LC-Tandem Mass Spectrometry (LC/MS/MS) in the Clinical Research Laboratory: A Selective, Sensitive, and Versatile Testing Technique for Measuring Endocrine Analytes

Introduction

Accurate measurement of steroids is important for research into a variety of endocrine disorders. Many techniques have been utilized to measure endogenous steroids including HPLC/UV, various immunoassays, and GC/MS. HPLC/UV methods lack the necessary sensitivity and specificity to distinguish low levels of target analytes from the biological matrix background. Immunoassays are widely used in many laboratories because of their ease of use and sensitivity. However, accurate and precise measurement of low levels of sex steroids, such as testosterone in women and children, has long been a concern and topic of discussion for both endocrinologists and clinical chemists. GC/MS methods are often sensitive and selective, but extensive sample preparation can make it difficult to implement these methods in today's high throughput laboratories. Because of its inherent sensitivity and selectivity, liquid chromatography with triple quadrupole tandem mass spectral detection (LC/MS/MS) is often considered a "gold standard" method for quantifying compounds in complex matrices. The use of LC/MS/MS in clinical research laboratories has continued to increase and is being applied to an expanding list of analytes, including a wide variety of steroids, vitamin D, and T3/T4. LC/MS/MS has the further advantage of having the capability to perform multi-analyte assays, even across compound classes.

Initial adoption of LC/MS/MS into routine clinical research laboratory use was limited because of its cost, complexity, and instrument footprint. However, technological advances have resulted in affordable benchtop instruments, and developments in software tools have made these systems user-friendly for even novice users. AB SCIEX offers a complete line of instruments that cover a range of performance depending on analytical needs. Our hybrid triple quadrupole/linear ion trap QTRAP® instruments deliver the quantitative performance of a triple quadrupole and add the qualitative capabilities of a linear ion trap – all in a single platform. Furthermore, unique MRM3 quantitation can be used when additional selectivity is required. Our LC/MS/MS hardware, combined with Cliquid® Software for easy routine analysis and our iMethod™ tests, deliver a complete solution to easily and quickly implement LC/MS/MS in the routine clinical research laboratory.

Testosterone (289/97) 1 ng/mL **Testosterone (289/109) 1 ng/mL**

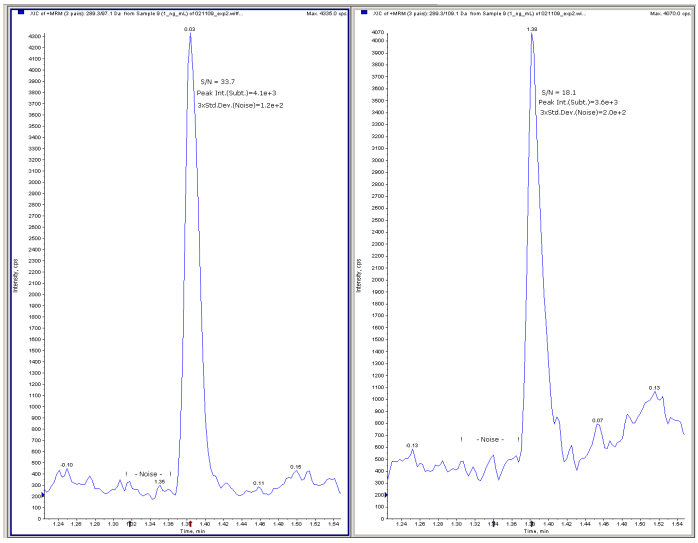

Figure 1. Data from a 1 ng/mL testosterone sample analyzed using an AB SCIEX API 3200™ LC/MS/MS System. This concentration represents the lower limit of quantification for this assay, which had a linear range from 1-400 ng/mL. The quantifier transition (289/97) is shown on the left, and the qualifier transition (289/109) is shown on the right.

