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Use of porous graphite packed columns in analysis of doping control samples for glucocorticosteroids

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Abstract

When preparing urine samples with glucocorticosteroids to an instrumental analysis using traditional C18 or C8 columns one can encounter some separation problems, especially with three difficult pairs: dexamethasone and betamethasone, prednisolone and cortisone, flunisolide and triamcinolone acetonide. Analysis of glucocorticosteroids by LC/MS/MS using porous graphitic carbon packed columns can solve this problem.

Gradient methods on different columns were tested. One was chosen for routine screening analysis. It allows obtaining full separation of all problematic compounds.

Introduction

Glucocorticosteroids were considered as doping agents by IOC Medical Commission in 1975. But until September 2003, the decision of banning them was in authority of the relevant sport federation. This year, WADA has set prohibition of this group, as mandatory rule for all in-competition samples. According to the WADA Prohibited List all glucocorticosteroids are prohibited when administered orally, rectally, intravenously or intramuscularly. Other routes of administration require an Abbreviated Therapeutic Use Exemptions, but topical preparations of glucocorticosteroids are permitted [1].

Minimum Requirement Performance Level (MRPL) for detection of glucocorticosteroids was set by WADA at the level of 30 ng/ml [2]. This was not named prohibited threshold to avoid full quantification analysis of all samples with glucocorticosteroids concentration values much lower than the MRPL. This single value has caused considerable controversies, because glucocorticosteroids - inside a group - have very diverse strength of action.

Problem description

When analyzing glucocorticosteroids using traditional C18 or C8 columns, one may encounter some separation problems. Despite the fact that LC/MS/MS is a very selective

technique it is always better to have compounds separated on HPLC prior to MS detection. Especially when they have the same molecular mass and produce similar spectra.
There are three difficult pairs, which should be well separated before detection: dexamethasone and betamethasone, prednisolone and cortisone, flunisolide and triamcinolone acetonide.
They can be difficult to resolve in reasonable time on traditional, silica based columns. Use of porous graphitic carbon packed columns can solve this problem. In comparison with silica based packing it has different retention mechanism and it is stable in very broad range of pH and temperature.
A ternary gradient method on Thermo Hypercarb column was developed. It allows obtaining full separation of all problematic compounds in reasonable time.

Porous graphitic carbon as HPLC column packing material.
PGC properties as a packing material for HPLC columns differ from those of traditional silica-based stationary phases. It can retain very polar compounds much stronger and has ability to separate closely related compounds. According to Thermo Hypercarb method development leaflet porous graphite has two main mechanisms of retention. First are the interactions between analyte – mobile phase and analyte – graphite surface. Retention increases as the hydrophobicity of the molecule increases. Second are the charge induced interactions of a polar analyte with the polarizable surface of graphite (Figure 1).

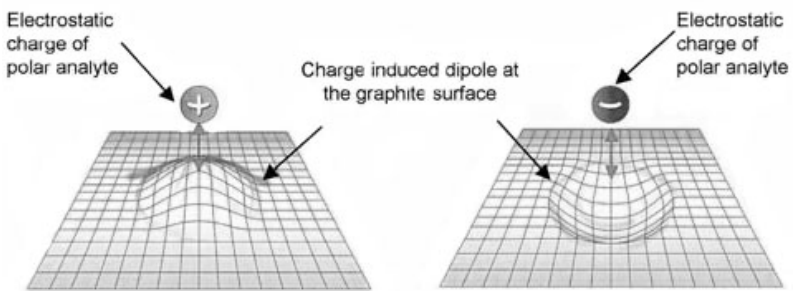


Fig 1. Scheme of charge induced interactions of a polar analyte (Drawing from Thermo Hypercarb leaflet).

The strength of analyte interactions with the Hypercarb depends very much on the molecular area in contact with the graphite surface (Figure 2) [3]. That gives us the possibility to separate compounds with similar hydrophobicity (like dexamethasone and betamethasone) if there is a little difference in three-dimensional arrangement of functional groups.

