Evidences of routinary clinical analysis in LC-MS/MS

Immunosuppressants and 25-OH vitamine D3 cases

Dr. Donzelli Simone

Health Sciences Business Development Manager
Southern Europe - Waters
Index

• Immunosuppresants
  ✓ Study of TDM
  ✓ Importance of TDM degli immunosuppressants
  ✓ Technics’ comparison

• Vitamine D3
  ✓ why measure 25-OH Vitamine D3
  ✓ Importance of 25-OH Vitamine D2
  ✓ Technics’ comparison
  ✓ State of arts
“Therapeutic Drug Monitoring”? 

- “Therapeutic Drug Monitoring (TDM) is a section of clinical chemsistry specialized in measurements of the levels of the drugs in blood. Its principal objectives is focused on drugs with a very small terapeutic margin: drugs that easily can be under or over estimated.” D. Holt

- The medicians will use the measurement of drugs’ concentration in the blood to modify il dosaggio del farmaco to obtain the best result for each different patient. This individualisation of the therapy is know as Therapeutic Drug Management.
Why is TDM necessary?

The biggest part of the therapeutic drugs can present toxic effects.

Ibuprofene

Sub-therapeutic

Therapeutic

Toxic

Immunosuppressants:
optimum of the therapeutic range is near to the toxic area

Sub-therapeutic

Therapeutic

Toxic

TDM
TDM of immunosuppressants

- TDM of ISD is a strategy that controls with high accuracy the level of the drugs’ concentration in blood for each patient with several analysis during its lifetime.

- Critical factors that can help to define an exact therapeutic index:
  - Age, sex, weight
  - Dosage of drug
  - Farmakocinetic
  - Co-medicaments
  - Different technics used to measure the drugs
Variability inter-individual

- For each different patient great differences in farmakocinetic and farmacodinamic. The right therapy depend on the right individualisation of the treatment for each patient

Patient 1

Patient 2

Patient 3

Patient 4
Why TDM for immunosuppressants?

- The therapeutic range is very small → the dosage must be controlled in an opportune way;

- The difficulty to individualize the right treatment due to the wide differences inter-patient in PK/PD;

- In some cases TDM is used even to assure that the patient is taking the right amount of the drug (compliance of the patient) AntiHIV.
Technics

- Immunoassay (automated / semi-automated)
- HPLC with UV / Fluorescence
- LC-MS/MS
Platforms for analysis of tacrolimus in US 2013

- Waters 58%
- AB Sciex 30%
- Thermo Fisher 8%
- Agilent 4%

- LC-MS/MS 29%
- Abbott Architet 44%
- Altre Piattaforme IA 5%
- Siemens Dimension 22%
Growth of LC-MS/MS in TDM of immunosuppressants - NeQAS

Number of results given by different laboratories

<table>
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<th>Year/Quarter</th>
<th>Total</th>
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<th>ACMIA</th>
<th>CMIA</th>
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Why is LC-MS/MS growing?

<table>
<thead>
<tr>
<th>Deficiency Immunoassay</th>
<th>Performance Immunoassay</th>
<th>Performance LC-MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Specificity</td>
<td>Interferences of metabolites of Cyclosporine and Tacrolimus are well documented. Low end performance for some IA questionable.</td>
<td>Highest specificity: the mass and the relative fragments for each drug</td>
</tr>
<tr>
<td>Low Sensibility</td>
<td>Most of the IA have sensibility problems</td>
<td>Eccellent: &lt; 0,5 ug/L</td>
</tr>
<tr>
<td>High costs*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Comparison between IA e LC-MS/MS
LC-MS/MS is more specific than IA

- Interferences with metabolites
- Interferences from endogenous antibodies\(^1\)
- Interferences from reumatoid factors\(^2\)
- Hematocrit effect\(^3\)
- Unknown Interferences\(^4\)

\(^1\) Ther Drug Monit 2009;31(2):269-272
\(^2\) Ther Drug Monit 2009;31(6):743-745
\(^3\) Ther Drug Monit 2007;27(1):94-97
\(^4\) Clinica Chimica Acta 2010;411:77-80
Why LC-MS/MS?

- Technic considered “Gold standard” for TDM of immunosuppressants

- High specificity for each drug
  - The mass and the characteristic fragments for each drug eliminate every possible cross-reaction with the metabolites

- Higher Accuracy and precision

- Higher sensitivity (0,5 ug/L)
Why LC-MS/MS?

Immunosuppressant Immunoassays - Company Assay % Metabolite Cross Reactivity

**Siemens**
- Dimension Systems SIRO Assay: 89%
- Emit 2000 Sirolimus Assay: 61%
- ADVIA Centaur Systems CsA Assay: 16%
- Dimension Clinical Chemistry System CSA/CSE Assay: 6%
- Emit 2000 Cyclosporine Assay: 8%
- Dimension Clinical Chemistry System Tacrolimus: 18%
- Emit 2000 Tacrolimus Assay: 21%

**Abbott**
- ARCHITECT Sirolimus Assay: 37%
- ARCHITECT Tacrolimus Assay: 94%
- ARCHITECT Cyclosporine Assay: 3.3%
- TDx Cyclosporine Monoclonal-Whole Blood Assay: 96%
- IMx Sirolimus Assay: 77%
- IMx Tacrolimus/Tacrolimus II Assay: 67%

**Thermo-Fisher**
- Thermo Scientific QMS Everolimus Immunoassay: 63%
- Thermo Scientific Innofluor Certican (Eve) Seradyn: 142%
- CEDIA Cyclosporine Plus (Microgenics): 32.5%
- CEDIA Tacrolimus (Microgenics): 38%

% Metabolite cross reactivity is the highest % of cross reactivity for a specific metabolite of the parent drug.

**References**
- Advances in Chromatographic Techniques for Therapeutic Drug Monitoring – Amitava Dasgupta 2010
LC-MS/MS vs. MEIA

ORIGINAL ARTICLE


surgery, hematocrit, and weakly to serum albumin levels. In summary, the LC-MS methods provide highly reliable and reproducible estimates of tacrolimus concentrations, whereas the performance of MEIA technology did not provide reliable long-term performance for longitudinal therapeutic drug monitoring of tacrolimus because it was effected by several inherent demographic factors and by factors that can change over time in transplant recipients.
Liquid Chromatography–Tandem Mass Spectrometry Outperforms Fluorescence Polarization Immunoassay in Monitoring Everolimus Therapy in Renal Transplantation

Dirk Jan A. R. Moes, PharmD,*† Rogier R. Press, PharmD,*† Johan W. de Fijter, MD, PhD, † Henk-Jan Guchelaar, PharmD, PhD,* and Jan den Hartigh, PharmD, PhD*

Background: There is a need to monitor everolimus blood concentrations in renal transplant recipients as a result of its high pharmacokinetic variability and narrow therapeutic window. However, analytical methods to determine blood concentrations often differ in performance. Therefore, we investigated whether two

Key Words: method comparison, everolimus, renal transplantation, therapeutic drug monitoring, LC-MS/MS, FPIA

(Ther Drug Monit 2010;32:413–419)
**LC-MS/MS vs. FPIA per everolimus**

- “The analytical methods FPIA and LC-MS/MS are not in agreement. . . . using FPIA results in higher everolimus concentrations compared with LC-MS/MS.”

