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Determination of long term metabolites of methylprednisolone using liquid chromatography tandem mass spectrometry

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Introduction

Glucocorticosteroids are potent anti inflammatory agents and are often misused in sports. Methylprednisolone (11-β, 17-α, 21-trihydroxy-6-α-methylpregna-1-4-dine-3,20dione) is a potent synthetic glucocorticosteroid mainly used for its anti-inflammatory and immunosuppressive actions. This synthetic glucocorticosteroid is readily metabolized in the liver and give rise to a number of metabolites. [1-4] (Figure-1) The aim of the present work is to explore the possibility of identifying long term marker metabolites of methylprednisolone to improve detection time window for its abuse.

Materials and methods:

The samples were prepared using enzymatic hydrolysis and liquid- liquid extraction [5] and analysis was performed on Agilent 1100 LC and an API 3200 TM tandem mass spectrometer (Table-1). The analytical method was developed and validated as per the requirement of WADA ISL (version 6.0) keeping in view sensitivity, recovery, accuracy, precision, linearity, specificity, reproducibility, and repeatability. [(6)] The excretion study was performed by administering drug to four healthy male volunteers (25±2 years, 70±5 kg) with their informed consent to participate in the study. The study protocol was reviewed and approved by the ethical committee of NDTL, India. Methylprednisolone was administered orally in 8 mg dose and urine samples were collected from 0-72 hours. The LC-MS/MS method was optimized to detect the target analytes, using the Multiple Reaction Monitoring (MRM) pair obtained after the precursor and product ion scan. The corresponding retention times, MS and MS/MS spectra were then used to obtain structural information.

Result and Discussion:

The parent compound and six urinary metabolites for methylprednisolone were identified in all the four excretion study samples namely, methylprednisone (11-oxo-metabolite) [M-1], 6-β-OH-methylprednisolone [M-2], 20-β-OH-methylprednisolone [M-3], 20-α-OH-methylprednisolone [M-4], 20-β-OH-methylprednisone [M-5] and 20- α -OH-methylprednisone [M-6] (Figure 2).M-1 is formed due to the C-11 hydroxyl group oxidation of parent drug and gives *m/z* 417. Hydroxylation at C-6 position leads to the formation of M-2 yielding *m/z* 391. M-3 and M-4 are the epimeric pairs formed due to reduction of C=O bond at C-20 position of methylprednisolone yielding *m/z* 377. Whereas, M-5 and M-6 are the epimers of M-1 with reduced C=O bond at C-20 position giving *m/z* 375. Out of the six metabolites M-1 and M-2 were detectable up to 48 hours while the parent drug methylprednisolone and other metabolites were detectable up to 24 hours after single oral dose (Figure 3).

The method was found to be specific and no significant matrix interference was observed. The calibration curve for methylprednisolone was found to be linear in range from 15-120 ng/ml. The calibration equation obtained was y = 1.1700+0.0005 r^2 = 0.9998. The percentage recovery at 30 ng/ml ranged from 80.5% to 93.3%. The method showed good precision and accuracy.

The reported literature shows identification of seven [3] and sixteen metabolites [4] after administration of intravenous dose [3] and single oral dose (8 mg). [4] The present study shows identification of six metabolites of methylprednisolone out of which 6-β-OH-methylprednisolone [M-2] is not reported earlier and is excreted for a longer period. Hence, it may further be used for confirmatory purposes.

References:

1. M. A. Popot, P. Garcia, Y. Bonnaire "Screening and confirmation method for the detection of synthetic corticosteroids in human urine" Recent advances In Doping Analysis, Sport und Buch Strauß, Koln,1998, 5, 185-189.
2. K. Deventer, F.T. Delbeke, "Excretion studies with corticosteroids" Recent advances In Doping Analysis, Sport und Buch Strauß, Koln, 2004, 12, 37-44
3. T. B. Vree, A. J. Lagerwerf, P.W.G.M Corrien, V. Wissen, P. J.H. Jongen "High-Performance liquid chromatography analysis, preliminary pharmacokinetics, metabolism and renal excretion of methylprednisolone with its C6 and C20 hydroxy metabolites in multiple sclerosis patients receiving high dose pulse therapy" J. Chrom B, 1999, 337-348.
4. A. Marina, Dikunets, A. Svetlana, A. Appolonova, M.R. Grigory "Determination of new long term metabolites of methylprednisolone in human urine by HPLC-ESI(-)/MS" Antidoping centre, Russia (unpublished work)
5. Reddy M.I., Beotra A., Jain S., Ahi S. (2009) "A simple and rapid ESI-LC-MS/MS method for simultaneous screening of doping agents in urine samples" Ind. J. Pharm., 41, 80-86
6. The World Anti Doping Code. WADA International Standard for Laboratories Version 6.0. Available online at http://www.wada-ama.org/rtecontent/document/International_Standard_for_Laboratories_v6_0_January_2009.pdf

