ARTICLE

Identification of Prednisolone, Methylprednisolone and Their Metabolites in Human urine using HPLC (+) ESI-MS/MS and Detection of Possible Adulteration in Indian Herbal Drug Preparations

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Abstract

Objective: To explore the possibility of identifying the maximum number of metabolites of prednisolone and methylprednisolone by LC-MS/MS and to further test the application of this developed method on six Indian herbal drug preparations. **Method and Materials:** The sample extraction procedure involves enzymatic hydrolysis and liquid-liquid extraction and further analysis using LC-MS/MS. The excretion profile was performed with four healthy male volunteers after administration of 40 mg and 8 mg of prednisolone and methylprednisolone, respectively. Six herbal drug preparations obtained from All India Institute of Medical Sciences, India were tested to detect the possible adulteration with prednisolone or methylprednisolone. **Results:** The Analytical method was validated as per the requirement of WADA International Standard of Laboratories (version 6.0). The parent compound and ten urinary metabolites of prednisolone could be identified. The parent, M-1, M-2 and M-3 could be detected up to 72 hours while rest of the metabolites were detectable up to 24 hours. In the case of methylprednisolone, the parent compound and six urinary metabolites were identified. M-1 and M-2 of methylprednisolone were detectable up to 48 hours while the parent drug methylprednisolone and other metabolites were detectable up to 24 hours. Out of the six herbal drugs tested, one showed the presence of prednisolone. **Conclusion:** The improved detection method developed for the identification and detection of prednisolone and methylprednisolone metabolites would prove highly beneficial in extending the time of detection of drug abuse in athletes and also detecting spiked ayurvedic, homeopathic and unani preparations.

Keywords: Doping, Excretion study, LC-MS/MS, Anti-doping Rule Violation, Prednisolone, Methylprednisolone

Introduction

Glucocorticosteroids are potent anti-inflammatory agents**.** These are often misused in sports due to their anti-inflammatory effect, which leads to decrease in pain and increases an athlete's ability to focus on competition. The sports medicine doctors use these drugs to help an injured

muscle or inflamed joint to recover, improving athlete's performance. The abuse of synthetic corticosteroids by medical practitioners and athletes led World Anti Doping Agency (WADA) to ban them in 2004 (1). This led WADA to develop the requirement to develop sensitive and specific detection methods by WADA accredited laboratories with minimal cost impact. At present there are 35 WADA accredited labs all over the world with India being one of them. Based on the testing protocol of various WADA accredited labs, each lab developed their methods to detect various corticosteroids (2-7). However, this method development was mainly focused on to the detection of parent drug.

As per WADA guidelines, use of oral and injectable glucocorticosteroids is banned whereas dermatological, inhalation, and intra-articular injections are permitted justifying the use of glucocorticosteroids for therapeutic purpose. In order to allow justified therapeutic use of alternative routes of glucocorticosteroids, WADA has put a Minimum Required Performance Limit (MRPL) level of 30 ng/ml (8). This means that if a urine sample shows a concentration above 30 ng/ml of glucocorticosteroid then it will be considered positive.

We carried out studies to investigate detectability of various Indian formulations of glucocorticosteroids in urine by detecting the parent form of various corticosteroids after different routes of administration (9). The detection of parent glucocorticosteroids gives a good idea of detectability for the drugs excreted as parent only. However, for the glucocorticosteroids which are extensively metabolized, identification of metabolites and their detection method is required to extend the detection time.

Prednisolone (pregna-1, 4-diene-11-β, 17-α, 21-triol-3, 20-diol) and methylprednisolone (11-β, 17-α, 21-trihydroxy-6-α-methylpregna-1-4-dine-3, 20dione) are potent synthetic glucocorticosteroid mainly used for their anti-inflammatory and immunosuppressive actions. Indian doctors prescribe these agents frequently. These synthetic corticosteroids are readily metabolized in the liver and give rise to a number of metabolites. Hence, an identification method is required to extend the detection time window for their abuse.

The aim of the present work was to explore the possibility of identifying the maximum number of metabolites of prednisolone and methylprednisolone by developing a sensitive and specific liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Based on the number of identified metabolites, excretion profile of various metabolites was studied in healthy volunteers. Further, the application of this developed method was tested on six Indian herbal drug preparations to detect possible adulteration.

