 β -hydroxysteroid dehydrogenase, 11 β -hydroxylase, 17 α -hydroxylase, and 21-hydroxylase deficiencies ²⁹
17180X	17-Hydroxyprogesterone, LC/MS/MS	Overcomes immunoassay overestimation in newborns ²⁷ Overcomes interference from steroids commonly seen in newborns, especially 17-hydroxypregnenolone ^{5,32} Overcomes method-dependent scatter at levels <165 ng/dL seen with extraction/RIA ²⁸	Helps overcome false-positive elevations in newborns ²⁷ Helps insure accurate monitoring of patients with CAH ²⁸ Permits accurate diagnosis of late-onset 21-hydroxylase deficiency ³⁰
17306X	Vitamin D, 25-Hydroxy, LC/MS/MS <i>Includes 25-hydroxyvitamin D₂, D₃, and total.</i>	Overcomes underestimation of 25(OH)D ₂ by immunoassay ³⁴	Permits evaluation of vitamin D status including vitamin D ₂ pharmacologic treatment
16558X	Vitamin D, 1,25-Dihydroxy, LC/MS/MS <i>Includes 1,25-dihydroxyvitamin D₂, D₃, and total.</i>	Overcomes lack of correction for extraction and chromatography losses ^{5,35} Overcomes interference from 1 α -hydroxylated vitamin D metabolites ^{5,36}	Permits accurate assessment of 1,25-dihydroxyvitamin D status

CAH, congenital adrenal hyperplasia.

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 TG2489-HS 06/2009



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