Testosterone

Testosterone is one of the most commonly measured serum hormones. Measurement of testosterone is used to research a variety of disorders, such as congenital adrenal hyperplasia and hypogonadism. Measurement of the typical levels in men is straightforward; however, measurement of the low levels present in women and children can be especially challenging. AB SCIEX provides solutions for routine analysis for both circumstances. Figure 1 shows representative data of the lower limit for the iMethod™ test for routine measurement of testosterone, analyzed on an API 3200™ LC/MS/MS system. The linear range established for this assay is 1-400 ng/mL. Figure 2 demonstrates the ability to measure very low levels of testosterone, such as the concentrations present in children and women, using an API 5000™ or AB SCIEX Triple Quad™ 5500 LC/MS/MS system. This highly sensitive method uses a small sample volume of 0.2 mL and has been tested across the 20-500 pg/mL range.

Estrogens

Estrogens play an important role in fertility and sexual development, as well as in cancer risk. The major estrogens in serum are estrone (E1), 17β-estradiol (E2), and estriol (E3), with E2 being the estrogen most often monitored. Serum levels typically range from 20-300 pg/mL in women, with lower concentrations in men and children. Cross-reactivity from estrone and/or estriol can be a problem in immunoassay testing, resulting in measured levels that are artificially elevated. LC/MS/MS-based techniques have the specificity to distinguish the three compounds from each other. And although E2 is the analyte most often monitored, increased interest in E1 has resulted in a growth in the number of samples requesting measurement of E1, which requires accurate quantitation down to 15 pg/mL. With LC/MS/MS, it is possible to simultaneously measure the low-level concentrations of both E1 and E2 in a single analytical test. Figure 3 shows calibration curves for analysis of E1 and E2 using the iMethod™ test for Analysis of Estrogens on an API 5000™ or AB SCIEX Triple Quad™ 5500 LC/MS/MS system. Linearity spanned 5-500 pg/mL for both analytes, and precision and accuracy were within ±10% across the entire range. Precision and accuracy were excellent, as shown in Table 1, which displays both inter- and intra-day statistics. A separate assay for measurement of E3 in serum has also been developed. Precision and accuracy are similar to results shown for E1 and E2, and the dynamic range for the E3 assay is 5-500 pg/mL using the API 5000™ or AB SCIEX Triple Quad™ 5500 instrument.

Testosterone (289/97) 20 pg/mL **Testosterone (289/109) 20 pg/mL**

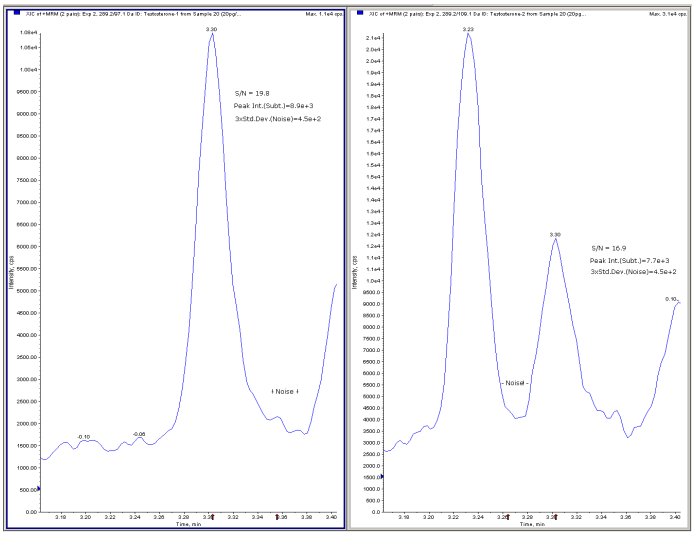

Figure 2. Data from a 20 pg/mL testosterone sample analyzed using an AB SCIEX API 5000™ LC/MS/MS System. This concentration represents the lower limit of quantification for this assay, which had a linear range from 20-500 pg/mL. The quantifier transition (289/97) is shown on the left, and the qualifier transition (289/109) on the right.