The outcome of the present work will improve the detectability window of these two drugs, which are extensively metabolized. It will provide relevant input to Indian doctors to carefully weigh the benefit of administering these drugs to the athletes for therapeutic purpose. It will also be beneficial to detect low concentration of these metabolites in contaminated ayurvedic, homeopathic and unani preparations.

Materials and Methods

Experimental Work

Reagents and chemicals

Reference standards of prednisolone, prednisone and methylprednisolone were purchased from Sigma-Aldrich (Germany). 20-β-OH-prednisolone was a gift from Anti-Doping Laboratory, Rome, Italy. The organic solvents and reagents were of HPLC grade. Acetonitrile, Ethanol and Ethyl acetate were obtained from Qualigens (Mumbai, India), Tertiary Butyl Methyl Ether (TBME) from Acros organics (New Jersey, USA), and Formic Acid from Merck (Mumbai, India). β-glucuronidase *(E.coli)* enzyme was purchased from Roche Diagnostics Corporation (Indianapolis, USA). Double distilled deionized water was prepared on Milli-Q laboratory plant (Millipore, Bedford, USA) installed in the laboratory.

Sample preparation

The sample extraction procedure involves enzymatic hydrolysis and liquid-liquid extraction (10). Two or four milliliters of urine sample aliquot (based on specific gravity), 500 ng/ml of internal standard (17-α methyl testosterone) was added. Hydrolysis was done at pH 7.0 using 0.2 M phosphate buffer by β –glucuronidase *(E.coli)* enzyme at 60°C for an hour. The pH was adjusted to 9-10 with 7% K_2CO_3 and liquid-liquid extraction was performed using 5 ml TBME. After mixing for 15 minutes and centrifugation for 10 minutes at 3000 rpm, the organic layer was separated. The pH of the aqueous layer was adjusted to 2-3 by 6 N HCL and second extraction was done using 4 ml ethyl acetate. After mixing for 15 minutes and centrifugation for 10 minutes at 3000 rpm, the organic layer was mixed with

the first one and evaporated under nitrogen gas at 60°C. Finally, the residue was reconstituted in 100 µl of mobile phase (1% Formic acid and Acetonitrile) (50:50) *(v/v)* and transferred into conical autosampler vials for analysis.

Instrumentation

All LC-MS/MS experiments were performed with Agilent 1100 series, high-pressure liquid chromatograph (Agilent Technologies, Waldron, Germany) for sample introduction and an API 3200™ tandem mass spectrometer (Applied Biosystems, Canada), operating with an electrospray ionization source. The internal standard and analytes were chromatographically resolved using column Inertsil® C-18 ODS-3 (3.0µm, 50mm x 4.6mm). The column temperature was set at 40 °C. The flow rate of mobile phase was 0.6 ml/min, consisted of Solvent A (1% Formic Acid in water) and Solvent B (Acetonitrile). The gradient program was: 0 min- 15% B; 10 min- 60% B; 15 min-75% B; 25 min-

Table 1. Calibration curve details for prednisolone, prednisone, 20-β-OH-prednisolone and methylprednisolone

Compound	Calculated Range(ng/ml)	Regression	Slope	Weighting factor
Prednisolone	15-120	0.9982	0.0447	1/x
Prednisone	15-120	0.9902	0.0674	1/x
20-β-OH-prednisolone	15-120	0.9984	0.0045	1/x
Methylprednisolone	15-120	0.9995	0.0012	1/x

Table 2. Quantitation details of prednisolone, prednisone, 20-β-OH-prednisolone and methylprednisolone

Compound	Expected Concentration [ng/ml]	Measured Concentration [Mean±SD]	Recovery Percentage [Mean±SD]
Prednisolone	15	13.9±0.6	92.6±4.3
	30	27.3±1.6	91.0±5.6
	60	57.2±1.8	95.2±3.1
	120	114.2±1.4	95.0±1.1
Prednisone	15	13.5±0.9	90.0±6.5
	30	29.1±0.2	97.3±2.0
	60	56.3±2.1	93.7±3.6
	120	118.0±1.2	97.9±1.0
20-β-OH-Prednisolone	15	13.8±0.8	92.1±5.6
	30	29.0±0.6	96.5±2.0
	60	56.8±1.8	94.7±3.1
	120	117.0±1.5	97.7±1.2
Methylprednisolone	15	13.6±1.2	90.6±8.0
	30	25.3±1.9	84.3±6.5
	60	56.3±0.8	93.8±1.4
	120	115.0±3.6	95.6±3.0

