

# LC-MS/MS in the clinical laboratory

## *Challenges and advances in steroid hormone analytics*

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IFCC Rome 2023



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EASTERN FINLAND



# Number of publications (LC-MS/MS) of steroids

- Significant amount of publications in steroid hormone analysis with LC-tandem mass spectrometry during the 21<sup>st</sup> century
- PubMed contains articles and reviews between years 2001 – 2023:  
LC-MS/MS methodology and
  - Steroids - 3127 publications
  - Testosterone - 671 publications
  - Estradiol- 495 publications
  - Progesterone – 368 publications
  - Cortisol – 498 publications
  - Aldosterone 138 - publications



# Should we use only LC-MS/MS for reliable steroid analysis ?

## **Requirement for Mass Spectrometry Sex Steroid Assays in the *Journal of Clinical Endocrinology and Metabolism***

D. J. Handelsman and L. Wartofsky

ANZAC Research Institute (D.J.H.), Concord Hospital, University of Sydney, Sydney, NSW 2139, Australia; and Department of Medicine, Washington Hospital Center (L.W.), Washington, DC 20010

J Clin Endocrinol Metab, October 2013, 98(10):3971–3973

It is timely to recognize that for high-impact clinical research, the steroid immunoassay era is gradually drawing to a close, and for direct immunoassays it is effectively over. Over recent years an increasing number of manuscripts submitted to the *JCEM* have been rejected, largely for their reliance on direct steroid immunoassays as major endpoints. Because the *JCEM* remains a leader in publishing clinical endocrinology research, reflected in consistently high bibliometrics (total citations, Eigenfactor score), the *Journal* is taking the next step (24) in setting acceptable assay standards for the field in upgrading its submission requirements for publication of studies using sex steroid measurements. Effective January 1, 2015, manuscripts reporting sex steroid assays as important endpoints must use MS-based assays including reporting or citing their methods with sufficient detail to allow them to be reproduced together with standard quality control, specificity, and reproducibility metrics. Some limited ed-



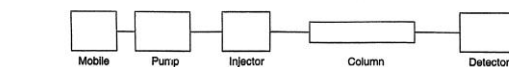
# Problems in direct immunoassays for steroid hormone

- Low specificity (cross reactions with metabolites and structurally similar compounds)
- Low sensitivity (estrogens, aldosterone, salivary cortisol)
- Inadequate accuracy and significant bias at low steroid levels (children, women)
- Interfering substances (heterophilic antibodies, HAMA, paraprotein, drugs, biotin etc.)
- Limited interest of manufacturers to produce and maintain immunoassays for some rare or new steroids (for ex. androstendiols, androgen and estrogen metabolites)

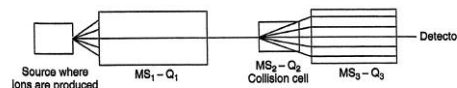


# Some features of steroid analytics with LC-MS/MS

- Steroid hormone extraction (serum, urine, tissue, other)
  - **Ultra pure**, borosilicate tubes (no plastic), deuterated and C13 internal standards
- Semi-automation (pipetting stations)
  - **Liquid-liquid extraction**
  - Solid-phase-extraction (C18) (SPE)
  - Solid-phase-liquid extraction (SLE)
- Chromatographic separation (LC)
  - **Chromatography** (separation)
  - HPLC vs. UPLC (fast run in LC, less than 3 min)
- Ionization and ion source
  - **ESI** (electrospray ionization)
  - APCI (atmospheric pressure chemical ionization)

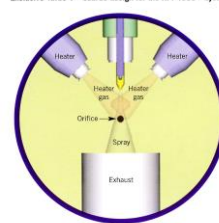


Block diagram of a typical HPLC system.



Schematic of a triple quadrupole mass spectrometer.

Exclusive Turbo V™ source design for the API 4000™ system



With the new Turbo V™ source, you can switch between APCI and TurbolonSpray™ probes in seconds. Determining the optimal ionization technique during method development is now simpler and faster.

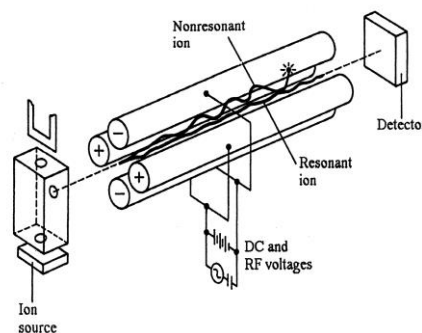


Figure 2.3: Linear quadrupole mass spectrometer.