- “FPIA can lead to clinically relevant differences in everolimus dosage advice and higher intra-patient variability.”

- “LC-MS/MS outperforms FPIA for clinical monitoring and intervention of everolimus therapy in adult renal transplant recipients...”
Why LC-MS/MS?

- Technic considered “Gold standard” for TDM of immunosuppressants

- High specificity for each drug
  - The mass and the characteristic fragments for each drug eliminate every possible cross-reaction with the metabolites

- Higher Accuracy and precision

- Higher sensitivity (0,5 ug/L)
Cyclosporine

Cut-off: heart: 350 ng/ml

Calibrazione
Intervallo di misura: 25 – 500 ng/ml (20.8 – 413.6 nmol/l)
Materiale di calibrazione: Calibratore CSA, Num. cat. DC89

Limitazioni della procedura
I campioni dei pazienti potrebbero contenere anticorpi eterofili potenzialmente in grado di reagire negli immunoassay fornendo risultati falsamente elevati o bassi. Questo test è stato sviluppato per ridurre al minimo l’interferenza da parte degli anticorpi eterofili. Giononostante, non è possibile garantire la completa eliminazione di tale interferenza da tutti i campioni dei pazienti. Eventuali risultati incompatibili con il quadro clinico e l’anamnesi dei pazienti devono essere interpretati con cautela.10,11

Questo test è stato sviluppato per ridurre al minimo l’interferenza da parte degli anticorpi anti-β-galattosidasi. Questi anticorpi possono essere presenti nei campioni in conseguenza di un’infezione batterica o possono produrre risultati falsamente elevati potenzialmente contraddistinti con la valutazione clinica. In casi rari, gli immunodiagnostici possono produrre risultati falsamente elevati o ridotti a causa di altre sostanze interferenti specifiche per il paziente.12

Nel paziente con funzionalità epatica compromessa e nei pazienti ai quali vengono somministrati altri farmaci che potrebbero indurre o inibire l’attività enzimatica microsomal, l’utilizzo di routine dell’analisi con test Dimension® della ciclosporina nel sangue intero potrebbe essere supportato da dati HPLC per valutare pause variazioni di biotrasformazione ed eliminazione.

Ciclosporina

Dimension®
clinical chemistry system

Flex® reagent cartridge

CSA

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ARCHITECT tacrolimus package insert

This package insert must be read carefully prior to product use. Package insert instructions must be followed. Reliability of assay results cannot be guaranteed if there are any deviations from the instructions in this package insert.

LIMITATIONS OF THE PROCEDURE

- Immunoassays are nonspecific and cross react with metabolites. When elimination of tacrolimus is impaired (e.g., during cholestasis), tacrolimus metabolites may accumulate. The immunoassay may overestimate the concentration of tacrolimus. In such cases, the use of a specific assay (e.g., Liquid Chromatography Mass Spectrometry/Mass Spectrometry [LC/MS/MS]) could be considered. Refer to the SPECIFICITY section below for estimates of cross-reactivity of ARCHITECT Tacrolimus to some metabolites of tacrolimus. Refer to the METHOD COMPARISON section below for representative data comparing patient results from the ARCHITECT Tacrolimus assay to the IMx Tacrolimus II assay and an LC/MS/MS method.

### Table

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Amount Added (ng/mL)</th>
<th>Mean Excess Concentration Detected (ng/mL, n=5)</th>
<th>% Cross Reactivitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-I (13-O-demethy/tacrolimus)</td>
<td>10</td>
<td>0.8</td>
<td>8</td>
</tr>
<tr>
<td>M-II (31-O-demethy/tacrolimus)</td>
<td>10</td>
<td>9.4</td>
<td>94</td>
</tr>
<tr>
<td>M-III (15-O-demethy/tacrolimus)</td>
<td>10</td>
<td>4.5</td>
<td>45</td>
</tr>
<tr>
<td>M-IV (12-hydroxytacrolimus)</td>
<td>10</td>
<td>0.8</td>
<td>9</td>
</tr>
</tbody>
</table>

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LIMITATIONS OF THE PROCEDURE
Samples with high background intensities should be investigated. Inconsistent results should be interpreted with caution. Clinically unexpected results should be interpreted with caution. If confirmation of a result is desired, an alternate methodology such as LC/MS is recommended.

A. Accuracy
1. Recovery of spiked samples to LC/MS
   Whole blood specimens negative for Certican® were spiked with Certican® (parent drug only) across the reportable range then assayed n=3 on three different analyzers. The mean value was compared to the LC/MS value and percent recovery calculated. The LC/MS method is specific for the parent compound and does not recognize metabolites.
Why LC-MS/MS?

- Technic considered “Gold standard” for TDM of immunosuppressants

- High specificity for each drug
  - The mass and the characteristic fragments for each drug eliminate every possible cross-reaction with the metabolites

- Higher Accuracy and precision

- Higher sensitivity (0.5 µg/L)
LC-MS/MS accuracy

Accuracy

- Concentration of Tacrolimus measured with Waters MassTrak kit related to the medium value of the Neqas report of all the laboratories that are using LC-MS/MS

- Linearity:
  \[ Y = 0.998X + 0.099; \quad r^2 = 0.996 \]

- Difference 1.1% in un range di 0.0 – 30.7 ng/mL
LC-MS/MS precision

Precision
Evaluation of the Waters MassTrak in analysis of Tacrolimus in different lab
- Evaluation done in 20 days
- 3 different pool of blood prepared
- 3 centres
- LC-MS/MS shows a total imprecision <8% in the tacrolimus range between 2.04 ng/mL e 29.9 ng/mL

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (ng/mL)</th>
<th>n</th>
<th>Within run SD</th>
<th>%CV</th>
<th>Between run SD</th>
<th>%CV</th>
<th>Total SD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>2.81</td>
<td>80</td>
<td>0.10</td>
<td>3.4</td>
<td>0.07</td>
<td>2.2</td>
<td>0.14</td>
<td>4.7</td>
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<tr>
<td>Medium</td>
<td>9.00</td>
<td>80</td>
<td>0.24</td>
<td>2.7</td>
<td>0.07</td>
<td>0.8</td>
<td>0.31</td>
<td>3.4</td>
</tr>
<tr>
<td>High</td>
<td>20.03</td>
<td>80</td>
<td>0.38</td>
<td>1.9</td>
<td>0.36</td>
<td>1.8</td>
<td>0.73</td>
<td>3.6</td>
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</table>