Table-1 Analytical Parameters

HPLC	**Agilent 1100 Series**
Column	ZORBAX Eclipse XDB-C-18 (3.0 µm, 50 mm x 4.6 mm)
Solvent A	1% aqueous solution of formic Acid
Solvent B	Acetonitrile
Flow	700 µl/minute
Gradient	0 min-15%B, 10 min-60%B, 15 min-75%B, 25 min-85%B, 30 min-15%B
Injection volume	10 µl
Mass Spectrometer	**Sciex API 3200, Triple Quadruple Mass Spectrometer**
Ionization	ESI Positive
Collision Gas	Nitrogen
Source Temperature	550°C
MRM Transition selection	Analysis in product ion scan mode followed by analysis in MRM mode at various collision energies.

Figure 1: Proposed metabolic pathway of methyl prednisolone

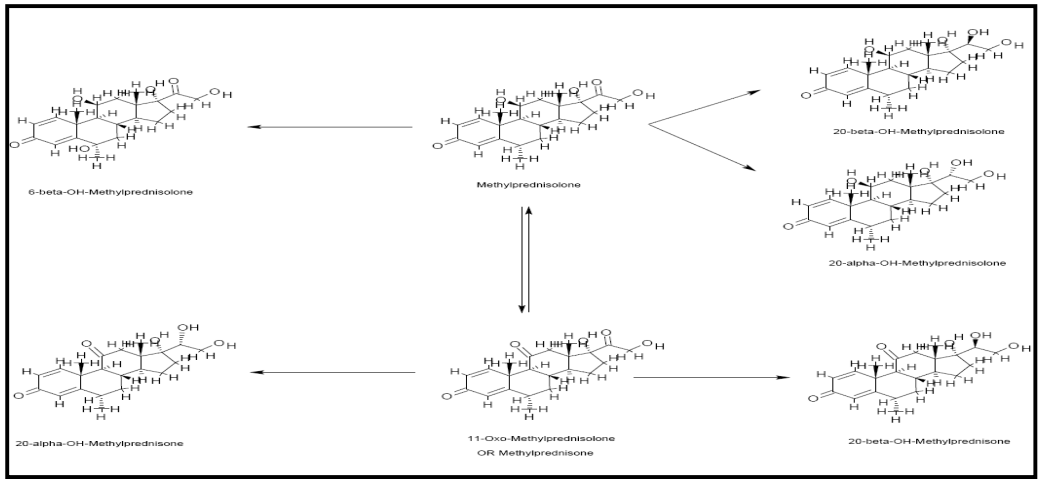

Figure-2 : Total Ion chromatogram of six metabolites of methylprednisolone

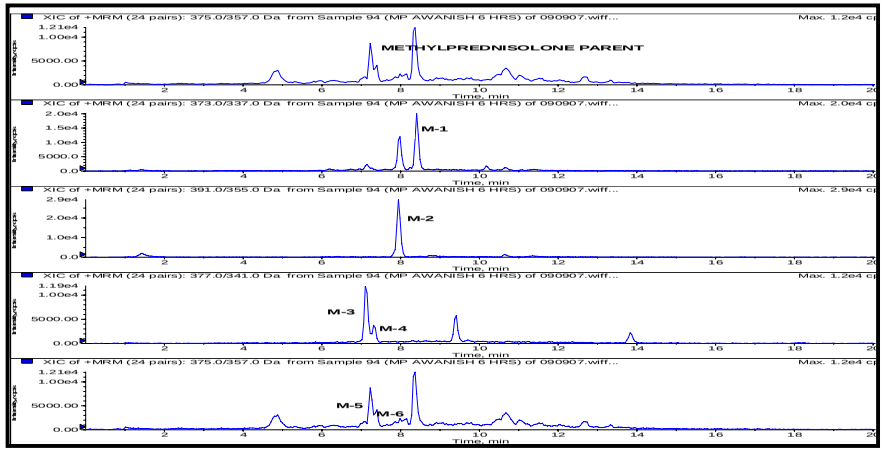

Figure-3: Excretion profile of methylprednisolone