- Separation of ions with mass analyzer and detection and quantification with mass detector
  - MS/MS-fragmentation – **specific parent and daughter ions**, low noise background
- Quantitative analysis
  - SIM (selective ion monitoring)
  - **SRM or MRM** (selected or multiple reaction monitoring)
- Sensitivity
  - Careful **tuning** of the analyzer and **validation and calibration** of the methods
  - **Sensitivity** (high S/N-ratio)



# LC-MS/MS equipment

API 3000™ LC/MS/MS System



API 2000™ LC/MS/MS System



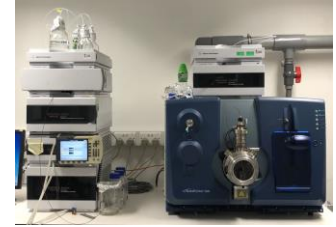
Gilson



Api Sciex 4000 QTRAP LC/MS/MS System



Sciex 5500 LC/MS/MS System



Sciex 6500 citrine LC/MS/MS System







# Steroid hormone LC-MS/MS assays in routine and research at Helsinki University and HUSLAB (since 2005)

## Steroid method project (LC-MS/MS)

Turpeinen Ursula, PhD

Hämäläinen Esa, MD

Stenman Ulf-Håkan, MD

More than 40 publications and reviews: New LC-MS/MS methods and their clinical applications (during the years 2005-2022)

Analyte	M <sub>r</sub>	Mode	Fragmentation	Adduct or derivative	Injected μl	API 2000 S/N = 10 nM	API 3000 S/N = 10 nM	API 4000 S/N = 10 nM
11-Deoxycortisol	346.5	ESI +	347.2/109.1	-	25	0.7	0.07	
17-OH-Pregnenolone	332.5	ESI +	350.4/297.5	NH <sub>4</sub>	25	n.d.		
17-OH-Progesterone	330.5	ESI +	331.2/97;109	-	25	0.2	0.05	0.05 (10 μl)
Aldosterone	360.5	ESI -	359.2/189;331	-	40	n.d.	0.02	0.015
Allotetrahydrocortisol	366.5	ESI -	365.2/335	-	5	1,3	0.03	
Androstendione	286.4	ESI +	287.1/97.2	-	25	0.1	0.05	0.02 (10 μl)
Corticosterone	346.5	ESI +	347.4/121.1	-	25	n.d.	0.1	
Cortisol	362.5	ESI -	361.1/331	-	25	0.3	0.07 (5 μl)	0.02
Cortisone	360.4	ESI -	359.1/328.8	-	5	2,3	0.03	
DHEA	288.4	ESI +	306.3/253.1	NH <sub>4</sub>	25	n.d.	1,3	1
DHEA	288.4	ESI +	271.2/213.1	-	40	n.d.	n.d.	0.6
Dihydrotestosterone	290.5	ESI +	291.1/255.1	-	40	21	0.1	0.05
Estrone	270.4	ESI -	269.4/145.0	-	40	n.d.	0.02	0.02 (25 μl)
Estrone	270.4	ESI +	504.1/156;171	dansylated	10	n.d.	0.01	0.005
Estradiol	272.4	ESI -	270.9/183.0;145.2	-	40	n.d.	n.d.	0.015
Estradiol	272.4	ESI +	506.3/156;171	dansylated	10	n.d.	n.d.	0.005
Pregnenolone	316.5	ESI +	334.2/281.1	NH <sub>4</sub>	40	20	1	n.d.
Pregnenolone	316.5	ESI +	317.2/159.2;299.3	-	40	n.d.	n.d.	0.8
Progesterone	314.5	ESI +	315.3/109.3	-	25	0.1	0.03	0.02
Testosterone	288.4	ESI +	289.1/97.2	-	25	0.1	0.03	0.01
Tetrahydrocortisol	366.5	ESI -	365.2/335	-	5	1	0.07	
Tetrahydrocortisone	364.5	ESI -	363.1/333.3	-	5	1	0.03	
25-OH-D3	400.6	ESI +	401.5/383.6	-	25	n.d.	0.7	1,5
25-OH-D2	412.7	ESI +	413.3/395.5	-	25	n.d.	0.7	2,1



# **Some practical examples in clinical steroid hormone analytics**

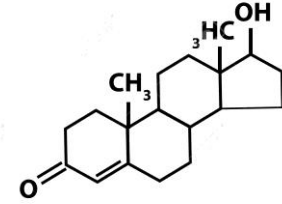
- Testosterone
- Estradiol-17-beta
- Aldosterone





# Testosterone analysis in clinical practice

Testosterone



## ■ Males:

- **Testicular function** (hypogonadism)
- Follow-up of **ProstateCa treatment** (chemical castration)

## ■ Females:

- Diagnosis and follow-up of treatment for hyperandrogenism
- **Diagnosis of hyperandrogenism**
- **Screening of hormonally active tumors**

## ■ Children:

- Hormonal dysfunction in puberty, delayed or premature adrenarche
- Treatment of CAH (21-OH-lase etc.)

## ■ Challenges (with immunoassay)

- Inadequate sensitivity at low testosterone levels (< 2 - 10 nm/l)
  - Children, women, hypogonadal men
- Specificity
  - Cross-reactions with androgens and drugs
- Precision at low levels
  - Poor precision considering clinically important cut-off limits