<table>
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<tr>
<th>Sample</th>
<th>Mean (ng/mL)</th>
<th>n</th>
<th>Within run SD</th>
<th>%CV</th>
<th>Between run SD</th>
<th>%CV</th>
<th>Total SD</th>
<th>%CV</th>
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<td>80</td>
<td>0.13</td>
<td>5.7</td>
<td>0.14</td>
<td>5.3</td>
<td>0.21</td>
<td>7.9</td>
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<td>Medium</td>
<td>11.33</td>
<td>80</td>
<td>0.39</td>
<td>3.5</td>
<td>0.22</td>
<td>2.0</td>
<td>0.57</td>
<td>5.1</td>
</tr>
<tr>
<td>High</td>
<td>27.78</td>
<td>80</td>
<td>0.66</td>
<td>2.4</td>
<td>0.39</td>
<td>1.4</td>
<td>0.76</td>
<td>2.7</td>
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<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (ng/mL)</th>
<th>n</th>
<th>Within run SD</th>
<th>%CV</th>
<th>Between run SD</th>
<th>%CV</th>
<th>Total SD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
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<td>2.04</td>
<td>80</td>
<td>0.11</td>
<td>5.6</td>
<td>0.04</td>
<td>2.1</td>
<td>0.16</td>
<td>7.6</td>
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<tr>
<td>Medium</td>
<td>10.91</td>
<td>80</td>
<td>0.35</td>
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<td>0.04</td>
<td>0.4</td>
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<td>29.90</td>
<td>80</td>
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<td>2.4</td>
<td>0.51</td>
<td>1.7</td>
<td>1.12</td>
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</table>
Why LC-MS/MS?

- Technic considered “Gold standard” for TDM of immunosuppressants

- High specificity for each drug
  - The mass and the characteristic fragments for each drug eliminate every possible cross-reaction with the metabolites

- Higher Accuracy and precision

- Higher sensitivity (0.5 ug/L)
Higher sensitivity

- The co-medication of different immunosuppressants carried to a decrement of active level for therapeutic dosage:
  - the sensibility, la specificity and precision of the IA to low level of therapeutic windows starts to become critical for the monitoring of.
  - With levels near to 1 ng/ml (active concentration for pediatric tacrolimus), LC-MS/MS has definitively better performances than IA
INTRODUCTION

Simple LC/MS/MS methods have been widely adopted in clinical chemistry laboratories for the therapeutic drug monitoring (TDM) of single immunosuppressants such as Tacrolimus and Cyclosporin A. For newer immunosuppressants, such as Everolimus, this may be the only method currently available to the clinical chemist. A demand for multi-analyte methods has arisen from the increasing use of combination therapy and the need to streamline laboratory workflows. Typically, these methods use on-line SPE and are not attractive to some laboratories in their present form because they tend to reduce sample throughput. We have therefore investigated the use of advanced LC and MS/MS technology to deliver a rapid multi-analyte immunosuppressant TDM method.

METHODS

Mass Spectrometry

A Waters ACQUITY® Tandem Quadrupole Detector (TQD) coupled to an ACQUITY UPLC (Waters Corporation, Manchester, UK) was used for all analyses. The full system configuration is shown in Figure 1. The instrument was operated in positive electrospray ionization mode. All data acquisition was performed using MassLynx™ software with auto data processing using the QuanLynx™ Application Manager. The compound-dependent cone voltage was optimized to maximize the abundance of the precursor ion entering the source and selected to pass through the first quadrupole to the collision cell. Collision-induced dissociation was facilitated by argon and collision energy to produce characteristic product ions. Using this information a specific Multiple Reaction Monitoring (MRM) experiment was created and is shown in Table 1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM Transition</th>
<th>Flow Rate (nL/sec)</th>
<th>Sensitivity (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tacrolimus</td>
<td>981.3 → 834.1</td>
<td>50</td>
<td>32</td>
</tr>
<tr>
<td>Everolimus</td>
<td>975.3 → 908.1</td>
<td>40</td>
<td>32</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>1213.5 → 1216.3</td>
<td>5</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 1. MRM transitions used for the mass spectrometer to monitor the four immunosuppressants and their internal standards.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linearity</th>
<th>Control</th>
<th>Mean Conc (ng/mL)</th>
<th>Std Dev</th>
<th>%CV</th>
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<td>Tacrolimus</td>
<td>0.9987</td>
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<td>3.6</td>
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<td>Medium</td>
<td>7.3</td>
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<td>2.7</td>
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<tr>
<td></td>
<td></td>
<td>High</td>
<td>14.2</td>
<td>0.2</td>
<td>1.4</td>
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<tr>
<td>Strimilus</td>
<td>0.9975</td>
<td>Low</td>
<td>1.9</td>
<td>0.7</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>12.9</td>
<td>0.8</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High</td>
<td>25.2</td>
<td>1.5</td>
<td>5.8</td>
</tr>
<tr>
<td>Everolimus</td>
<td>0.9978</td>
<td>Low</td>
<td>3.6</td>
<td>0.3</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>12.0</td>
<td>0.5</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High</td>
<td>20.1</td>
<td>1.3</td>
<td>6.4</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>0.9966</td>
<td>Low</td>
<td>2.2</td>
<td>0.7</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>105.5</td>
<td>4.4</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High</td>
<td>217.8</td>
<td>9.2</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Table 2. Summary of the intra-day precision (n=5) determined using Chromatogram software.

Figure 5. Passing-Bablok linear regression analysis between single and multi-analyte methods is shown in Figure 5.
25(OH)-Vitamin D3/D2
25 OH - Vitamin D

What is Vitamin D?

• We cannot consider Vitamin D as a real Vitamin (not like Vitamin C that participate to the biological reaction helping the body to operate in a proper way)

• Vitamin D is quite different: the effects that produces in the body are alligned to hormones that acts to influence the metabolic pathways...

• The active metabolic form of Vitamin D, 1,25 dihydroxyvitamin D (or calcitriol) is called secosteroid hormon (pro hormon)
Metabolic pathway of 25 (OH) Vitamin D3

Cholesterol → 7-Dehydrocholesterol → UV → Previtamin D₃ → Skin temp. → 7-Dehydrocholesterol

Sunlight → Skin → 7-Dehydrocholesterol

dietary intake → Cholecalciferol (vitamin D₃) → Liver → Vitamin D₃ (fish, meat) → Vitamin D₂ (supplements) → 25-hydroxyvitamin D₃

Kidney → 25-(OH)-Vitamin D₃

CYP24 → 24-Hydroxylase → 24,25-(OH)₂-Vitamin D₃

Kidney → 1α-Hydroxylase → 1α,25-(OH)₂-Vitamin D₃

Kidney → 25-Hydroxylase → 25-hydroxyvitamin D₃

Maintains calcium balance in the body

calcitriol
Benefits of Vitamin D

- Osseus Metabolism: prevent osteoporosis, rachitism, broken bones

- Cellular health: prevent tumor (breast, prostate, pancreas, ovary, colon)

- Body health in general: prevent cardiac diseases, strokes, diabetes...