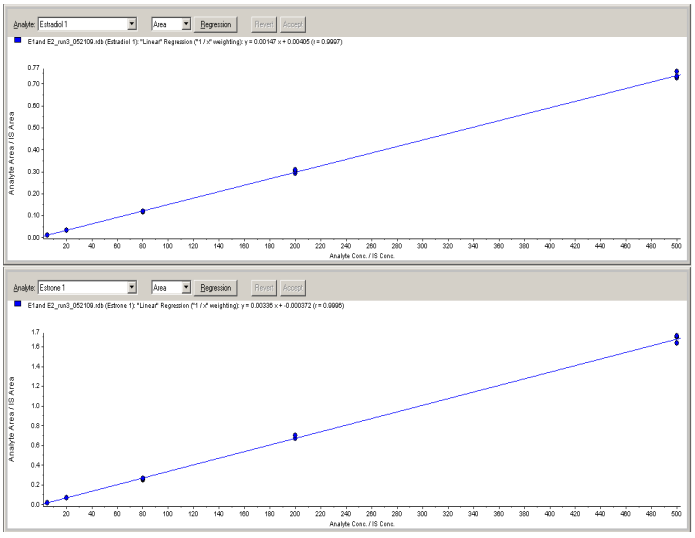

Figure 3. Calibration curves for estradiol (E2, top) and estrone (E1, bottom). The linear dynamic range for both compounds was 5-500 pg/mL as analyzed on an AB SCIEX API 5000™ LC/MS/MS System. The r value for each was >0.999, and precision and accuracy were ±10% across the range.

Table 1. Precision and accuracy for estrone and estradiol. Data were collected over three days; the % CV and accuracies are as listed.

Estrone (E1) Sample (pg/mL)	% CV	% Accuracy	Estradiol (E2) Sample (pg/mL)	% CV	% Accuracy
	Day 1-3	Day 1-3		Day 1-3	Day 1-3
LLOQ (5)	9	104	*LLOQ (5)*	10	101
Low QC (15)	3	104	*Low QC (15)*	4	96
High QC (100)	3	101	*High QC (100)*	5	102
ULOQ (500)	3	100	*ULOQ (500)*	4	101

Estrone

- LLOQ CV < 9%, QC CV < 3%
- Accuracy deviation < 4%

Estradiol

- LLOQ CV < 10%, QC CV < 5%
- Accuracy deviation < 4%

Vitamin D

Vitamin D has long been known to play a key role in calcium homeostasis, and therefore bone formation and strength. However, further research into the role of vitamin D in the body has shown that it affects many other health factors, such as heart disease, immune response, type I diabetes, and various cancers. Because of this expanded understanding of the role of vitamin D in overall health, interest in vitamin D has significantly increased.

The metabolites most commonly monitored to determine vitamin D levels are the 25-OH metabolites of vitamins D2 and D3. iMethod™ tests for LC/MS/MS analysis of 25-OH-vitamin D2 and D3 have been developed and can be used on AB SCIEX 3200 and 4000 series instruments. These methods use commercial calibrators and controls or kits, helping ensure accurate calibration and QC checks, as well as saving time and simplifying workflows. Total run time is 4 min per sample, keeping up with the high-throughput demands placed on today's laboratory. The lower limit of quantitation using an API 3200™ LC/MS/MS system is 4 ng/mL (Figure 4). This method also has the ability to distinguish between 25-OH-vitamin D2 and vitamin D3 and provides quantitative results for each form, which can be useful when researching the effectiveness of vitamin D supplementation. The linear ranges for 25-OH-vitamin D2 and D3 are 4-70 ng/mL and 4-100 ng/mL, respectively. Linearity extended beyond 100 ng/mL for each analyte but was not relevant for this test. Representative calibration curves are shown in Figure 5.