# Problems with direct immunological testosterone methods

*Good correlation, but significant positive bias ( below 3,47 nmol/l)*

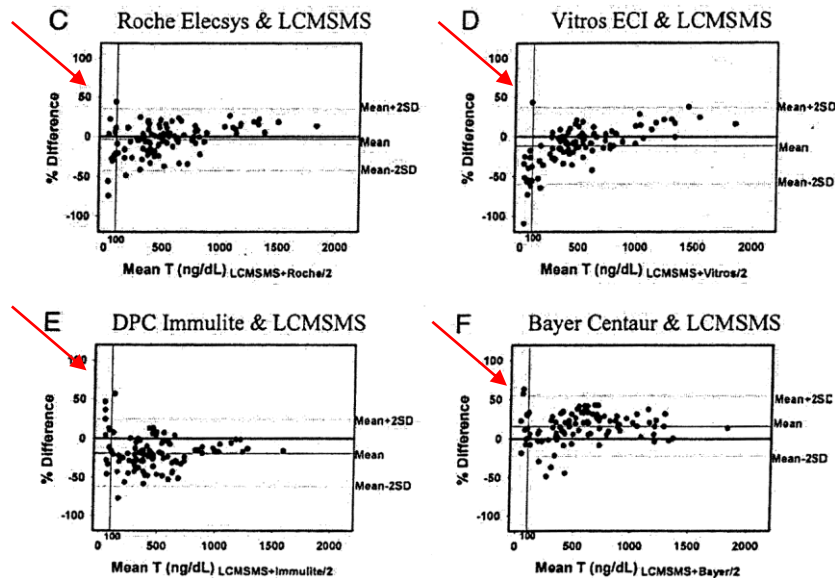
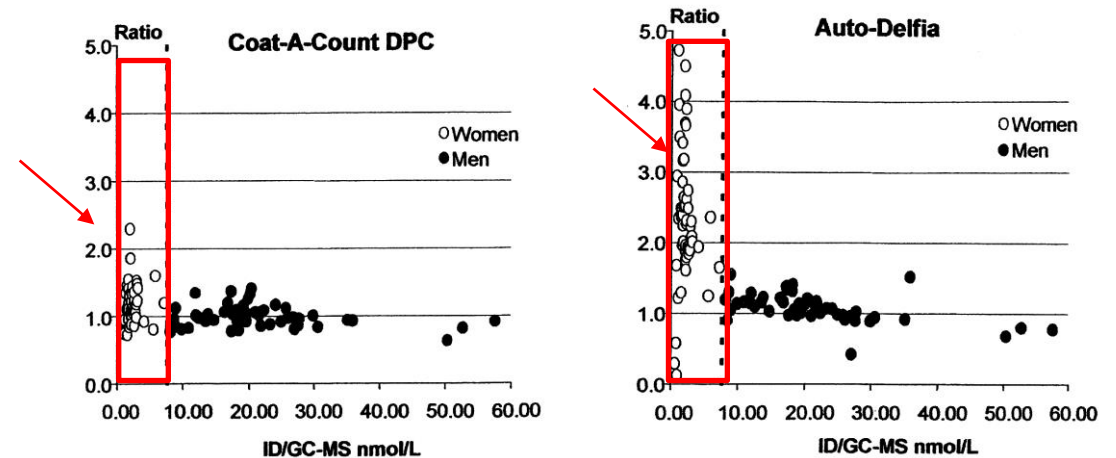


FIG. 3. Plots of percentage differences in serum T levels (test minus LC-MSMS) against the average of the two methods. The **bold solid line** represents 0%, the **light solid line** the mean percentage difference between the methods, and the **dashed lines** 2 SD of the mean percentage difference.

*Overestimation of testosterone (2,0 nmol/l) in hypogonadal men and women and significant bias below 1,7 nmol/l*



Wang C. et al., J. Clin Endocrinol Metab 2004; 89:534-543

Taieb J. et al. Clin. Chem. 2003, 49:1381-1395



**Herold D.A. and Fitzgerald R.L., Clin. Chem. 49:1250-51, 2003**

**” Laboratory professionals should not be associated with a test where an educated guess would provide an equivalent or better result”.**

- Bias in serum testosterone at female range is 200-500% !
- If testosterone immunoassay results is compared with random number-generator, the generator gives better correlation with ID-GC-MS than some of the immunoassays
- If a clinician knows the patient anamnesis, the clinical guess about testosterone level correlates better with ID-GC-MS than most of the immunomethods !!

**Bhasin S., et al.. Testosterone therapy in men with hypogonadism: an Endocrine Society clinical practice guideline. J. Clin. Endocrin. Metab. 2018;103:1715–44, LC-MS/MS or CDC approved method is suggested.**