- Muscular health

- Mental health: prevent depression (stimulate production of serotonin), schizophrenia, Alzheimer,...
25 OH - Vitamin D

Measurements of Vitamin D

- In the body there are about 30 metabolites of Vitamin D. Mostly of them don’t conduct any physiological function (are only degradation products)

- Better indicator of the state of vitamin D (reservoir):
  25-OH-vitamin D₃

  - It’s the principal metabolite (product in the liver);
  - Half cicle life of 8 weeks;
  - The concentration are enough to be measured in serum/plasma: 20 – 70 µg/L;
  - For this reason is the principal indicator of the presence of Vitamin D in the body

Why the active form 1,25-(OH)₂-vitamin D₃ is not considered as principal indicator of the deficiency of Vitamin D?

- really instable (half cicle life of 8 minutes)
- amount of about 1000 times lower to 25-OH-vitamin D
Due to the fact that synthesis of vitD3 is activated by solar UV ray the medium value of Vit D3 different between summer and winter and a very low level value in zones not exposed to the sun (UK, Scandinavian, Middle East,

- Dark skinned → less Vit.D3; Pale skinned → more Vit.D3
- In UK more than 50% of the population has deficiency in the level of VitD3
- Even in Middle East → high level of the sun but the women has very low level of Vit D3. The women have serious deficiency of Vit D3 at 40 years old!)

Therepeutic Range of Vitamin D:

- Serious Deficiency: < 10 ug/L (ng/mL)
- Deficiency: 10 ug/L < Vit D < 20 ug/L
- Optimal: 20 ug/L < Vit D < 70 ug /L
25(OH)-Vitamin D3 or D2?

➢ Vitamin D3 is not present in nature (produced by the body with the metabolism process seen before). To supplement it they need to synthentized it in the lab. Expensive.

➢ Vitamin D2 has a vegetal origin. Through diet we can improve a little bit an higher concentration of Vit D2 (salads, mushrooms...).

→ this was of supplementation is cheaper because you can extract in nature.
Why is important to separate and quantify the 2 forms?

- The are significative differences in the biological activities and in the toxicity of the 2 forms:
  - The intestinal absorbance of Vit D2 is lower than Vit D3
  - The clearance of 25OH-VitD2 is faster than 25OH-VitD3 one. Due to the fact to the minor affinity of 25OH-VitD2 to the proteic D-bond (DPBV)

There are significative differences in the metabolism of these 2 forms:

Vit D2 cannot be considered efficient as Vit D3!
State of the arts of Vitamin D

Evaluation of:
- Roche
- Diasorin RIA
- Diasorin Liaison
- IDS EIA
- HPLC UV
- LC-MS/MS

- Capability to analyze 25OH vitD2 and 25OH vit D3 separately
- Capability to measure Vit D2 and vit D3 total
- Capability to see the epimer
- LOQ
- Precision
- Productivity
- Flexibility to move from one method to another
State of the arts of Vitamin D

**Evaluation of Immunoassay:**

**Diasorin:**  
Sensitivity: 4 ug/L  
Linearity Range: 4 – 150 ug/L

**IDS:**  
Sensitivity: 2.5 ug/L  
Linearity Range: 2.5 – 144 ug/L

**Abbott:**  
Sensitivity: 8.0 ug/L  
Linearity Range: 8 – 120 ug/L

**All the immunoassay:**  
- Measure Total Vitamin D  
- They cannot separate epimer of Vit. D

**Evaluation of HPLC:**

**HPLC-UV:**  
Sensitivity: 3 ug/L  
Linearity Range: 3 – 250 ug/L

**HPLC-MS/MS:**  
Sensitivity: 1.0 ug/L  
Linearity Range: 1.0 - 250 ug/L

**All the methods:**  
- Can separate and measure 25-OH vitamin D₃ e D₂  
- The LC-MS/MS can separate and quantify the epimer of the Vit. D
**State of the arts of Vitamin D**

Current assays overestimate 25-hydroxyvitamin D₃ and underestimate 25-hydroxyvitamin D₂ compared with HPLC: need for assay-specific decision limits and metabolite-specific assays

P Glendenning¹, M Taranto¹, JM Noble², AA Musk¹, C Hammond³, PR Goldswain², WD Fraser⁴ and SD Vasikaran¹

*Ann Clin Biochem* 2006; 43: 23–30

- Compares DiaSorin RIA, Nichols CPBA, IDS RIA with LC/UV
- Vitamin D₂ is a common supplement worldwide for the treatment of vitamin D deficiency. Consequently, assay recognition of 25OHD₂ is critically important
- Treatment with vitamin D₂ may not be accurately monitored with any of the three commercial assays studied
State of the arts of Vitamin D

State-of-the-Art Vitamin D Assays:
A Comparison of Automated Immunoassays with Liquid Chromatography–Tandem Mass Spectrometry Methods

Christopher-John L. Farrell,1,2 Steven Martin,1 Brett McWhinney,3 Isabella Straub,4 Paul Williams,5 and Markus Herrmann1,4*

BACKGROUND: Vitamin D testing is increasing worldwide. Recently several diagnostic manufacturers including Abbott and Siemens have launched automated 25-hydroxy vitamin D (25OH-D) immunoassays. Furthermore, preexisting assays from DiaSorin and Roche have recently been modified. We compared the performance of 5 automated immunoassays, an RIA and 2 liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods.

CONCLUSIONS: Automated immunoassays demonstrated variable performance and not all tests met our minimum performance goals. It is important that laboratories be aware of the limitations of their assay.

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An increasing recognition of the high prevalence and manifold consequences of vitamin D deficiency (1–3)

Comparison of different methods:
- RIA (DiaSorin)
- Diasorin Liaison (CLIA)
- IDS – ISYS (CLIA)
- Abbott (CMIA)
- Siemens (CLIA)
- Roche (ECLIA)
- 2 LC-MS/MS

Analysis of 170 patients

→ Diasorin R&D: Waters Acquity TQD
→ Pathology Queensland: Waters Acquity XE premiere (Tecan)
State of the arts of Vitamin D

Work done:
- Passing-Bablock Regression → Y = a + bX
- Intrassay precision
- CCC = Concordance Correlation Coefficient
- Bias

In conclusion, several automated 25OH-D immunoassays have recently been launched. The DiaSorin Liaison premarket evaluation assay demonstrated the best performance characteristics. Liaison, IDS, and Siemens met minimum performance goals for the measurement of 25OH-D at concentrations >8 μg/L (>20 nmol/L) and can be recommended for routine use. None of the automated immunoassays can reliably quantify 25OH-D concentrations <8 μg/L (<20 nmol/L). Regardless of the assay employed, it is of con-
State of the arts of Vitamin D