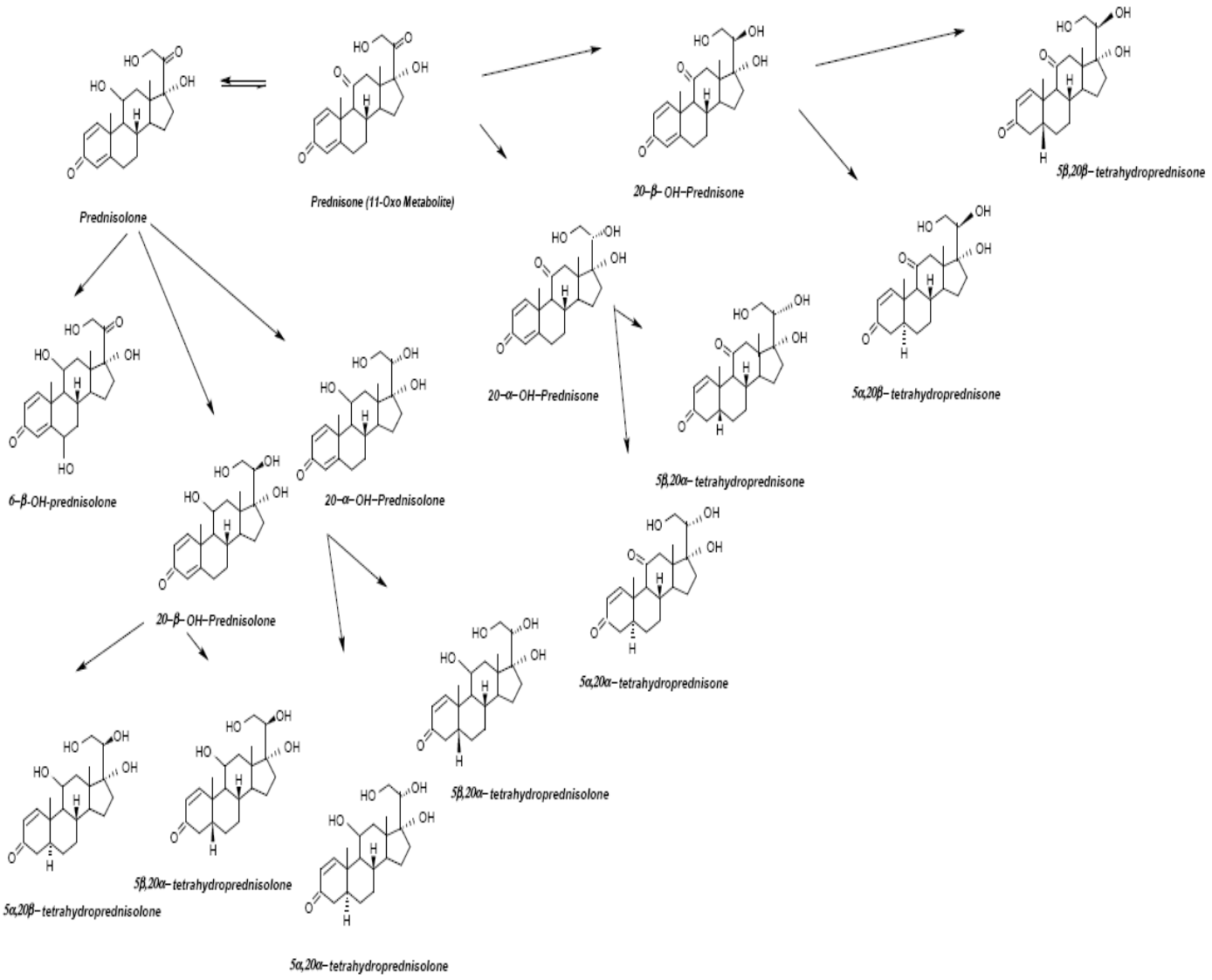

Figure 1a: Proposed metabolic fate of prednisolone

85% B; 30 min-15% B. The injection volume was 10µL. The mass spectrometer operating conditions consisted of a source heater probe of 550 °C, with a TurboIonspray voltage of 5500 V, entrance potential of 10, curtain gas setting of 15 and CAD setting of 3. Collision energies were different for different analytes. The ions for all the analytes were generated in the positive-ion mode. Data acquisition and quantitative processing were accomplished using PE Sciex Analyst™ 1.4.2 software.

