

## Sex Hormone Testing by Mass Spectrometry

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### Learning Objectives

After this presentation you should be able to:

1. Describe the analytical reasons for measuring sex hormones by mass spectrometry.
2. Determine the concentration of standards using extinction coefficients.
3. Describe approaches to measuring estradiol using mass spectrometry.
4. Describe harmonization efforts for improving utility of sex hormone measurements.

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### Sex Steroids

- Interact with androgen and estrogen receptors, present in all tissue
- Testosterone promotes secondary male sexual characteristics
- Estrogens promote secondary female sexual characteristics

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## Why Measure Testosterone

- Males: hypogonadism, delayed or precocious puberty, monitor testosterone therapy, anti-androgen therapy
- Females: idiopathic hirsutism, congenital adrenal hyperplasia, polycystic ovarian syndrome, androgen secreting tumors, bone health

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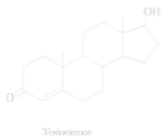
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## Testosterone

- First isolated in June 1935 by Laqueur
- Isolated 10 mg from 100 kg of steer testes
- $C_{19}H_{28}O_2$
- Molecular weight 288.41
- Insoluble in water
- Soluble in alcohol, ether, etc.
- UV molar absorptivity 15100 @ 241 nm

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## History of Measurements

- 1960s - Bio-assays used for Testosterone
- Late 60s - Electron capture GC (20 mL)
- SHBG ligand binding assays (< 10 mL)
- 1970s - RIA for testosterone (< 1 mL)
- 1990s - Automated direct immunoassays
- 1995ish - Mass spectrometry-based assays

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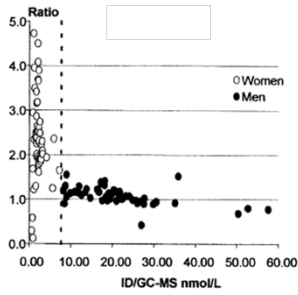
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### Direct Total Testosterone -Worst



Taieb et al. *Clin Chem* 2003;49:1381-95.

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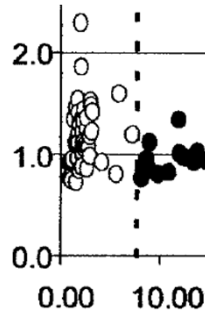
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### Direct Total Testosterone - Best



Taieb et al. *Clin Chem* 2003;49:1381-95.

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0021-9725/03/4911-000  
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89:2-520-524  
Copyright © 2003 by The Endocrine Society  
doi: 10.1210/2003-02275

#### Editorial: Serum Testosterone Assays—Accuracy Matters

*Clinical Chemistry* 49, No. 8, 2003

#### Editorial

#### *Immunoassays for Testosterone in Women: Better than a Guess?*

**Conclusion:** None of the immunoassays tested was sufficiently reliable for the investigation of sera from children and women, in whom very low (0.17 nmol/L) and low (<1.7 nmol/L) testosterone concentrations are expected.

© 2003 American Association for Clinical Chemistry

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## Standard Preparation

- Analytical balance (10 to 100 mg)
- Quantitative transfer to class A volumetric
- Dilute to 1 mg/mL with methanol
- Dilute 1:100, verify concentration by UV
- Analyze vs standards in use
- Store stock as 1 mg/mL, dilute for working standards

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## UV concentration check

Absorbance =  $\epsilon \times \text{concentration} \times \text{length}$

$$\epsilon = 15100 \text{ L/mol} \cdot \text{cm}$$

Dilute stock standard from 1 mg/mL to 10 ng/ $\mu$ L. Measure absorbance at 241 nm.

Ex: 10 ng/ $\mu$ L solution has  $A_{241} = 0.540$  using a 1 cm cuvette. The concentration by UV would be 10.3 ng/ $\mu$ L. Therefore the prepared standard is within 3% of the target value.

Chem Pharm Bull 1964;12:1217, USP vol 21 page 1024

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## Comparison of ID GC/MS for Women

GC/MS	GC/MS	% Error
1.08	1.15	6.5
3.84	4.00	4.2
6.29	6.70	6.5
1.00	0.99	1.0
1.57	1.56	0.6
1.81	1.86	2.7
1.34	1.30	3.0

Concentrations in nmol/L

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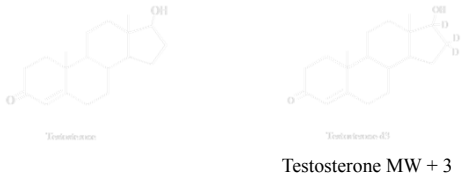
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## Internal Standard

- Each deuterium adds mass of 1 to molecule
- Typically 3 deuteriums is sufficient



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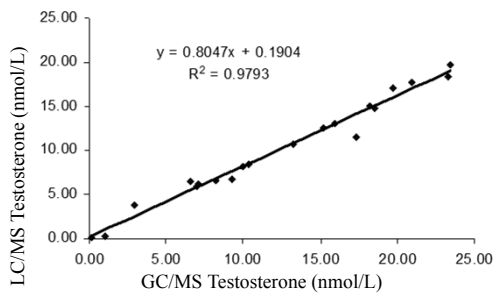
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## GC/MS and LC/MS



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## MS Is NOT Perfect

- GC/MS and LC/MS well correlated
- Methods differ by 20% consistently
- Difference explained with UV check of LC/MS standards
- Reinforces need for thorough validation
- When properly validated, MS is accurate and precise

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## Methods for analysis

- Most assays use an extraction step
- EI, NCI, ESI, APCI, and APPI all work
- GC/MS requires derivatization
- LC/MS successful w/ and w/o derivatization

Kushnir et al. *Clinical Chemistry* 2006;52:120-8  
Fitzgerald et al. *Methods in Molecular Biology* 2010;603:489-500

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## ESI Product Ion Spectra

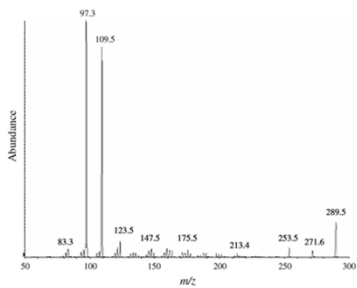


Fig. 48.4. Full scan ESI MS-MS spectra of underivatized testosterone. Prominent ions that are monitored for selected reaction monitoring are 97 and 109  $m/z$ .