Supplemental Data Table 1: Characteristics of all 25OH-D methods based on package inserts and information provided by the manufacturers and laboratories.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Principle</th>
<th>Instrument</th>
<th>Specificity D$_2$ &amp; D$_3$ (% Recovery)</th>
<th>Antibody used</th>
<th>Label</th>
<th>Specimen Type</th>
<th>Sample Volume $\mu$L</th>
<th>LoB$^b$ nmol/L</th>
<th>LoD$^b$ nmol/L</th>
<th>LoQ$^b$ nmol/L</th>
<th>Reportable range $\mu$mol/L</th>
<th>Precision Studies</th>
<th>Intra-assay CV %</th>
<th>Inter-assay CV %</th>
<th>Range Tested$^b$ nmol/L</th>
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<tbody>
<tr>
<td>RIA</td>
<td></td>
<td></td>
<td>2.8</td>
<td>2.1 to 3.6</td>
<td>0.87</td>
<td>0.85 to 0.90</td>
<td>6.4</td>
<td>123.4</td>
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<td>0.983</td>
<td>≤4.6</td>
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<tr>
<td>Abbott</td>
<td></td>
<td></td>
<td>5.8</td>
<td>2.9 to 8.5</td>
<td>1.1</td>
<td>1.03 to 1.19</td>
<td>16.5</td>
<td>195.1</td>
<td>32.4</td>
<td>No</td>
<td>0.848</td>
<td>0.809 to 0.880</td>
<td>0.931</td>
<td>0.910</td>
<td>≤16.9</td>
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<tr>
<td>IDS</td>
<td>-2.6</td>
<td>-4.5 to -0.6</td>
<td>1.2</td>
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<td></td>
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<td>0.874 to 0.919</td>
<td>0.954</td>
<td>0.942</td>
<td>≤12.9</td>
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<tr>
<td>LIAISON</td>
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<td>2.8 to 5.8</td>
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<td>0.84 to 0.93</td>
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<td>24</td>
<td>Yes</td>
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<td></td>
<td>0.949</td>
<td>0.933 to 0.962</td>
<td>0.954</td>
<td>0.995</td>
<td>≤&lt;11.0</td>
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<td>0.942</td>
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<td>209</td>
<td>28.5</td>
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<td></td>
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<td>0.968</td>
<td>&lt;&lt;11.2</td>
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<td>-1.3 to -0.4</td>
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<td>0.995</td>
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<td>≤6.6</td>
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<td>0.992 to 0.996</td>
<td>0.996</td>
<td>0.998</td>
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Table 1. Passing–Bablok and concordance correlation analysis of all 25OH-D methods against the mean of the 2 LC-MS/MS methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Intercept</th>
<th>95% CI</th>
<th>Slope</th>
<th>95% CI</th>
<th>Minimum</th>
<th>Maximum</th>
<th>SD</th>
<th>Cumul test</th>
<th>CCC</th>
<th>95% CI</th>
<th>r</th>
<th>Cb</th>
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<td>22.6</td>
<td>Yes</td>
<td>0.968</td>
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<td>0.985</td>
<td>0.983</td>
</tr>
<tr>
<td>Abbott</td>
<td>5.8</td>
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Samples >8 μg/L (20 nmol/L)

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<th>Slope</th>
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<th>Minimum</th>
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<th>CCC</th>
<th>95% CI</th>
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<th>Cb</th>
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<td>2.9</td>
<td>1.5 to 4.4</td>
<td>0.87</td>
<td>0.83 to 0.90</td>
<td>17.9</td>
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<td>-1.3 to -0.4</td>
<td>1.05</td>
<td>1.04 to 1.06</td>
<td>4.5</td>
<td>153.5</td>
<td>27.6</td>
<td>Yes</td>
<td>0.992</td>
<td>0.989 to 0.994</td>
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<tr>
<td>LC-MS/MS PQ</td>
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<td>0.94 to 0.97</td>
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<td>0.991</td>
<td>0.988 to 0.994</td>
<td>0.995</td>
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</tbody>
</table>
An Offline Automated Solid-Phase Extraction Method for 1 of Serum 25-Hydroxyvitamin D for Clinical Research

Billy Molloy, Lisa Calton, and Donald Cooper
Waters Corporation, Manchester, UK

APPLICATION BENEFITS
- Automated sample preparation for high volume 25-hydroxyvitamin D measurements
- Minimal operator intervention
- Method demonstrates good agreement with an EQAS scheme

INTRODUCTION
The demand for serum 25-hydroxyvitamin D (25(OH)D) has dramatically increased in recent years. While the role of 25(OH)D in established, comparably little is known about its role in recently retrospective analysis of clinical trials of vitamin D deficiency and a variety of diseases, and funds are being applied to randomized, pro

Accuracy
The accuracy of the assay for 25(OH)D3 was determined by the analysis of sixteen external quality control samples from the International Vitamin D External Quality Assessment Scheme (DEQAS; www.deqas.org). The calibration curve generated using the Chromsystems calibrators was used to calculate the DEQAS sample concentrations. All results were within 10.8% deviation of the 25(OH)D3 LC-MS method mean.

Recovery
The recovery of 25(OH)D2 and 25(OH)D3 was > 80% (analyte response to blank spiked horse serum pre- and post-extraction expressed as a percentage) over the analytical range of the assay.

Analytical Sensitivity and Specificity
A chromatogram of a serum sample with a calculated concentration of 4.7 ng/mL for 25(OH)D3 is shown in Figure 4. The quantification transition (m/z 401.35:159.1) enables reproducible peak integration and the quantification of 25(OH)D3 in samples having very low concentrations of this vitamin D metabolite.

<table>
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<th>Medium QC</th>
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<td>Intra-assay</td>
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<td>Inter-assay</td>
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<tr>
<td>% CV</td>
<td>11.93</td>
<td>8.67</td>
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</tbody>
</table>

Table 2. Intra- and inter-assay precision at the three levels of QC.

Figure 1. System configuration of the Waters Offline Automated Sample Preparation System (OASPS).

Figure 4. Chromatogram of a low level 25(OH)D3 serum sample.
Thanks
for
Attention
Questions
Steroidi
Via metabolica degli Steroidi

Mineralocorticoids (21 carbons)
- Aldosterone
  - Aldosterone synthase
  - Cholesterol side-chain cleavage enzyme

Glucocorticoids (21 carbons)
- Corticosterone
- Cortisol

Androgens (19 carbons)
- Dehydroepiandrosterone
- Androstanediol
- Androstenediol
- Testosterone
- Estrone
- Estradiol
- Estriol

Estrogens (18 carbons)
- Dihydrotestosterone
- Mitochondria
- Smooth endoplasmic reticulum

Cellular location of enzymes
Mineralcorticoidi: mantenimento dell’equilibrio elettrolitico
promuovono il riassorbimento di Na⁺(e indirettamente del Cl⁻) dal filtrato urinario e l’eliminazione di K⁺
Aldosterone, Progesterone, Pregnenolone, Corticosterone