Drug administration and excretion study

Excretion study of prednisolone and methylprednisolone were each performed with four healthy male volunteers. The dosage was 40 mg and 8 mg for prednisolone and methylprednisolone, respectively. The study protocol was reviewed and approved by the ethical committee of NDTL, India. Each volunteer signed a consent form prior to drug administration. Samples were collected before and after drug administration for 80 hours and stored at -20 °C prior to analysis.

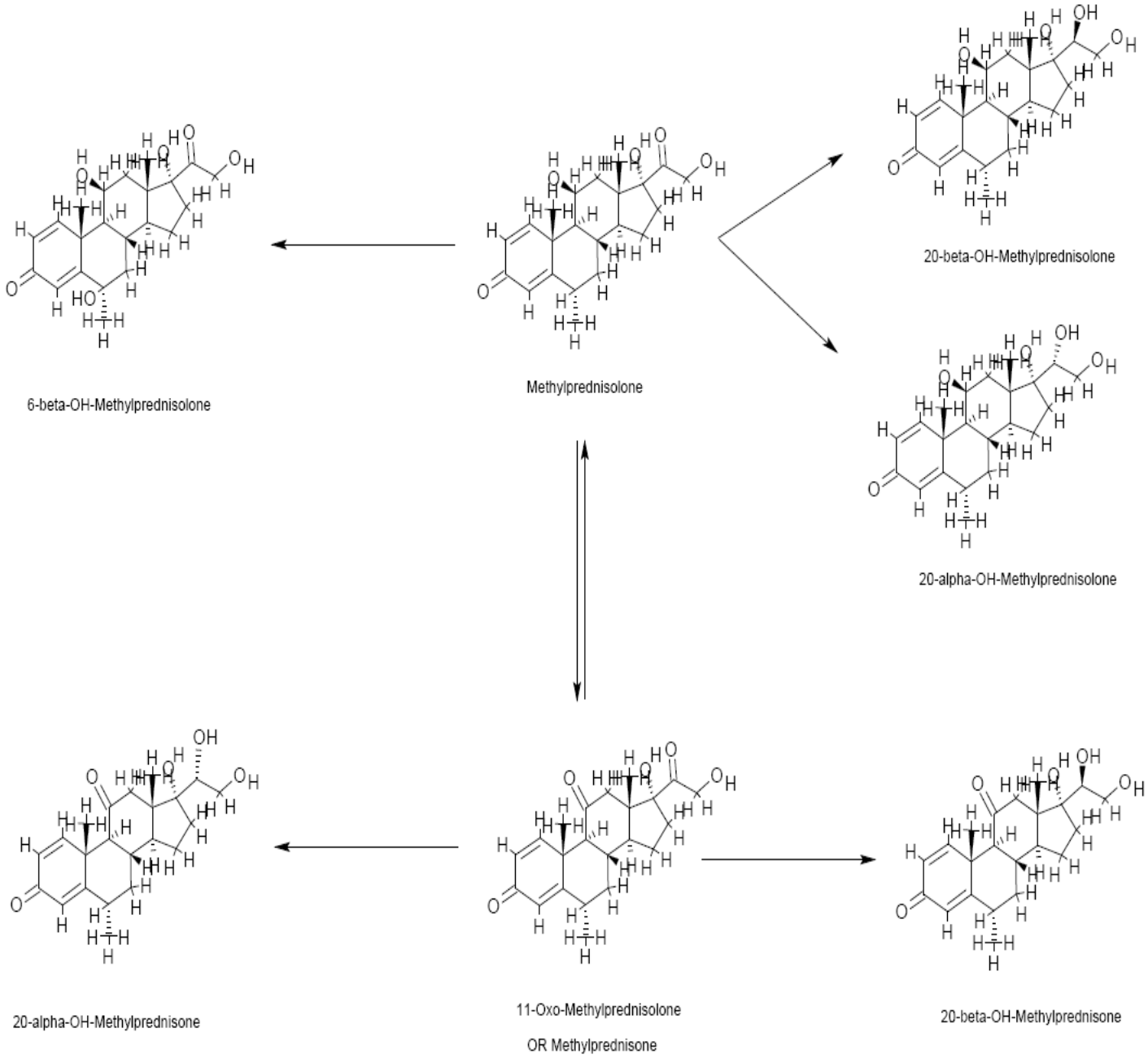

Figure 1b. Proposed metabolic fate of methylprednisolone

Method validation

The Analytical method was validated as per the requirement of WADA International Standard of Laboratories (version 6.0) (11). Method validation was performed under the following heads: sensitivity, recovery, accuracy, precision, linearity, specificity, reproducibility, repeatability, limit of detection (LOD) and limit of quantitation (LOQ). Quantitation of prednisolone, prednisone, 20-β-OH-prednisolone and methylprednisolone were done, as certified reference materials for these compounds were available in the lab. Structural assignment of metabolites was based on changes in molecular masses and retention

