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# The Separation of Betamethasone from Dexamethasone by Using LC/MS/MS Technique with Triple Quadrupole and Excretion Studies of Dexamethasone after Intramuscular Administration

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## Introduction

Glucocorticosteroid drugs are synthetic analogues of hormones, which are anti-inflammatory agents, alleviate pain and enhance the athlete's concentration capacity during strength and endurance competitions [1].

Initially, corticosteroids have been detected through GCMS [2,3]; the main inconvenience has been the low volatility of most corticosteroids, which requires an additional derivatization step. This derivatisation step is time-consuming and, due to relatively great number of polar functions from a corticosteroid's molecule, does not have the best results. Therefore, for the corticosteroids' analysis, it has been used LCMS technique [4,5,6,7,8]; this technique has been proven to be a sensitive method, even to low concentration of corticosteroids, and which does not require derivatization.

A specific issue is raised by betamethasone and dexamethasone: they are epimers; the only structural difference between them is the orientation of the methyl group on carbon 16. As a result, the two corticosteroids have very closed retention times and very similar mass spectra. Using regular LC/MS ionization techniques (such as ESI or APCI), their tandem mass spectra reveal the same fragmentation ions, which vary only in abundance [4,7,9,10,11].

Together with Doping Control Center from Ankara, Turkey, we have developed an excretion study of dexamethasone after intramuscular administration.

## Materials and Methods

*Reagents*: the standards of dexamethasone, betamethasone and desoximetasone were purchased from Sigma-Aldrich, USA; the methanol, gradient grade for liquid chromatography, from Merck, Germany; the β-glucuronidase from E. Coli K12, from Roche

Diagnostics, Germany. The water was supplied by a Simplicity 185 ultrapure water system, from Millipore, Great Britain; all other chemicals are pro analysis or HPLC grade.

*Equipment*s:

Romanian Doping Control Laboratory:

- Instrument: Varian 1200L triple quadrupole LC-MS/MS with APCI source;
- Column: OmniSpher 3 C18, 2.0x100mm, particle size 3µm;
- Eluents: A = 5mM ammonium acetate in water with 0,1% acetic acid,
  B = methanol;
- Flow: 0.25ml/min;
- Gradient A: 1min 70%→50%, 15min 50%→30%, 1min 30%, 5min 70%;

(the separation of dexamethasone from betamethasone was performed in isocratic mode with a mixture of 30% acetonitril / 70% A)

- Injection volume: 10µl;
- Interface: APCI 400$^0$C; corona current 5µA; negative ionization mode;
- Collision gas: Argon, 1.5mTorr;
- MRM parameters are presented in the table below:

| Substance | RT (min) | RRT | Precursor Ion | Product Ion (collision energy, relative abundance) |
|---|---|---|---|---|
| Dexamethasone | 11.49 | 0.866 | 451.2 | 361 (19V, 100%), 325 (35V, 10.04%), 307 (33V, 24.55%), 292 (41V, 12.27%) |
| ISTD-Desoximetasone | 13.27 | 1.000 | 435.2 | 355 (16V) |

Turkish Doping Control Center:

- Instrument: Finnigan LCQ Advantage Max ion-trap LC-MS/MS with ESI source;
- Column: Synergi 4µ MAX-RP 80A, 2.0x100mm, particle size 4µm;
- Eluents: A = 1% acetic acid in water,
  B = acetonitril;
- Flow: 0.2ml/min;
- Gradient A: 5min 65%→42%, 7min 42%, 1min 42%→65%, 7min 65%;
- Injection volume: 25µl;
- Interface: ESI 300$^0$C; negative ionization mode;
- Collision gas: Helium;
- MS/MS settings are presented in the table below:

| Substance | RT (min) | RRT | Precursor Ion | Product Ion (relative abundance) | CE (V) |
|---|---|---|---|---|---|
| Dexamethasone | 5.88 | 0.658 | 451 | 361 (100%), 390 (18.42%) | 20 |
| ISTD-Mefruside | 8.93 | 1.000 | 381 | 345 (100%), 317 (29.04%) | 35 |

*Excretion Study*: Dexamethasone Sodium Phosphate (EIPICO) vials of 2ml, containing 8mg dexamethasone phosphate have been administered intramuscular to hospital patients, both males and females. The samples have been collected before administration and 3, 6, 9, 12 and 24 hours after administration.

*Sample Preparation*: 2ml urine samples were spiked by a 40µl aliquot of a 10µg/ml desoximetasone (as internal standard) and prepared by a conventional liquid-liquid extraction procedure [4,5]. The residue was dissolved in 100µl mobile phase (20:80, solventA/solventB).

*Quantification and validation:* For quantitative determination of dexamethasone it has been used a calibration curve on range 25-800ng/ml. For the elaboration of calibration curves it has been prepared a solution of 10µg/ml dexamethasone in methanol, from which volumes of 5, 10, 15, 20, 40, 60, 80, 160µl were dropped over 2ml blank urine aliquots, spiking them on concentration levels of 25, 50, 75,

100, 200, 300, 400, 800ng/ml. The spiked urines have been processed following the same extraction scheme used for excretion samples' preparation. For validation of the method, the following parameters have been determined: linearity (25-800ng/ml), identification capability (MRPL 30ng/ml), recovery, limit of detection, matrix interferences and specificity. Certified reference material has been used.