Glucocorticoidi: parte del glucosio viene convertito in glicogeno ed immagazzinato nel fegato e nei muscoli. La maggior parte viene rilasciato in circolo.
Cortisolo, 11-deossicortisolo, 17 OH progesterone

FEGATO
• aumento sintesi di enzimi che promuovono la gluconeogenesi,
• deamminazione degli amminoacidi rilasciati dai muscoli e conversione in glucosio

MUSCOLI
diminuzione dell’assunzione di glucosio,
riduzione dell’assunzione di AA che vengono rilasciati in circolo e deamminati nel fegato a dare glucosio

ADIPOCITI (tessuto adiposo)
mobilizzazione degli acidi grassi che possono essere utilizzati per la gluconeogenesi o direttamente metabolizzati nei muscoli per fornire l’energia necessaria alla contrazione muscolare

Sessuali: Androstenedione, DHEA, DHEAS, Testosterone, Estradiolo
androgeni (maschio):
sviluppo dei caratteri sessuali primari (pene, vasi deferenti, vescicole seminifere, ghiandola prostatica, epididimo),
sviluppo dei caratteri sessuali secondari (barba),
accrescimento generale e sintesi proteica (sviluppo della massa muscolare).

estrogeni (donna):
sviluppo dei caratteri sessuali primari (utero, ovario, vagina),
sviluppo dei caratteri sessuali secondari (mammelle),
regolazione dei cicli riproduttivi (fase follicolare (estrogeni) e fase luteinica (estrogeni e progesterone)
<table>
<thead>
<tr>
<th>Ormone</th>
<th>Tessuto di origine</th>
<th>Tessuto bersaglio</th>
<th>Azione principale</th>
<th>Regolazione</th>
<th>Carenza</th>
<th>Aumento</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone</td>
<td>corticale del surrene</td>
<td>tubuli renali distali e dotti collettori</td>
<td>promuove il riassorbimento di Na⁺ (e indirettamente del Cl⁻) dal filtrato urinario e l’eliminazione di K⁺</td>
<td>la sezione è stimolata dall’angiotensina II e dall’aumento della concentrazione plasmatica di K⁺</td>
<td>ipertensione; ipotassiemia; iperaldonismo primario e secondario¹</td>
<td></td>
</tr>
<tr>
<td>Corticosterone</td>
<td>corticale del surrene</td>
<td>fegato, tubuli renali</td>
<td>nell’uomo ha solo una debole attività: 1. glucocorticoide: contribuisce all’assorbimento e all’utilizzo del glucosio e alla formazione del glicogeno nel fegato; 2. mineralcorticoide: stimola il riassorbimento del Na⁺ e l’eliminazione del K⁺ E, importante perché prodotto intermedio tra la sintesi del pregnenolone e dell’aldosterone</td>
<td>la sezione è stimolata dall’ACTH</td>
<td>morbo di Addison o iposurrenalismo cronico²</td>
<td>ipercorticosurrenalismo o ipercortisolismo o sindrome di Cushing³</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>corticale del surrene, cervello</td>
<td>sistema nervoso centrale</td>
<td>regola la funzionalità sinaptica; promuove la mielinizzazione, miglioramento cognitivo e la memoria. Si pensa possa avere un ruolo importante nella terapia della schizofrenia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>ovaie (corpo luteo), corticale del surrene</td>
<td>utero, ghiandole mammarie,</td>
<td>mantiene il trofismo dell’endometrio; stimola la formazione dei dotti galattofori</td>
<td>la sezione è stimolata dall’aumento dei livelli di LH e prolattina</td>
<td>ciclo mestruale anticipato, irregolare, doloroso e molto abbondante o, addirittura, una emorragia e, per lo più, non avviene l’ovulazione</td>
<td>endometrio non adatto all’impianto dell’ovulo, con ghiandole scarse e atrofiche e con un muco troppo spesso e denso che rende difficile il passaggio dello sperma</td>
</tr>
</tbody>
</table>

¹ Iperaldonismo primario e secondario
² Morbo di Addison o iposurrenalismo cronico
³ Ipercorticosurrenalismo o ipercortisolismo o sindrome di Cushing
<table>
<thead>
<tr>
<th>Cortisolo</th>
<th>corticale del surrene</th>
<th>fegato, tessuto adiposo</th>
<th>aumenta la gittata cardiaca; aumenta la glicemia favorendo la gluconeogenesi epatica a partire dagli aa provenienti dal catabolismo proteico muscolare e favorendo la mobilizzazione degli acidi grassi a livello del tessuto adiposo; riduce la difese immunitarie diminuendo le reazioni infiammatorie; inibisce la sintesi di collagene e della matrice ossea aumentando l’osteoporosi</th>
<th>la sezione è stimolata da stress fisiologici, dal CRH (corticotropin releasing hormone) e dall’ACHT, l’orologio biologico ne controlla le variazioni diurne (secrezione massima al mattino)</th>
<th>morbo di Addison o iposurrenalismo cronico</th>
<th>ipercorticosurrenalismo o ipercortisolismo o sindrome di Cushing</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-deossicortisolo</td>
<td>ghiandole surrenali</td>
<td>precursore della biosintesi del cortisolo</td>
<td>la sezione è stimolata dall’ACTH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17-idrossiprogesterone</td>
<td>ghiandole surrenali, gonadi</td>
<td>non ha un ruolo fisiologico definito tranne come molecola del precursore: è il punto di partenza per la biosintesi di ormoni maschili (androgeni)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

iperplasia surrenalica congenita o sindrome adreno genitale: elevati valori indicano un deficit dell’enzima 21-idrossilasi causato da alterazioni genetiche del relativo gene.