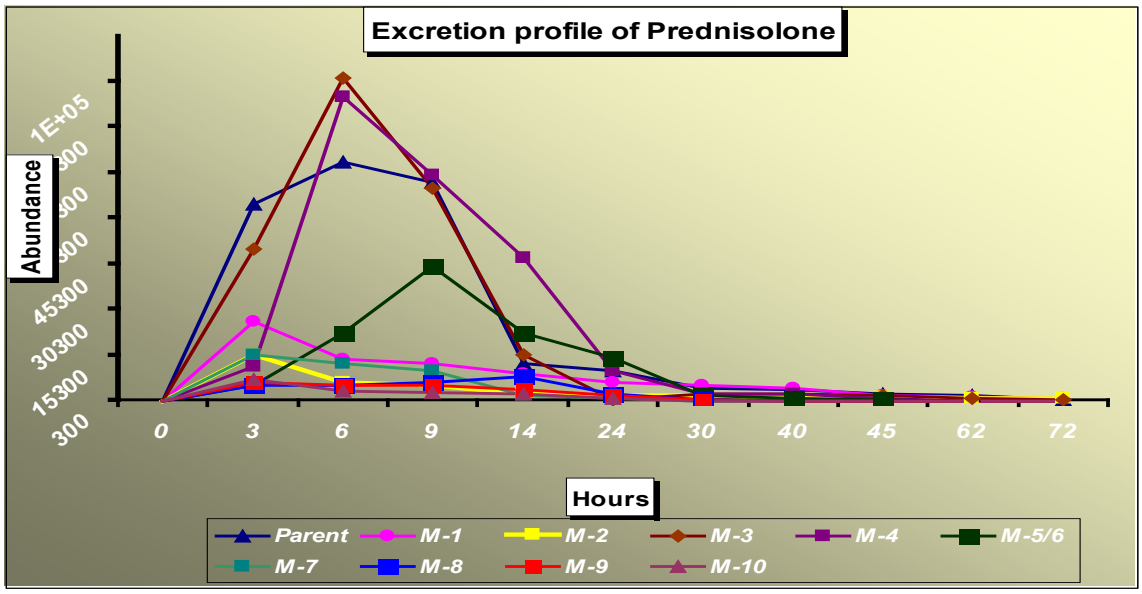

Figure 2. Excretion profile of prednisolone

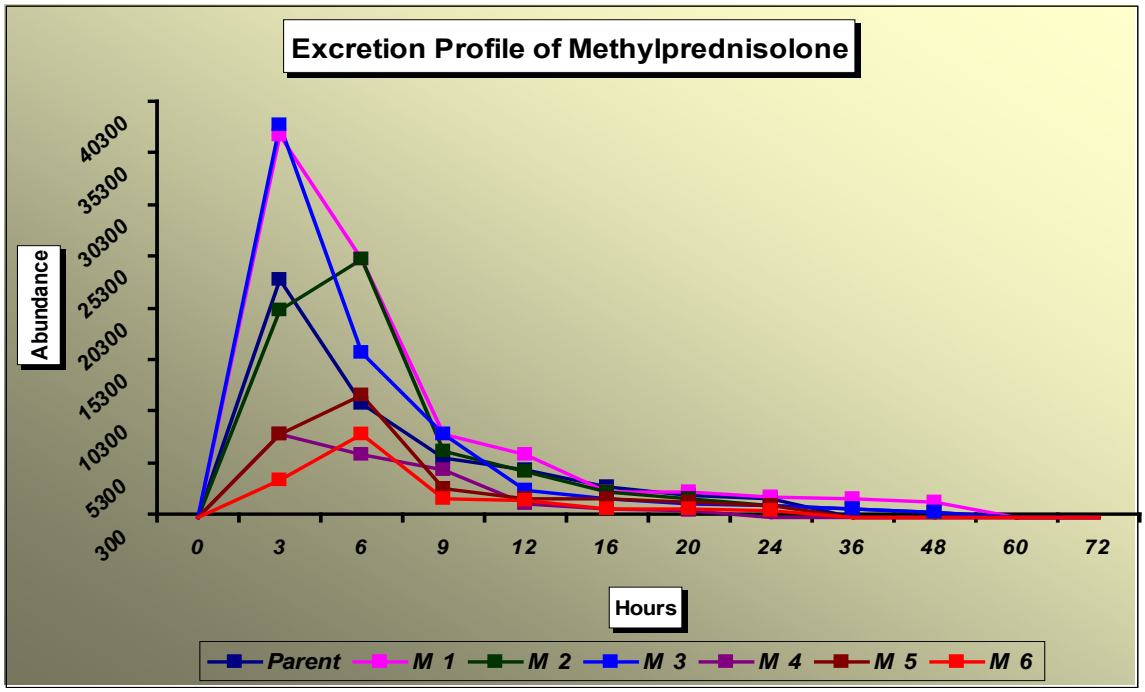

Figure 3. Excretion profile of methylprednisolone

times.

The four level calibration curve was made by adding defined volumes of ethanolic solution of the reference standards of prednisolone, prednisone, 20-β-OH-prednisolone and methylprednisolone. Quality control samples (spiked) were prepared alike in five replicates at four concentration levels. The concentrations of calibration standard and quality control samples were 15 ng/ml, 30 ng/ml, 60 ng/ml, and 120 ng/ml. The inter batch coefficient of variation had to be <15% for precision, and for accuracy the mean value had to be within ±15% of the actual value. A linear regression was used with a weighting factor of 1/x. The coefficient of correlation has to achieve a degree of certainty of R=0.99. Recovery was determined by comparing a 10 µL injection of unextracted calibrator vs a 10 µL injection of extracted