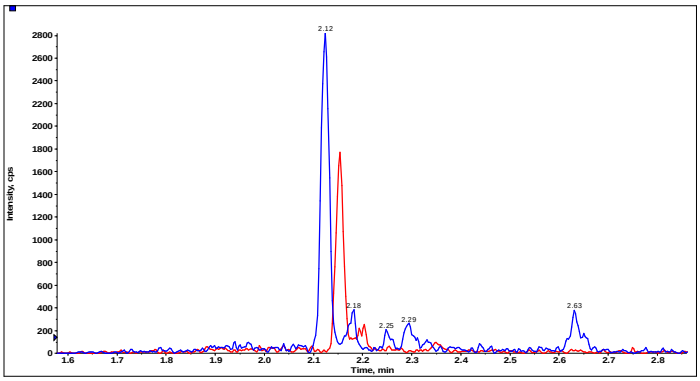

Figure 4. Chromatograms from a 4 ng/mL sample of 25-OH-vitamin D2 and D3 analyzed on an AB SCIEX API 3200™ LC/MS/MS System. The linear ranges were from 4-70 ng/mL for 25-OH-vitamin D2 and 4-100 ng/mL for 25-OH-vitamin D3. Linearity extended beyond 100 ng/mL but was not relevant for this test.

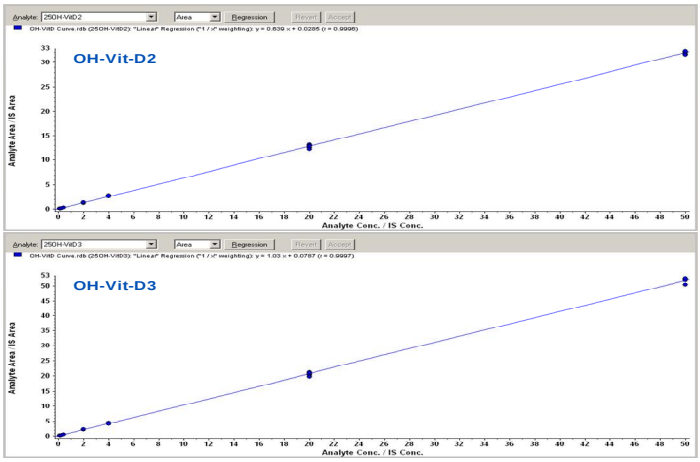

Figure 5. Calibration curves for 25-OH-vitamin D2 (top) and 25-OH-vitamin D3 (bottom). The linear dynamic range for both compounds was 4-70 ng/mL and 4-100 ng/mL, respectively, as analyzed on an AB SCIEX API 3200™ LC/MS/MS System. Linearity extended beyond 100 ng/mL but was not relevant for this test. Precision and accuracy were both within 10% across the range.

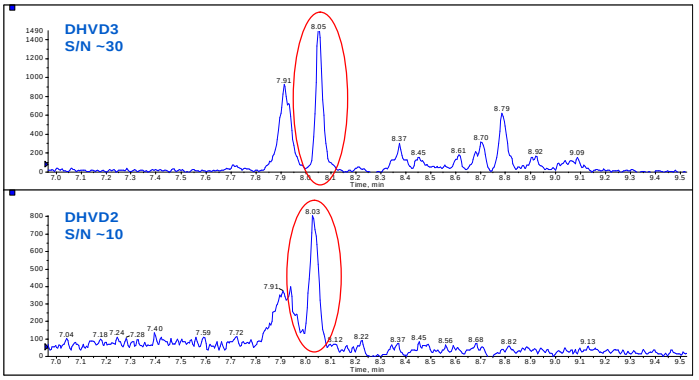

Figure 6. Representative data from 1,25-di-OH-vitamin D analysis of a 25 pg/mL sample. Data were acquired using an AB SCIEX API 5000™ LC/MS/MS System.