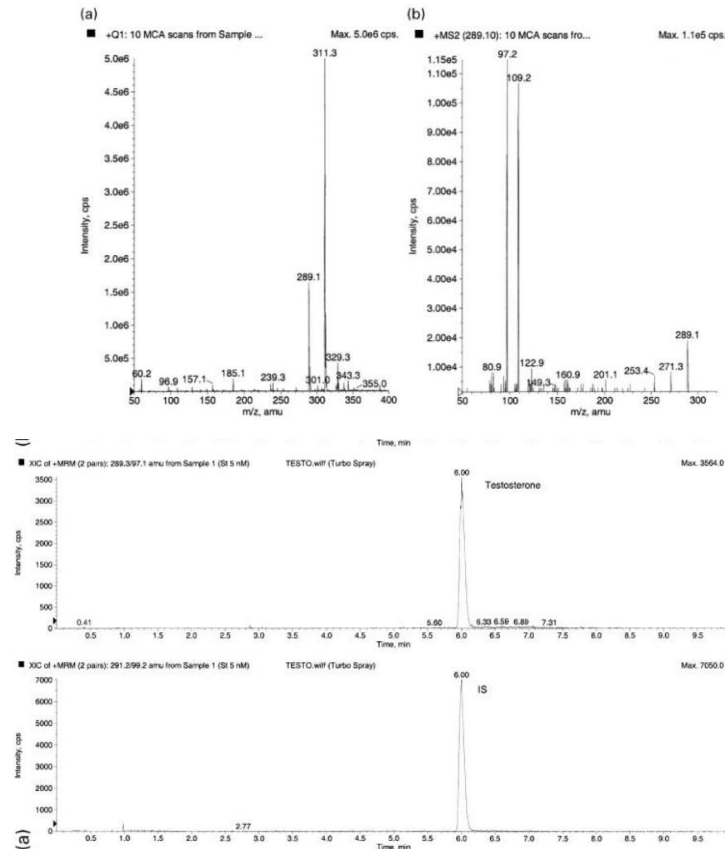


# Serum testosterone with LC-MS/MS

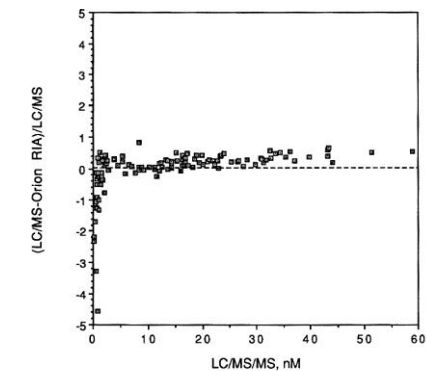
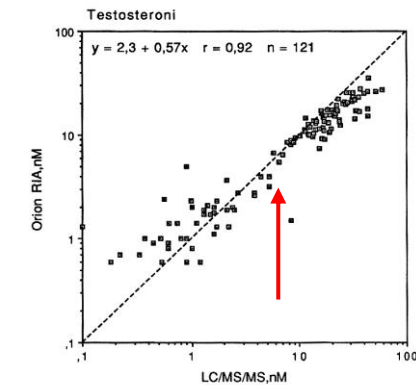
Turpeinen U, et al., Determination of testosterone in serum by liquid chromatography-tandem mass spectrometry. Scand J Clin Lab Invest. 2008;68(1):50-7.

## ■ Principle: DEE-ethylacetate-extraction and LC-MS/MS

- Serum sample: 250 - 500 ml + d<sub>2</sub>-testosterone
- Chromatography: MeOH, C18-column (2,1x50 mm)
- LC-MS/MS: ESI positive ion mode,
  - testosterone 289.3/97.1
  - D<sub>2</sub>-testo 291 /99.2
- Run-time 10 min
- Linearity **0,1 - 50** nmol/l
- Sensitivity (S/N=10) =
  - API 3000: **0.030** nmol/l



## Spectria (Orion Diagnostica) vs. LC-MS/MS





# Testosterone in EQA-rounds (Labquality Ltd) 2020

LC-MS/MS

Serum Testosterone interlaboratory range  
15,1 – 33,8 nmol/l (2020)

## ■ Testosterone Mean ( range, nmol/l)

•

*LC-MS/MS* 23,6 (21,9 - 26,2)

•

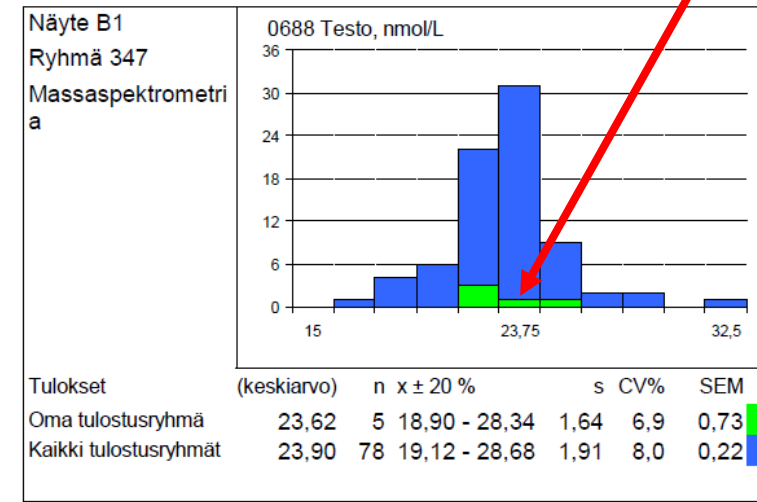
## • Immunoassays:

- Roche 24,4 (22,3 – 33,1)
- Siemens Atellica/ 22,31 (19,8 - 25,0)
- Advia Centaur
- Architect 24,1 (23,1 – 26,1)

•

• 23,90 nmol/l - bias  $\pm$  6,4 % = 1,53 nmol/l ( 22,4 – 25,42)

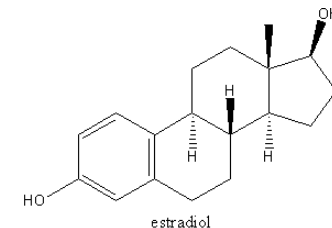
• 8,00 nmol/l - bias  $\pm$  6,4 % = 0,512 nmol/l (7,49 – 8,51)