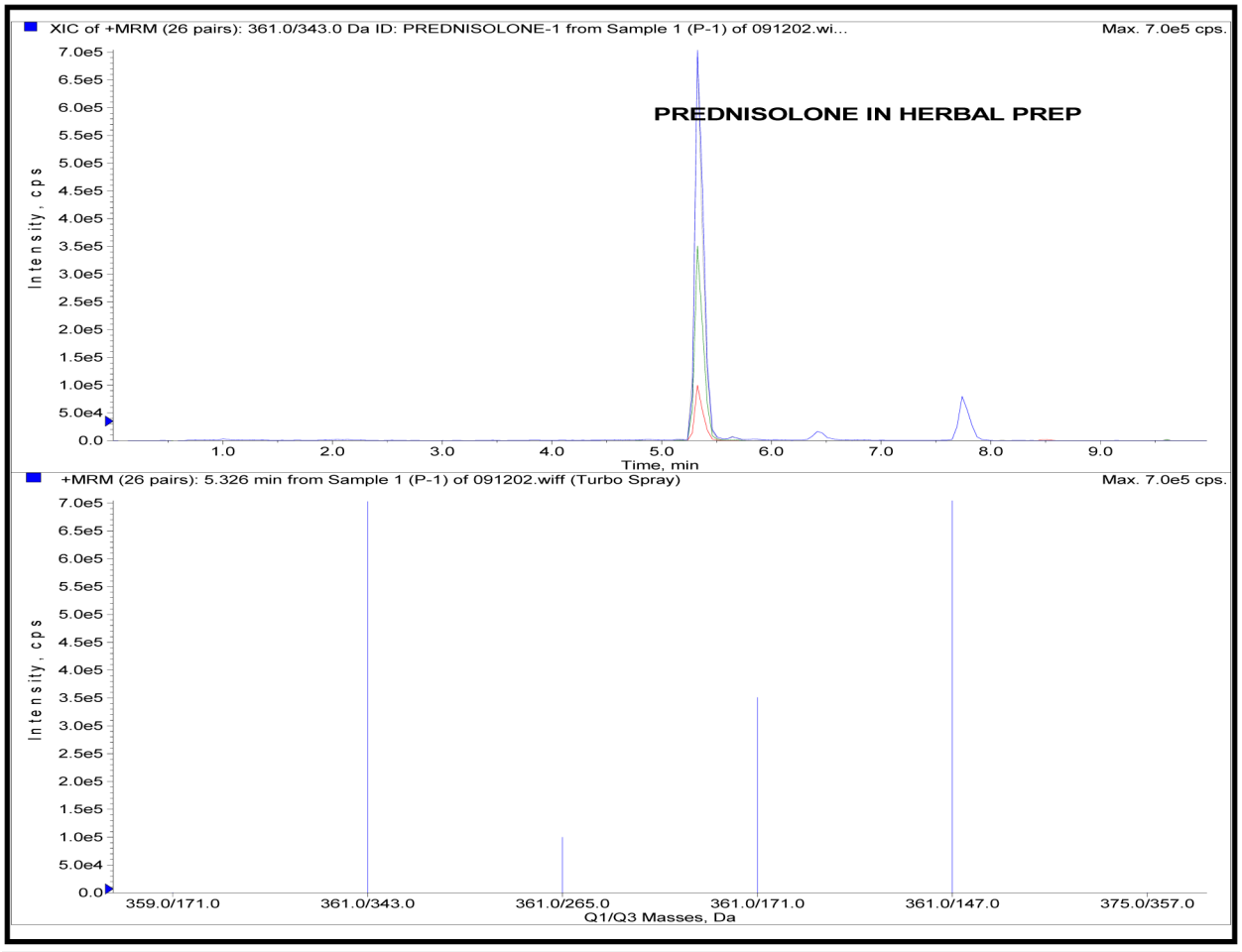

Figure 4. Herbal drug preparation tested positive for prednisolone

calibrator in a range of 15 ng/ml, 30 ng/ml, 60 ng/ml, and 120 ng/ml. Specificity was defined as area of possible interferences in blank urine samples. Blank urine samples had to be below 1/3 of the area of calibration standard of 1ng/ml or not detectable. Repeatability and reproducibility was determined in multiple measurements of the samples under the same analytical conditions.

Herbal drugs testing

Six herbal drugs in powder form were obtained from All India Institute of Medical Sciences, India. For each herbal product 500 mg of powder was dissolved in 5 ml methanol and was centrifuged at 3000 rpm for 15 minutes. The supernatant was separated and was subjected to LC-MS/MS analysis.

Results

The chromatographic conditions (column and mobile phase) were chosen in an appropriate way and were found to be compatible with the API source. Prednisolone and methylprednisolone and their metabolites (10 for prednisolone and 6 methylprednisolone) could be eluted within 30 minutes of runtime. Ionization of mobile phase components (Acetonitrile, 1% formic acid) and endogenous compounds were the main source of background noise from which the compounds of interest were successfully differentiated. With the MS/MS method, the signal intensities for all of the metabolites were found better in positive mode as compared with the negative mode because of better electrospray ionization of positively charged steroids. The corresponding retention times, MS and MS/MS spectra were then used to obtain structural information.

Method validation

Ten different blank urine samples were prepared by the same procedure. The total ion chromatogram of the excretion study samples was compared with that of blank urine sample to detect suspicious peaks. No significant

interferences from matrices at the retention times of the targeted ion transitions were observed.

The method was validated under WADA ISL (6.0) guidelines, which is a mandatory requirement for all WADA accredited laboratories. The reference standards of prednisolone, prednisone, 20-β-OH-prednisolone and methylprednisolone were available in the laboratory and were used for the purpose of method validation. The calibration range used was from 15 ng/ml to 120 ng/ml. The calibration curve was linear in the range from 15 ng/ml to 120 ng/ml as shown in Table-1.

The recoveries of all the four analytes were determined at 15, 30, 60, and 120 ng/ml. Five replicates of urine samples were spiked with the target analytes in the beginning of the sample preparation and the mean peak area of the respective analyte was compared with the mean peak area of direct standard solutions of same concentration. The details of recovery percentage and calculated concentration are tabulated in Table-2. The method showed very good precision and accuracy (reproducibility). The acceptance criterion was ± 15%.