L’eccesso di androgeni in entrambi i sessi si manifesta con accelerazione della maturità ossea, acne, irsutismo, fino ad avere virilizzazione.
<table>
<thead>
<tr>
<th>Androstenedione</th>
<th>surrene, gonadi</th>
<th>è il precursores degli estrogeni: estradiolo ed estrone (ormoni sessuali femminili), e dell'androgeno: testosterone (ormone sessuale maschile per eccellenza)⁷ nella donna viene prodotto, sotto lo stimolo delle gonadotropine ipofisarie e dalle cellule della teca. La sintesi surrenalica di androstenedione avviene sotto stimolo dell'ACTH ipofisario (menopausa) insufficienza surrenale</th>
<th>tumori delle ovaie, dei testicoli o delle ghiandole surrenali; ovaio policistico⁸; irsutismo (peluria eccessiva); virilismo (comparsa nella donna di peluria, timbro di voce ed altri caratteri sessuali secondari tipici del sesso maschile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deidroepiandrostone (DHEA)</td>
<td>corticale del surrene, ovaie, testicoli</td>
<td>la maggior parte dei tessuti</td>
<td>regolazione e stimolazione delle funzioni sessuali, della libido e dei caratteri sessuali secondari; regolazione e stimolazione della produzione di mielina, una sostanza importante per la protezione ed il funzionamento del sistema nervoso; aumento della forza e delle masse muscolari, mantenimento del trofismo osseo; regolazione del metabolismo lipidico (lipolisi = utilizzazione dei grassi a scopo energetico)⁹. Se ne effettua il dosaggio nel sangue per valutare la funzionalità delle ghiandole sessuali (sia maschili, sia femminili)</td>
</tr>
<tr>
<td>Deidroepiandrostonesolfato (DHEAS)</td>
<td>corticale del surrene, ovaie, testicoli</td>
<td>gonadi, ghiandole sessuali</td>
<td>sviluppo dei caratteri sessuali e secondari della pubertà, può essere metabolizzato in androgeni più potenti, come testosterone ed androsterone o può essere convertito in estrogeni (ormoni femminili). Considerato che il DHEAS è prodotto principalmente dalle ghiandole surrenali, è utile come marcatore per la funzione surrenale la sintesi regolata dall'ACTH e dagli altri fattori pituitari</td>
</tr>
<tr>
<td>Testosterone (androgeni)</td>
<td>Testicolo (cellule di Leydig), corticale del surrene</td>
<td>La maggior parte dei tessuti</td>
<td>Promuove lo sviluppo ed il mantenimento delle caratteristiche e del comportamento maschili e la spermatogenesi</td>
</tr>
<tr>
<td>------------------------</td>
<td>---------------------------------</td>
<td>-----------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Estradiolo-17-β (estrogeni)</td>
<td>Follicolo ovarico, corpo luteo, corticale del surrene</td>
<td>La maggior parte di tessuti</td>
<td>Promuove lo sviluppo e il mantenimento delle caratteristiche e del comportamento femminili, la maturazione degli oociti e la proliferazione della mucosa uterina</td>
</tr>
</tbody>
</table>
Case report
Plasma poltergeists: A negative cortisol adrenal insufficiency

4. Conclusions

A COMPARISON BETWEEN EXTRACTION RIA AND TANDEM MASS SPECTROSCOPY (TMS) FOR THE MEASUREMENT OF BLOOD STEROIDS

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Martyn Egerton¹ and John Hopkins²

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2. Waters Corporation, Atlas Park, Manchester, M22 5PP

Introduction and study aims
Evaluation of the Perkin Elmer MSMS Steroids Kit was performed using patient and NEQAS samples by comparing results with both extraction RIA and NEQAS TMS method means. The kit contains 10 steroids allowing analysts a choice of desired steroids. We chose those that were previously performed by extraction RIA: DHEAS, 17-Hydroxyprogesterone (17PG), Testosterone (TES), Androstenedione (AD2), and were to be replaced by TMS following a local service reorganisation.
4. Conclusions

Over the past five decades, we have learned that immunoassays can be used to help diagnose human disease. Sadly, we have also learned that immunoassays can hurt patients. It is important for clinicians to remember how immunoassays can give inaccurate results in many clinical situations. It is also important for the community as a whole to look for solutions, which may reside in novel detection technologies like tandem mass spectrometry. And as with all novel technologies, we must be prepared for unexpected complications from the infinitely complex reservoir of human biology.

Table 1
Summary of common problems

<table>
<thead>
<tr>
<th>Problem</th>
<th>Brief description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay performance</td>
<td>Assay does not perform as expected</td>
<td>Sapin (2002)</td>
</tr>
<tr>
<td>Autoantibodies</td>
<td>Epitopes from reagent antibodies bind endogenous immunoglobulins</td>
<td>Willman et al. (1999); Rotmensh and Cole (2000); Morgan and Tarter (2001); Giovanello et al. (2007)</td>
</tr>
<tr>
<td>Anti-reagent antibodies</td>
<td>Endogenous non-specific/multi-specific immunoglobulins bind reagent antibodies</td>
<td>Furuya et al. (2001); Fleseriu et al. (2006); McCudden et al. (2009)</td>
</tr>
<tr>
<td>Hook effect</td>
<td>Reagent antibodies are saturated with analyte preventing sandwich formation</td>
<td>Falsey low results in the setting of extremely high analyte concentrations</td>
</tr>
</tbody>
</table>
Analisi del profilo steroideo utilizzando kit diagnostici CE-IVD
Obiettivo:
Valutazione del kit per analisi degli steroidi CHS su strumentazione ACQUITY UPLC/Xevo TQ-S

Il metodo usato utilizza la preparazione del campione del kit diagnostico: Precipitazione proteica del siero + evaporazione del surnatante + ricostituzione

Il kit presenta calibratori in matrice a 7 livelli (L1-L7) e 3 controlli di qualità sempre in matrice (QC1-QC3)

Abbiamo utilizzato la colonna Waters ACQUITY UPLC HSS T3 1.8μm 1.0x100mm
Analiti misurabili nel PE MSMS Steroidi Kit

- Testosterone
- Cortisolo
- Corticosterone
- DHEA, Deidroepiandrosterone
- Progesterone
- 4-androsten-3,17-dione
- 11-deossicortisolo
- DHEAS, Deidroepiandrosterone solfato
- 17-alfa-idrossiprogesterone
- Aldosterone
Grazie a Acquity UPLC la cromatografia è stata migliorata portando il tempo a 12 minuti di corsa da iniezione ad iniezione (aumentando produttività e salvando circa il 25% di fase mobile). Inoltre è stata migliorata la separazione tra i picchi del 11-deossicortisolo e del corticosterone.
Il migliore valore di precisione (2.3% RSD) è stato ottenuto sul controllo di qualità alto del cortisolo e del testosterone (Q3).

Il peggiore sul controllo di qualità basso (Q1) del DHEAS (7.25 RSD).
Questi dati ottenuti sono migliori di quanto dichiarato sul technical note della Perkin Elmer.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>QC1 %RSD</th>
<th>QC2 %RSD</th>
<th>QC3 %RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone</td>
<td>3.73</td>
<td>2.50</td>
<td>2.39</td>
</tr>
<tr>
<td>Androstendione</td>
<td>1.78</td>
<td>1.26</td>
<td>0.84</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>2.01</td>
<td>1.15</td>
<td>1.71</td>
</tr>
<tr>
<td>Cortisol</td>
<td>2.66</td>
<td>2.33</td>
<td>2.70</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>2.88</td>
<td>4.74</td>
<td>2.49</td>
</tr>
<tr>
<td>DHEA</td>
<td>5.09</td>
<td>2.36</td>
<td>3.43</td>
</tr>
<tr>
<td>DHEAS</td>
<td>1.03</td>
<td>1.03</td>
<td>2.75</td>
</tr>
<tr>
<td>Progesterone</td>
<td>4.57</td>
<td>3.53</td>
<td>3.41</td>
</tr>
<tr>
<td>17-alpha hydroxyprogesterone</td>
<td>4.93</td>
<td>3.42</td>
<td>4.30</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1.52</td>
<td>2.65</td>
<td>0.98</td>
</tr>
</tbody>
</table>
**Linearità**