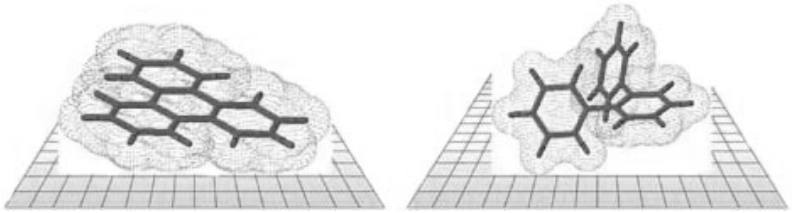


Fig 2. Scheme of the influence of molecule shape on interaction with graphite surface (Drawing from Thermo Hypercarb leaflet).

As it is shown (Figure 3), the only difference between dexamethasone and betamethasone is the orientation of methyl group relative to almost flat steran structure. If this group is on the same side as fluorine (like in dexamethasone), it works as a spatial obstacle. It weakens the second type interaction (dipole inducting) between electronegative fluorine and the graphite surface. As a result dexamethasone has shorter retention time than betamethasone.

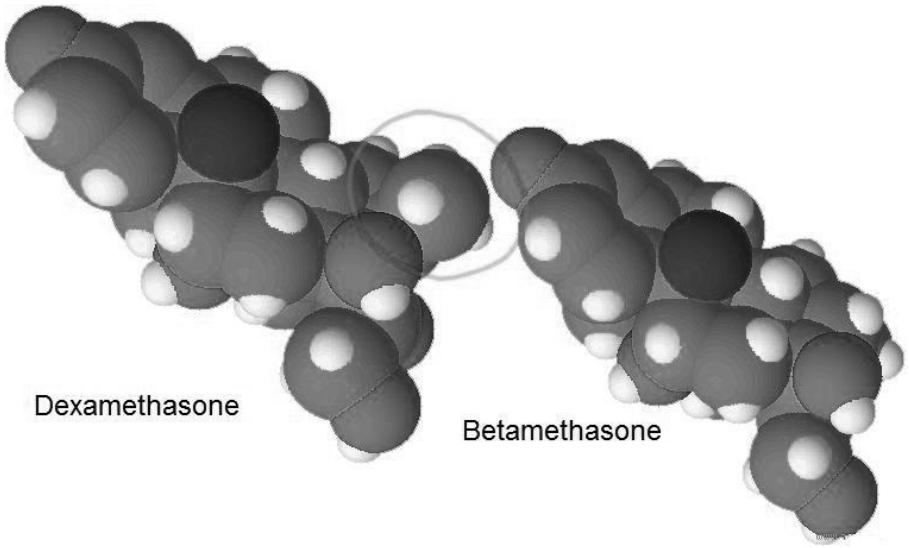


Fig 3. 3D model showing structural difference between dexamethasone and betamethasone.

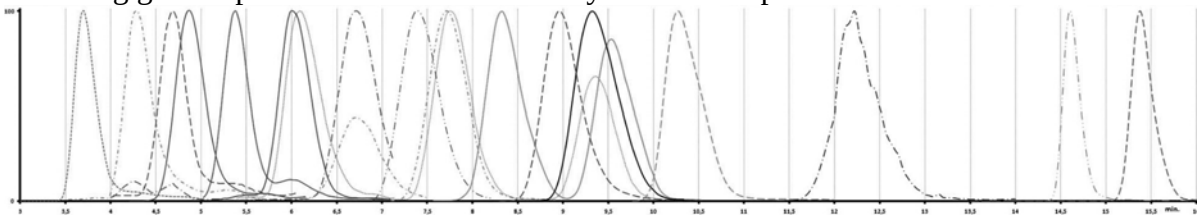
Materials and Methods

HPLC separation: Instrument: Waters Alliance 2695
Column: Thermo Hypercarb 5µm 2,1mm x 100mm (with 10mm guard column)
Column temperature: 58 ^{O}C Flow: 400 µl/min Injection volume: 10µl
MS/MS detection Instrument: Micromass QuattroMicro
Ionization: ESI(-)
All compounds detected as an acetate ion adducts.
Two transitions for each compound monitored.

Acetonitrile (LC/MS grade), isopropanol (LC/MS grade) and acetic acid (HPLC grade) were purchased from Baker (Polish supplier - Witko). Water was purified using Millipore MiliQ.