Tutkimus	Tulostusryhmä	x	med	s	CV%	n	Min	Max	Hyläkearvo	Maanpäälis	gkm
Hormonilaboratorien yhteinen EQA-ryhmä B1											
Testo, nmol/L											
Abbott Alinity		26,96	27,3	2,19	8,1	0,77	23,6	29,9	20,7 - 33,8	0	8
Abbott Architect		24,07	23,5	1,41	5,8	0,70	23,1	26,1	19,3 - 27,8	0	4
Beckman Coulter Access & Unicel		21,44	22,5	1,94	9,1	1,12	19,2	22,6	16,7 - 28,3	0	3
bioMerieux Vidas		17,05	17,0	-	-	-	-	-	17,0 - 17,0	0	1
CIS-Bio CT		20,70	20,7	0,42	2,0	0,30	20,4	21,0	19,4 - 22,0	0	2
Massaspektrometria		23,62	23,5	1,64	6,9	0,73	21,9	26,2	18,6 - 28,4	0	5
Roche cobas e, Elecsys, & Modular E		24,41	24,5	0,98	4,0	0,20	22,3	33,1	22,9 - 25,9	0	7
Siemens Advia Centaur & Atellica		22,31	22,0	1,76	7,9	0,53	19,8	25,0	16,7 - 27,3	1	11
Siemens Immulite 1000, 2000, 2500		21,07	20,1	1,67	7,9	0,97	20,1	23,0	15,1 - 25,1	0	3
Snibe Diagnostic Maglumi		22,94	22,9	-	-	-	-	-	22,9 - 22,9	0	1
Tosoh AIA		24,84	24,8	1,47	5,9	1,04	23,8	25,9	20,4 - 29,3	0	2
Kaikki		23,90	24,0	1,91	8,0	0,22	17,0	33,1	21,0 - 26,8	1	15



# Estradiol analysis in clinical practice



## ■ Females:

- Screening of ovarian function during IVF
- Follow-up of antiestrogen (antiaromatase) treatment in breast ca patients

## ■ Children:

- Hormonal dysfunction in puberty, delayed or premature adrenarche

## ■ Males:

- Hypogonadism

## ■ Challenges (with immunoassay)

- Immunoassay works with high E2 levels (f.ex. IVF patients)
- Inadequate sensitivity and precision at low E2 levels (LLOQ varies from **19 to 40 pmol/l**, significant increase in CV% below 100 pmol/l)
  - Post-menopausal women and Brca patients with antiaromatase treatment
  - Pediatric and male estrogen levels
- Specificity
  - Cross-reactions with estrogen metabolites (catechol estrogens, estrogen conjugates)

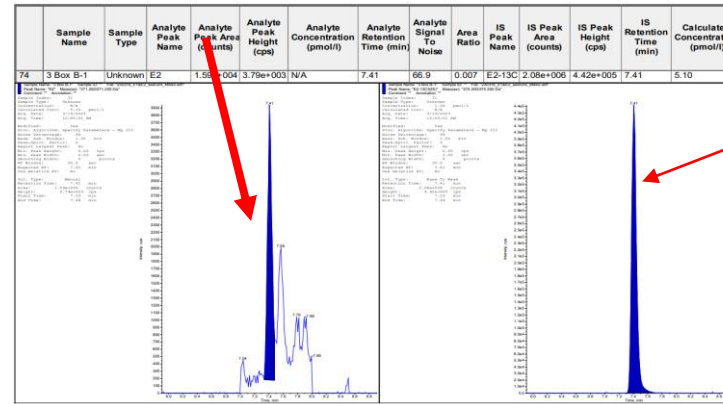




# Serum Estradiol-17beta with LC-MS/MS

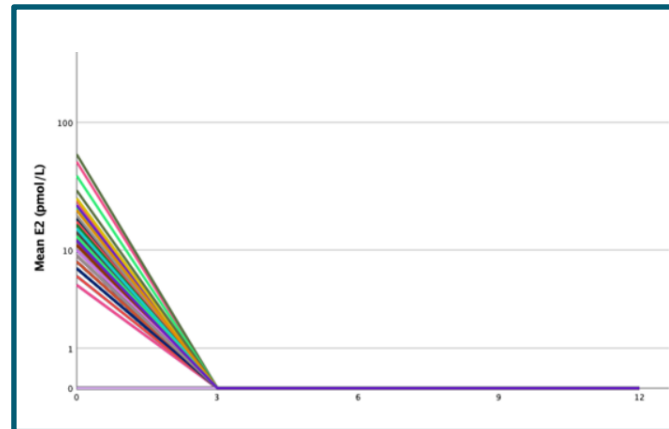
- **Principle of LC-MS/MS** (*Haanpää M, Turpeinen U, Hämäläinen E*)
- Agilent 1200 HPLC, Sciex triple quad 5500 MS/MS
- $^{13}\text{C}_3$ -int.std. E2 and E1
- DEE extraction
- Discovery HS F5-3 column (2.1 × 100 mm, 3  $\mu\text{m}$  and SunFire C18 column (2.1 × 50 mm, 3.5  $\mu\text{m}$ )
- Gradient : 40  $\mu\text{mol/l}$  ammonium fluoride in water (A) and methanol (B) at a flow rate of 300  $\mu\text{L/min}$
- Run time 10 min, MRM
- LLOQ < 5 pmol/l

Serum **estradiol** , post-menopausal women (5,1 pmol/l)



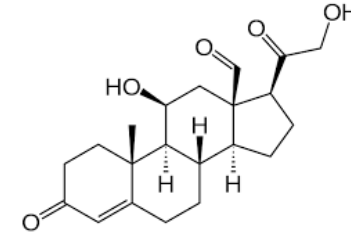
Int-Std.