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## Interferences with testosterone

- Epi-testosterone, and DHEA are isomers of testosterone
- DHEA does not ionize well with ESI
- Epi-testosterone chromatographically separated
- Plasma separator gel tubes can interfere

Shim, et al. *Clinical Biochemistry* 2012;45:1706-9

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## Testosterone Reference Ranges

- Framingham data best to date
- 348 to 1196 ng/dL is inner 95% for total testosterone
- Healthy young men 19 – 40 yo
- Men with low testosterone had higher prevalence of sexual dysfunction, physical problems, and diabetes

Bhasin, et al. *J Clin Endocrinol Metab* 2011, 96(8):2430-9

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## How good is good enough?

If pre-analytical error is minimized, and the analytical variation ( $CV_a$ ) is considerably less than the biological variability ( $CV_b$ ), serial testing can be used to determine if a patient has improved or not.

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Table 2. Analytical performance goals for testosterone measurements on the basis of biological variability.<sup>a</sup>

CVa goal	Bias goal	TE goal
Minimal (0.75 CV), 8.0%	Minimal $[0.375 (CV^2 + CV_b^{1/2})]$ , 9.5%	Minimal acceptable performance, 25.1%
Desirable (0.50 CV), 5.3%	Desirable $[0.25 (CV^2 + CV_b^{1/2})]$ , 6.4%	Desirable acceptable performance, 16.7%
Optimal (0.25 CV), 2.7%	Optimal $[0.125 (CV^2 + CV_b^{1/2})]$ , 3.2%	Optimal acceptable performance, 8.4%

<sup>a</sup> Mean CVi in the literature review, 10.6%; mean CVg in the literature review, 23.1%.

Yun et al. *Clinical Chemistry* 2012;58(12):1703-10

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## Estradiol

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## Why Measurement Estradiol?

- Evaluate female reproductive status
- Gynecomastia due to estrogen tumors
- In vitro fertility
- Hormone replacement therapy
- Anti-estrogen therapy (aromatase inhibitors)

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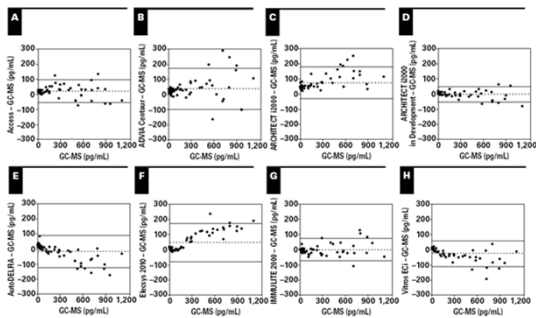
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## How are we doing?



Am J Clin Pathol 2004;112:332-337  
DOI: 10.1309/A2R4H4GMAACPBY

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## Endocrine Society Position

- Modern immunoassays and LC/MS/MS are reasonably well-suited for infertility...
- ...but the very low concentrations that appear to be crucial in nonreproductive tissues are a separate and more difficult issue.
- Such levels are too low to be routinely measured accurately or precisely, and further evolution of analytical methods required.

*J Clin Endocrinol Metab* 2013; 98(4):1376-87

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## Practical Issues

- Estradiol present at low concentrations
- Ranges from 1 pg/mL to 3000 pg/mL
- Extractions required
- Derivatization often utilized
- Dansyl derivative increases sensitivity 1000 fold

Tai, et al. *Analytical Chemistry* 2005;77:6359-63

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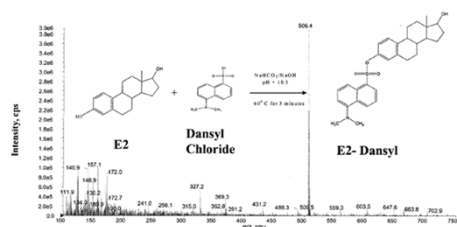
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## Derivatization Reaction for Estradiol



Nelson, et al. *Clinical Chemistry* 2004;50(2):373-84

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## Direct analysis of Estradiol

- Add IS and ppt proteins
- Heat at 95°C for 3 minutes
- Inject into 2 dimensional LC
- ESI with negative ion mode
- Fluorine in mobile phase
- Summed 5 identical MRMs
- No comparisons with RMP...

Pauwels, et al. *Anal Bioanal Chem* 2013. DOI 10.1007/s00216-013-7259-5.

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## Harmonization of Measurements

- CDC HOrmone STandardization HOST (<http://www.cdc.gov/labstandards/hs.html>)
- PATH Pathway for Accurate Testing of Hormones
- NIST SRM 971-2 Testosterone
- CRM BCR-576 Estradiol
- CAP accuracy based survey (Testosterone and Estradiol)

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## Best practices

- Measure testosterone using well validated isotope dilution mass spectrometry methods
- Begin with single assays and move to multiplex
- Gain experience prior to implementing estradiol assays
- Base calibration on NIST materials
- Participate in accuracy based PT

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Thanks for your attention.

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