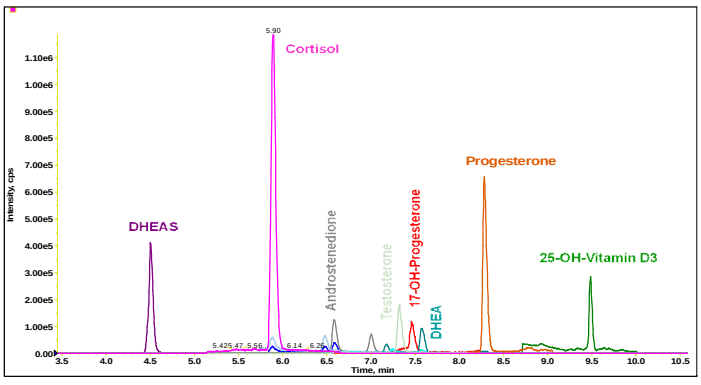

Figure 7. Representative chromatogram of analysis of steroids in plasma using an AB SCIEX Triple Quad™ 5500 system. Concentrations were 25-250 pg/mL for most analytes except for cortisol (25 ng/mL) and DHEAS (125 ng/mL).

Table 2. Routine tests and recommended AB SCIEX LC/MS/MS Systems.

Application	Recommended Instrument
Routine Testosterone	API 3200™ System
Low-level Testosterone	API 5000™ System or Triple Quad™ 5500 System
Estrogens: Estradiol, Estrone, and Estriol	API 5000™ System or Triple Quad™ 5500 System
25-OH-Vitamin D2 and D3	API 3200™ System
1,25-di-OH-Vitamin D2 and D3	API 5000™ System or Triple Quad™ 5500 System
Multi-analyte Steroid Panel	API 5000™ System or Triple Quad™ 5500 System

Although the 25-OH-vitamin D metabolite is most often utilized as an indicator of vitamin D levels, interest in quantifying the active metabolite, 1,25-di-OH-vitamin D, is growing. Measurement of the di-OH metabolite is very challenging because of the low concentration at which it is present, requiring quantification at the very low pg/mL level. Furthermore, distinguishing the 1,25-di-OH metabolite from other metabolites is also a challenge. The selectivity and sensitivity of LC/MS/MS again proves an effective tool in this difficult analysis. Figure 6 shows the ability to separate and detect the 1,25-di-OH metabolite from other vitamin D metabolites in a 25 pg/mL sample. This concentration represents the limit of quantitation for analysis of 1,25-di-OH-vitamin D using an API 5000™ or AB SCIEX Triple Quad™ 5500 instrument.

Multi-Analyte Test

One key aspect of LC/MS/MS analysis is its ability to analyze a wide variety of compounds, even across different compound classes. The tests previously described for testosterone, estrogens, and mono-OH-vitamin D can easily be combined into a single analytical method. Other steroids, such as OH-progesterone, cortisol, and DHEA – to name just a few – can also be easily added into an LC/MS/MS method for quantitation of a panel of steroids in a single test. Figure 7 shows a chromatogram for a method that measures the levels of a wide variety of compounds in a single analytical run. Combining multiple analytes into a single analysis increases throughput and minimizes sample consumption when sample volume is limited.

Summary

The need for increased specificity and sensitivity introduced the clinical research laboratory to LC/MS/MS as a technique to quantify low levels of endogenous steroids in complex biological matrices. The precision, accuracy, and linearity of results presented here demonstrate why triple quadruple LC/MS/MS has become the “gold standard” for quantification. In addition, the flexibility of this technique and low per-test costs have contributed to the rapid growth in the use of LC/MS/MS in the clinical research laboratory, and the types of tests performed using LC/MS/MS continue to expand. AB SCIEX provides a variety of tools to facilitate easy implementation of LC/MS/MS into new laboratories. Our state-of-the-art hardware provides unparalleled sensitivity and rugged instrumentation results in maximum uptime. Cliquid® Software combined with iMethod™ tests can get your laboratory running the most commonly requested routine assays quickly and effortlessly. Table 2 is an overview of various clinical research applications and the solutions AB SCIEX provides. Whether you are seeking an ultrasensitive assay for quantification of a single analyte or a high-throughput, multi-component method, AB SCIEX can customize a solution to meet the needs of your laboratory.

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Headquarters
353 Hatch Drive | Foster City CA 94404 USA
Phone 650-638-5800
www.absciex.com

International Locations
For our office locations please call the division headquarters or refer to our website at
www.absciex.com/offices