Results and Discussion

As can be seen on following figures (4 and 5) porous graphite packed column allows achieving good separation of structural closely related compounds.



Graph 1. Model of the obtained separation (numerical data from MassLynx imported to Excel).

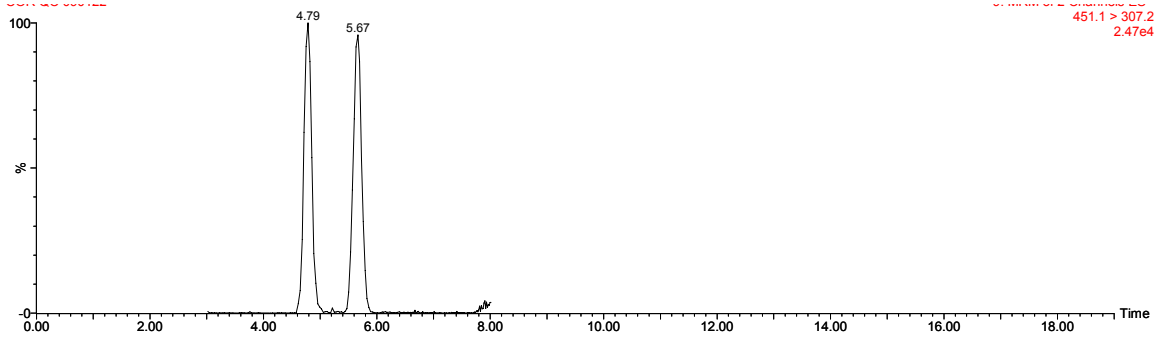


Fig 4. Chromatogram of urine spiked with 30 ng/ml of dexamethasone (RT 4.79) and betamethasone (RT 5.63).

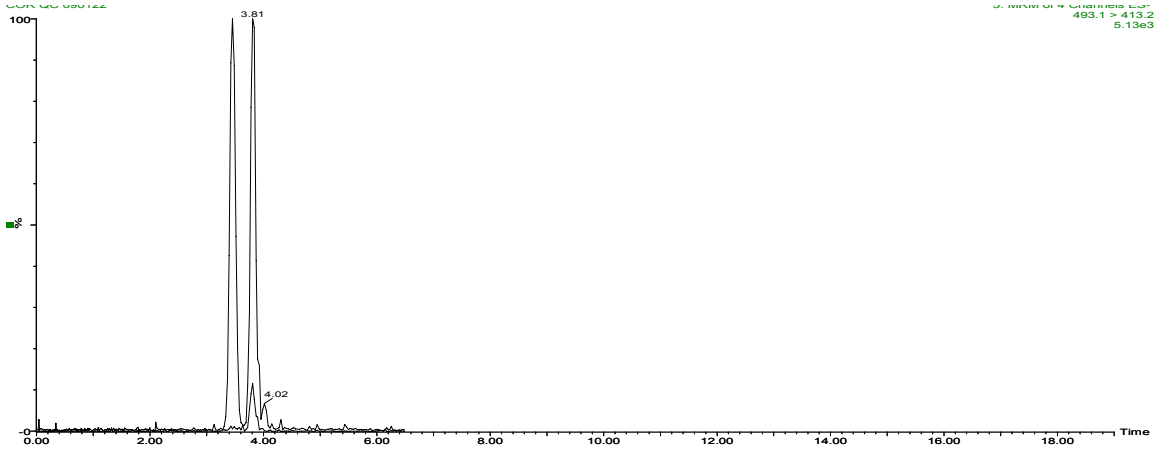


Fig 5. Chromatogram of urine spiked with 30 ng/ml of flunisolide (RT 3.45) and triamcinolone acetonide (RT 3.81).

Use of graphite packed column in routine screening analysis allows obtaining full separation of all problematic glucocorticosteroids. It lasts 19 minutes, including column equilibration and injection cycle.

References

(1) The World Anti-Doping Code. The 2008 Prohibited List. International Standard, September 22, 2007.
(2) WADA Technical Document – TD2004MRPL. January 15, 2004.
(3) Thermo Hypercarb column leaflet “Hypercarb Method Development Guide” http://www.thermo.com/eThermo/CMA/PDFs/Product/productPDF_28871.pdf