Serum Estradiol-17beta levels below 5 pmol/l in post-menopausal BrCa patients with letrozole treatment (Faltinova et al. 2023, submitted)





# Aldosterone and LC-MS/MS



## ■ Females and males:

- Diagnosis of endocrine hypertension (primary hyperaldosteronism, PHA)
- Screening of PHA is based on serum aldosterone to renin –ratio (ARR) and salt loading test
- AV-difference by catheterization

## ■ Challenges in immunoassay

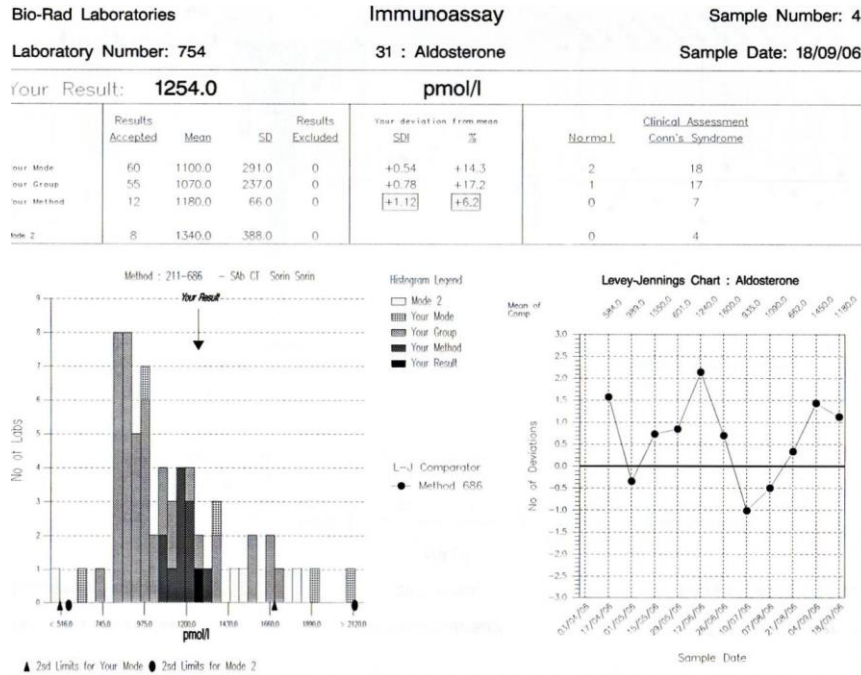
- Cross-reaction / overestimation of S-Aldos in immunoassay
- Large variation in serum/plasma aldosterone levels
  - low when suppressed (20 – 100 ng/l)
  - Extremely high in adrenal vein catheterization (5000 ng/l or more)



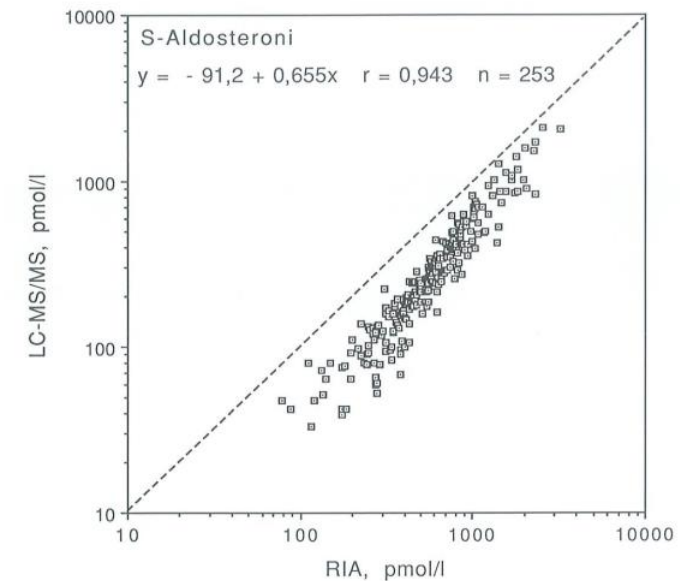
# Aldosterone with LC-MS/MS

Stowasser M. and Gordon R.D. "Aldosterone Assay: An urgent Need for Improvement", Clinical Chemistry, Editorial, 2006; 52: 1640-42

## Bio-Rad EQAS



LC-MS/MS vs Liason RIA 35 % lower, LLOQ = 30 pmol/l



Turpeinen U, Hämäläinen E, Stenman UH. Determination of aldosterone ... J Chromatogr B Analyt Technol Biomed Life Sci. 2008; 862(1-2):113-8.