Excretion Study

Identification of Prednisolone metabolites

The parent compound and ten urinary metabolites of prednisolone could be identified. These were prednisone [M-1], 6-β-OH-prednisolone [M-2], 20-β-OH-prednisolone [M-3], 20-α-OH-prednisolone [M-4], 20-α-OH-prednisone [M-5], 20-β-OH-prednisone [M-6], 2 epimers of 20-β-OH-prednisolone (5-α-20-β–tetrahydroprednisolone & 5-β-20-β–tetrahydroprednisolone) [M-7], 2 epimers of 20-α-OH-prednisolone (5-α-20-α–tetrahydroprednisolone & 5-β-20-α–tetrahydroprednisolone) [M-8], 2 epimers of 20-β-OH-prednisone (5-α- 20-β –tetrahydroprednisone & 5- β- 20-β–tetrahydroprednisone) [M-9], 2 epimers of 20-α–OH-prednisone (5-α-20-α–tetrahydroxyprednisone & 5-β-20-α–tetrahydroprednisone) [M-10]. (Figure: 1a)

The parent, M-1, M-2 and M-3 could be detected up to 72 hours while rest of the metabolites were detectable up to 24 hours. The excretion profile of prednisolone and its metabolites is shown in Figure 2.

Identification of Methylprednisolone metabolites

The parent compound and six urinary metabolites for methylprednisolone were identified and these included: methylprednisone [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: 1b)

M-1 and M-2 of methylprednisolone were detectable up to 48 hours while the parent drug methylprednisolone and other metabolites were detectable up to 24 hours. The excretion profile of methylprednisolone and its metabolites is shown in Figure 3. All the metabolites of methylprednisolone were also detected in samples of a patient after intraarticular injection of methylprednisolone for 72 hours while the parent was detectable up to 48 hours.

Herbal drugs testing

Out of the six herbal drugs tested one showed the presence of prednisolone (Figure 4). This may be due to the adulteration of the herbal drug which could have been done intentionally. The presence of prednisolone may be insignificant clinically but inadvertent use of the herb can lead to a positive drug test.

Discussion

The synthetic glucocorticosteroids are often misused in sports. The use of glucocorticosteroids is quite prevalent in India by the allopathic medical practitioners. Ayurvedic, homeopathic and unani doctors misuse it sometimes. They spike their preparations with the glucocorticosteroids for fast recovery of patients. In sports, it is permitted to use glucocorticosteroids if medically justified. The local, intra-articular injections or dermatological preparations are allowed with the approval of a therapeutic use exemption granted by the committee constituted for the said purpose (11). The adverse analytical findings (positive reports) of 35 WADA accredited labs are statistically evaluated by WADA on yearly basis. The evaluation for the year 2008 by WADA shows a total 316 adverse analytical findings for glucocorticosteroids all over the world. Out of 316 adverse analytical findings of glucocorticosteroids, there were 11.7% and 4.4% positives reported for prednisolone and methylprednisolone, respectively (12). These synthetic glucocorticosteroids are readily metabolized in the liver and give rise to a number of metabolites. The qualitative identification of multiple metabolites in the present study was done by the HPLC-MS/MS method. The excretion profile of the identified metabolites was further studied by doing an excretion study of both drugs in four healthy volunteers each. It was possible to identify ten urinary metabolites of prednisolone and six urinary metabolites of

methylprednisolone after oral administration of drug. The study explores newer metabolites of the drugs, which can further be used for the confirmatory purposes.

The present study revealed that after the administration of prednisolone, the parent drug, prednisone [M-1], 6-β-OH-prednisolone [M-2] and 20-β-OH-prednisolone [M-3] were detectable up to 72 hours and can be treated as additional markers for prednisolone abuse. After the administration of methylprednisolone, parent drug disappears in 24 hours but the two metabolites methylprednisone [M-1] and 6-β-OH-methylprednisolone [M-2] were detectable up to 48 hours, which can be used as marker for methylprednisolone abuse.

Out of the six herbal drugs tested one was found positive for prednisolone. This proves that all herbal preparations may not be safe and may lead the athlete to get caught under anti-doping rule violation. It is suggested that medical practitioners and athletes should be aware of the possibility of testing positive with the use of the oral preparations of prednisolone and methylprednisolone due to the possibility of detection of metabolites for longer period than the parent drug. The medical fraternity needs to be advised to use these drugs more judiciously than using them as a magic drug for fast recovery. It is also essential to educate Indian doctors and athletes about the possibility of getting caught under the anti-doping rule violation, either knowingly or unknowingly.

It is therefore concluded that improved detection method developed for the identification and detection of metabolites of prednisolone and methylprednisolone would prove highly beneficial in extending the time of detection of drug abuse in athletes and also detecting spiked ayurvedic, homeopathic and unani preparations.

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