**Compounds and Calibration Curves:**
- **Aldosterone**
  - Correlation coefficient: $r = 0.999677$, $r^2 = 0.999355$
  - Calibration curve: $0.682043 \times x + 0.0095455$
  - Response type: Internal Std (Ref 11), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

- **Androstenedione**
  - Correlation coefficient: $r = 0.999838$, $r^2 = 0.999675$
  - Calibration curve: $0.605509 \times x + 0.00262052$
  - Response type: Internal Std (Ref 15), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

- **Cortisol**
  - Correlation coefficient: $r = 0.999617$, $r^2 = 0.999234$
  - Calibration curve: $0.0339563 \times x + 0.00774359$
  - Response type: Internal Std (Ref 12), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

- **DHEA**
  - Correlation coefficient: $r = 0.9997390$, $r^2 = 0.994787$
  - Calibration curve: $0.00692836 \times x + -0.00609587$
  - Response type: Internal Std (Ref 19), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

- **Progesterone**
  - Correlation coefficient: $r = 0.999890$, $r^2 = 0.997962$
  - Calibration curve: $1.04123 \times x + -0.0094292$
  - Response type: Internal Std (Ref 17), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

- **11-Deoxycortisol**
  - Correlation coefficient: $r = 0.9999494 \times x + 0.01299063$
  - Calibration curve: $0.00199494 \times x + 0.009556$
  - Response type: Internal Std (Ref 16), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

- **DHEAS neg**
  - Correlation coefficient: $r = 0.999556$, $r^2 = 0.999113$
  - Calibration curve: $0.188787 \times x + 0.0119618$
  - Response type: Internal Std (Ref 18), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

- **Corticosterone**
  - Correlation coefficient: $r = 0.999358$, $r^2 = 0.998717$
  - Calibration curve: $0.188787 \times x + 0.00745066$
  - Response type: Internal Std (Ref 13), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

- **Androstenedione**
  - Correlation coefficient: $r = 0.999809$, $r^2 = 0.996426$
  - Calibration curve: $0.0126422 \times x + 0.00113332$
  - Response type: Internal Std (Ref 14), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

- **17-alpha hydroxyprogesterone**
  - Correlation coefficient: $r = 0.999759$, $r^2 = 0.999518$
  - Calibration curve: $0.188787 \times x + 0.00745066$
  - Response type: Internal Std (Ref 13), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

- **Corticosterone**
  - Correlation coefficient: $r = 0.999838$, $r^2 = 0.999675$
  - Calibration curve: $0.605509 \times x + 0.00262052$
  - Response type: Internal Std (Ref 15), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

- **Testosterone**
  - Correlation coefficient: $r = 0.999759$, $r^2 = 0.999518$
  - Calibration curve: $0.188787 \times x + 0.00745066$
  - Response type: Internal Std (Ref 13), Area * (IS Conc. / IS Area)
  - Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None
Sovrapposizione di 8 curve di calibrazione:

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Lowest cal (nmol/L)</th>
<th>Highest Cal (nmol/L)</th>
<th>r^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone</td>
<td>0.087</td>
<td>21.50</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td>Androstendione</td>
<td>0.290</td>
<td>63.60</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0.844</td>
<td>190.00</td>
<td>&gt;0.998</td>
</tr>
<tr>
<td>Cortisol</td>
<td>3.960</td>
<td>853.00</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>0.190</td>
<td>50.60</td>
<td>&gt;0.998</td>
</tr>
<tr>
<td>DHEA</td>
<td>1.290</td>
<td>254.00</td>
<td>&gt;0.994</td>
</tr>
<tr>
<td>DHEAS</td>
<td>34.300</td>
<td>7564.00</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.364</td>
<td>77.20</td>
<td>&gt;0.997</td>
</tr>
<tr>
<td>17-alpha hydroxyprogesterone</td>
<td>0.376</td>
<td>70.70</td>
<td>&gt;0.996</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.113</td>
<td>27.80</td>
<td>&gt;0.999</td>
</tr>
</tbody>
</table>
Nota: Risoluzione Cromatografica mantenuta!
MEASUREMENT OF SERUM CORTISOL, ANDROSTENEDIONE AND 17-HYDROXYPROGESTERONE BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Heather A Brown PhD, Lisa J Calton PhD, Scott D Gillingwater PhD, Mike Morris PhD (Waters Corporation, Manchester) and Claudia Rossi (G. d’Annunzio University, Italy)

INTRODUCTION

Current direct immunoassay procedures for measuring 17-hydroxyprogesterone (17-OHP) in newborn dried bloodspots for screening the neonatal population for congenital adrenal hyperplasia (CAH) is hampered by an unacceptably high false positive rate. This is due to immunoassay antibody cross-reactivity with adrenal steroid intermediates, which may be transiently raised due to pre-maturity or acute illness. A second tier investigation of an elevated bloodspot 17-OHP level with a more analytically selective and diagnostically specific plasma adrenal steroid profile may be beneficial in many cases. The biochemical

EXPERIMENTAL

A Waters® Xevo™ TQ MS coupled to an ACQUITY UPLC® was used for all analyses. The full system configuration is shown in Figure 1. The instrument was operated in positive electrospray ionization mode using MassLynx™ 4.1 software with auto data processing by the TargetLynx™ Application Manager. The compound-dependent cone voltage was optimized to maximize the abundance of the precursor ion entering the source and selected to pass through the first quadrupole to the collision cell. Collision-induced dissociation was facilitated by argon and collision energy to produce characteristic product ions. Using this information, specific Multiple Reaction
Rapid steroid hormone quantification for congenital adrenal hyperplasia (CAH) in dried blood spots using UPLC liquid chromatography–tandem mass spectrometry

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ABSTRACT

Newborn screening for congenital adrenal hyperplasia (CAH) is usually done by quantifying 17α-hydroxyprogesterone using immunoassay. However, this test produces high rates of false positive results caused by cross reacting steroids. Therefore we have developed a selective and specific method with a short run time (1.25 min) for quantification of 17α-hydroxyprogesterone, 21-deoxycortisol, 11-deoxycortisol, 11-deoxycorticosterone and cortisol from dried blood spots. The extraction procedure is very simple and steroid separation is ensured on a BEH C18 column and an ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). Analysis was done in positive ionization mode (ESI+) and recorded in multiple reaction monitoring mode (MRM). The method gave linear results for all steroids over a range of 5–200 (cortisol: 12.5–500) nmol/L with coefficients of regression >0.992. Absolute recovery was >64.1%. Across the analytical range the inter-assay coefficient of variation (CV) was <3%. Newborn blood samples of patients with confirmed 21-CAH and 11-CAH could clearly be distinguished from samples of unaffected newborns falsely positive on immunoassay. The method is not influenced by cross reactions as found on immunoassay. Analysis of dried blood spots shows that this method is sensitive and fast enough to allow rapid analysis and can therefore improve the newborn screening program.

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