# Problems in direct serum 17OH-progesterone immunoassay and immunoassay of serum cortisol and urinary cortisol HPLC-UV

- **Problems in Cortisol immunoassay**
- Cross-reactions (steroids and drugs)
  - Prednisone, prednisolone (S-, U-)
  - Carbamazepine (U-)
- Limited sensitivity (20 nmol/l)
- Commercial immunoassays not suitable for salivary cortisol (cut-off less than 3 nmol/l)
- Reference intervals
- Adjusted to pediatric age

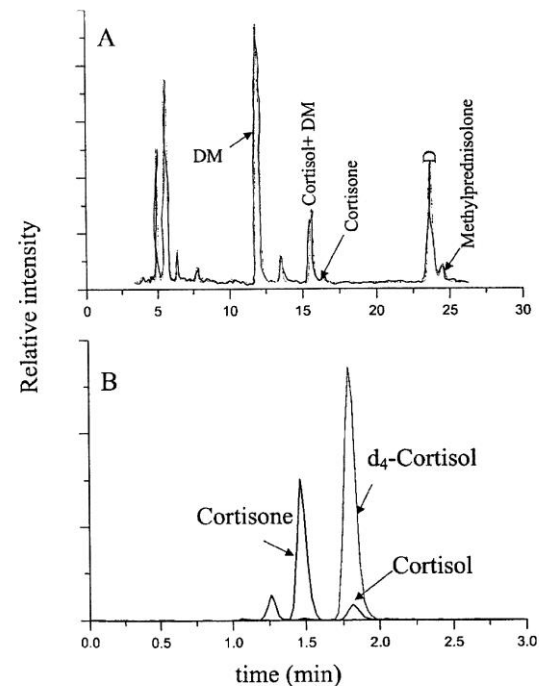


Fig. 5. Chromatograms demonstrating the presence (A) or absence (B) of interference by carbamazepine and its metabolites in the LC-UV and LC-MS/MS methods, respectively.

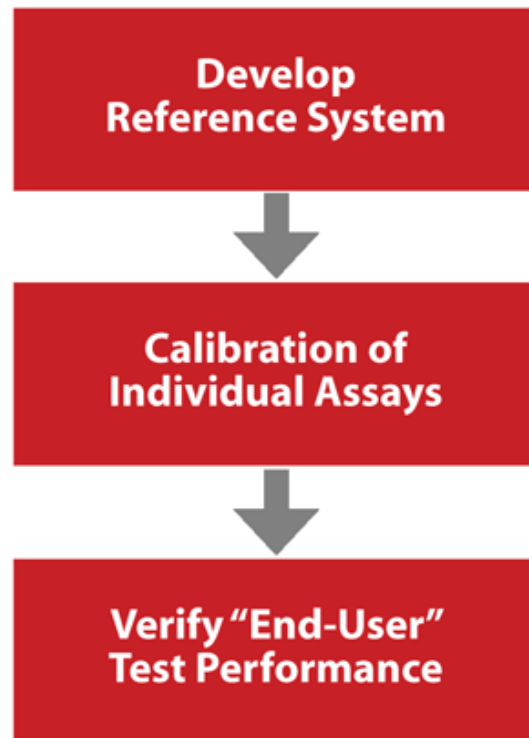
(A), chromatographic interference attributable to carbamazepine (D) and its metabolites (DM) can be seen in the LC-UV method for UFC, which also has a longer run time. (B), the presence of carbamazepine and its metabolites in the same specimen has no effect on the quantification of cortisol by the LC-MS/MS method.

- **Problems in 17OH-Progesterone immunoassays**
- High amount of false positive in CAH screening
  - Cross-reactions (steroids ) in newborns
  - Sulphate and glucuronide conjugates
- Methodological variation