**Results and discussions**

The excretion study's results are shown in figure 1. The concentrations measured by the two LC-MS/MS equipments were similar. With one exception, the maximum concentration of excreted dexamethasone (around 1000ng/ml) has been detected in the first urine sample, collected 3 hours after administration; subsequently, the concentration of excreted dexamethasone decreases steeply 24 hours after administration, reaching a level lower than 30ng/ml (WADA's MRPL). The different excretion curve has been noticed on an immobilized female subject, from whom the sample has been collected through a urinary drain; the excreted concentration has been relatively constant in the first 12 hours after administration (around 100ng/ml), and then decreases slowly, 24h after administration being around 30ng/ml. A sample collected from one of the subjects 21 days after administration did not show any dexamethasone traces.

The calibration curve used for dexamethasone quantification was linear for the selected range with a correlation coefficient of 0.9987. The dexamethasone was easily identified in urine spiked at the WADA MRPL for corticosteroids (30ng/ml). The recovery was 30 % for dexamethasone concentration of 30ng/ml. The limit of detection (LOD) was defined as the sample concentration which produces a peak with a signal-to-noise ratio >3 for, at least, the first three of the four MRM transitions. LOD was determined at <5ng/ml for dexamethasone. Neither the urine matrix nor the other corticosteroids usually screened (except for betamethasone) gave significant interferences with the dexamethasone signals.

The attempts for betamethasone and dexamethasone separation by modifying the methanol / ammonium acetate-acetic acid buffer ratio in mobile phase, the mobile phase flow or the chromatographic column's temperature didn't have satisfactory results. The separation of those compounds has been accomplished by replacing the mobile phase based on methanol and ammonium acetate-acetic acid buffer with a mixture of 30% acetonitril / 70% ammonium acetate-acetic acid buffer (Figure 2). The separation's resolution may be improved through the use of a mobile phase with 27% or 25% acetonitril, but at the cost of increasing retention times (to 14-15 minutes, respectively to 19-20 minutes), increasing peaks' broadening and decreasing peaks' height.

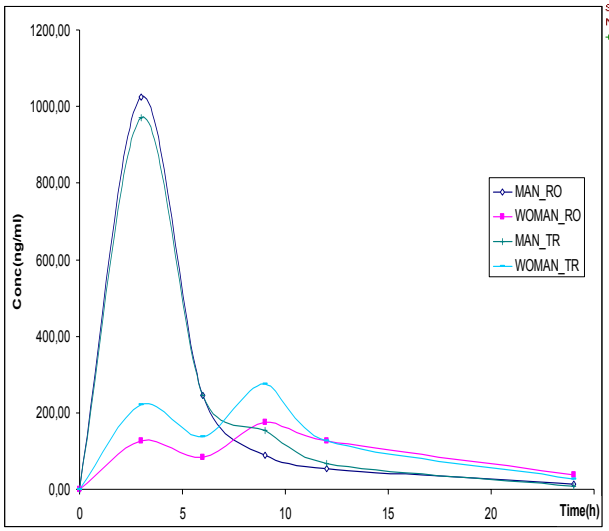


Figure 1. Renal excretion curve of dexamethasone after intramuscular administration of an 8 mg dose.

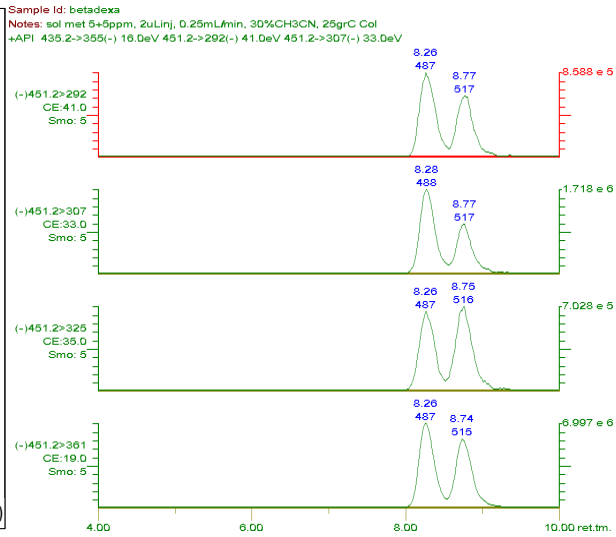


Figure 2. LC-Separation of betamethasone from dexamethasone.

## Conclusions

- Dexamethasone excretion is fast; after 24 hours after administrating an intramuscular dose of dexamethasone (8mg), its urinary concentration decreases to a level lower than 30ng/ml (WADA's MRPL).
- The optimization of the LC-parameters has made the separation of betamethasone and dexamethasone possible with a runtime of less than 10 minutes and showing a good resolution.

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