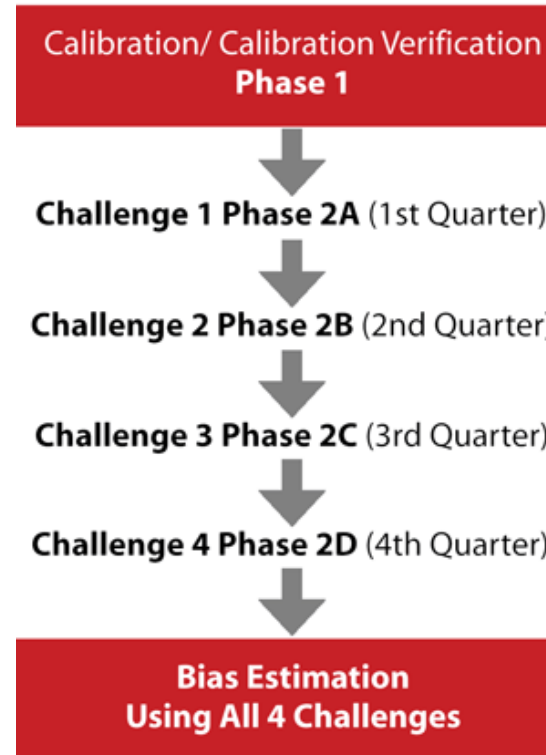


# CDC HoSt Program (hormone standardization program)

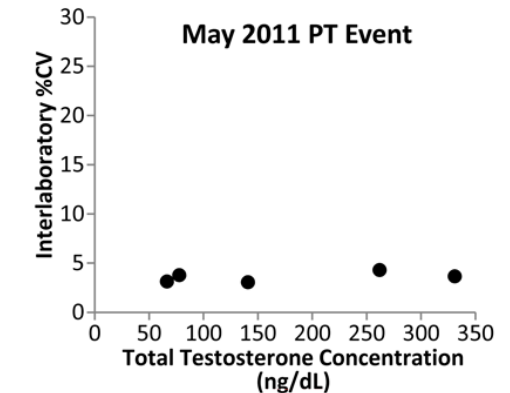
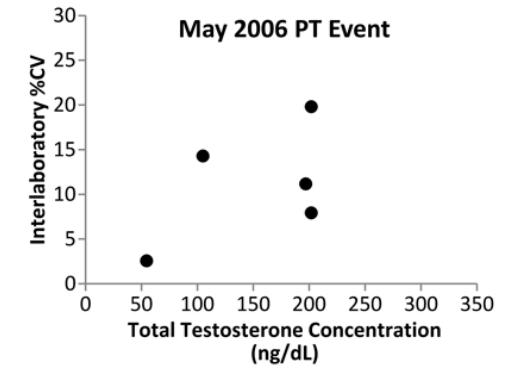
Hubert W. Vesper and Julianne Cook Botelho, Clinical Laboratory News, 2012



**Figure 1** Steps for Achieving Accurate and Comparable Patient Results



**Figure 2** CDC Hormone Standardization (HoSt) Program Process



**Figure 3** Testosterone Interlaboratory Variability



# LC-MS/MS of steroid hormones in future

- Commercial LC-MS/MS applications and protocols for main clinically important steroid hormones
- Automation prior to LC-MS/MS (SPE, 96-well plate technique, pipetting equipment, batch-analysis)
- Multiplexing techniques to improve capacity
- Metabolomics and steroidomics coming to routine
- Fully automated analyzers with UPLC-MS/MS technology
- Laboratory robotics



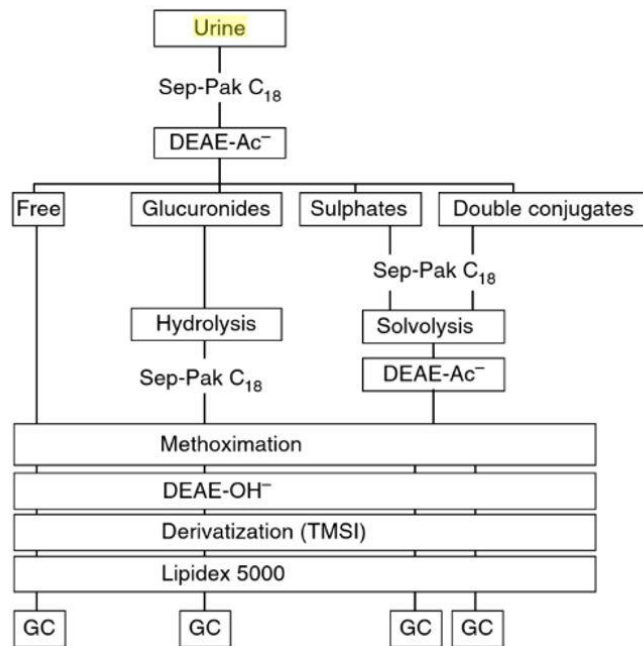




# Steroid hormone profiling in the 1990s (GC-MS)

[Hämäläinen E et al.](#)

In Book: **Steroid Analysis**, Edit. Hugh L. J. Makin, D.B. Gower,  
Springer Science 2010



**Fig. 3.10** An example of the use of modified Sephadex for the fractionation of urinary neutral steroids prior to GLC analysis (from Hamalainen *et al.*, 1991., with permission)

[Hämäläinen E<sup>1</sup>](#), [Fotsis T](#), [Adlercreutz H](#)

A gas chromatographic method for the determination of neutral steroid profiles in urine, including studies on the effect of oxytetracycline administration on these profiles in men. [Clin Chim Acta](#). 1991; 14;199(2):205-20



# Summary: **Challenges** and **advances** of LC-MS/MS in steroid analysis

- Price of the equipment (> 300 000 euros)
- Education of personal and specialists
- LC-MS/MS equipment and IT-programs are designed for R & D, not for routine
- Capacity and turn-around time (TAT)
- Ionization problems with some steroids (matrix effect, ion suppression, low sensitivity)
- Stable isotope internal standard ( $^{13}\text{C}_3$  /deuterated) is expensive or not available
- Lack of certified calibration standards for several analytes (large interlaboratory variation)

- High specificity and sensitivity !
- Inherited problems in immunological methods can be avoided
  - Cross-reactions, no interference (heterophilic and HAMA, drugs) and Quality fluctuations in commercial immunoassay kits
- Cost benefit can be achieved, if sample load is large (batch) or using preanalytical automation, multiplexing technique and simple pre-purification procedures and short run times with UPLC (3-5 min)
- Several applications for clinically important steroids
- Multiple steroid profiling, metabolomics-steroidomics
- Fully automated, random access analyzers are coming



# Thank you for your interest !

- **List of collaborators**
- At the Departments of Clinical Chemistry, Helsinki University (HUSLAB) and University of Eastern Finland (UEF, ISLAB)
  - Esa Hämäläinen, MD, PhD, professor emeritus
  - Ursula Turpeinen, PhD, Adj. professor emeritus
  - Mikko Haanpää, Bc.Sci, laboratory technician
  - Ulf-Håkan Stenman, MD, professor emeritus
  - Sofia Valtonen, Bc.Sci, student
  - Aleksanteri Petsalo, PhD, biochemist
  - Anu Olkku, PhD, clin. biochemist