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Simultaneous identification of prednisolone and its ten metabolites in human urine by high performance liquid chromatography-tandem mass spectrometry

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The use of prednisolone and prednisone is prohibited by the World Anti-Doping Agency (WADA) due to their performance-enhancing effect. The purpose of the present work was to explore the possibility of identification and detection of various metabolites of prednisolone by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in excretion study samples. Ten metabolites of prednisolone could be identified namely prednisone (11-oxo metabolite) [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 tetrahydro epimers of 20-β-OH-prednisolone [M-7], 2 tetrahydro epimers of 20-α-OH-prednisolone [M-8], 2 tetrahydro epimers of 20-β-OH-prednisone [M-9], and 2 tetrahydro epimers of 20-α–OH-prednisone [M-10]. Prednisolone was administered in 10-, 20-, and 40-mg dosage to healthy volunteers to study detection of various metabolites. The parent, M-1, M-2, and M-3 could be detected up to 72 h while rest of the metabolites were detectable up to 24 h after drug administration. The detection of newer metabolites of the drug can further be used for confirmatory purposes. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: prednisolone; LC-MS/MS; WADA; glucocorticosteroids

Introduction

Glucocorticosteroids are naturally produced steroid hormones, or synthetic compounds, that inhibit the process of inflammation. Cortisol (hydrocortisone) is the standard of comparison for glucocorticosteroid potency. The pharmacological activity of different synthetic glucocorticosteroids is calculated on the basis of pharmacological scale designed in comparison to hydrocortisone. All the synthetic glucocorticosteroids are potent anti-inflammatory agents as compared to hydrocortisone. These are often abused in sports due to their anti-inflammatory effect, which leads to decrease in pain and increases athletes ability to focus on competition. Synthetic glucocorticosteroids are a subclass of steroids, which are analogues to cortisol with respect to their chemical and pharmacological properties. They are involved in a wide range of physiological functions, such as the breakdown of protein, fat, and carbohydrate, and regulation of inflammation, blood electrolytes level, and behaviour. The misuse of glucocorticosteroids in sports has been banned by the World Anti-Doping Agency (WADA) since 2004.[1] According to WADA guidelines, use of oral and injectable corticosteroids is banned in sports whereas, dermatological, inhaler, and intra-articular injections are permitted justifying the use of corticosteroids for therapeutic purpose. In order to allow justified therapeutic use of dermal/inhaler forms of corticosteroids, WADA has put a Minimum Required Performance Limit (MRPL) of 30 ng/ml, which means that the adverse analytical finding for glucocorticosteroids would only be reported if the concentration of glucocorticosteroids is found above 30 ng/ml.[2–4] Prednisolone (pregna-1, 4-diene-11-β, 17, 21-triol-3, 20-diol) is a synthetic corticosteroid widely used to treat various clinical conditions because of its anti-inflammatory and immunosuppressive actions.

The gas chromatography-mass spectrometry (GC-MS) methods for the analysis of corticosteroids are not successful due to low volatility of these compounds as MO-TMS or TMS derivatives, thermal instability, long analysis time, and time-consuming derivatization.[5–10] High performance liquid chromatography coupled with mass spectrometry (LC-MS) presents the condition well suited for the separation of these compounds with good specificity and sensitivity.[11–13]

The detectability of various Indian formulations of glucocorticosteroids was studied in urine by detecting the parent form of various corticosteroids after different routes of administration.[14] The detection of parent glucocorticosteroids in human urine provides relevant information for doping control analysis of these substances as a parent drug. Few of glucocorticosteroids are extensively metabolized in humans; the detection of their metabolite/s and identification of long-term marker metabolite/s may prove beneficial to extend the detection time window to catch their abuse in sports.

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Prednisolone is excreted to a much lesser extent as a parent compound in human urine and is metabolized to a larger extent in to various metabolites.[15–21] The purpose of the present work was to explore the possibility of identification of prednisolone and its maximum number of metabolites in human urine by high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC/ESI/MS/MS). Structural assignment of metabolites was based on changes in molecular masses and retention times. The study explored identification of newer metabolite/s of the drug which may be further used for confirmatory purposes in dope testing.

Experimental

Materials and chemicals

Certified reference standard of prednisolone, prednisone and 17 -α methyl testosterone were purchased from Sigma-Aldrich (Munich Germany) and 20-β-OH-prednisolone was a gift from the Anti-Doping Laboratory, Rome, Italy. Acetonitrile, ethanol and ethyl acetate were obtained from Qualigens (Mumbai, India). Tertiary butyl methyl ether (TBME) was obtained from Acros Organics (New Jersey, USA), and formic acid was obtained from Merck (Mumbai, India). β-glucuronidase *(E.coli)* enzyme was purchased from Roche Diagnostics Corporation (Indianapolis, IN, USA). Double distilled deionized water was prepared on Milli-Q laboratory plant (Millipore, Bedford, MA, USA) installed in the laboratory.

Sample pretreatment

The sample preparation procedure involves enzymatic hydrolysis and liquid-liquid extraction.[27] Based on the specific gravity (SG) of the urine samples, 2 ml (SG ≥ 1.010) or 4 ml (SG < 1.010) of urine sample aliquot was taken; 500 ng/ml of internal standard (17-α methyl testosterone) was added to each sample aliquot. Hydrolysis was done at pH 7.0 using 0.2 M phosphate buffer by β –glucuronidase *(E.coli)* enzyme at 60 °C for 1 h. 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 min and

Table 1. MRM transitions, eclustering potential, collision energy and retention time for prednisolone and ten metabolites.

S.No.	Compound	MRM (m/z)	Declustering Potential (DP)	Collision Energy (CE)	Retention Time (RT) (minutes)
1.	Prednisolone	361-343.4	40	20	9.5
		361-175.1	40	30	
		361-147.3	40	32	
2.	Prednisone [M1]	359-341.2	40	20	9.5
		359-322.7	40	30	
		359-295.4	40	35	
3.	6-β-OH-Prednisolone [M-2]	377-359.3	45	20	9.0
		377-341.1	45	30	
		377-266.2	45	35	
4.	20-β-OH-Prednisolone [M-3]	363-345	40	20	8.2
		363-267.3	40	35	
		363-171.5	40	40	
5.	20-α-OH-Prednisolone [M-4]	363-345	40	20	8.3
		363-267.3	40	35	
		363-171.5	40	40	
6.	20-α-OH-Prednisone [M-5]	361-265.5	40	33	8.2
		361-147.3	40	40	
		361-343.3	40	20	
7.	20-β-OH-Prednisone [M-6]	361-265.5	40	33	8.2
		361-147.3	40	40	
		361-343.3	40	20	
8.	5-α-20-β–tetrahydroprednisolone / 5-β-20-β–tetrahydroprednisolone) [M-7]	365-347.1	43	20	8.3
		365-329.3	43	28	
		365-147.1	43	45	
9.	5-α-20-α–tetrahydroprednisolone / 5-β-20-α-tetrahydroprednisolone [M-8]	365-347.1	43	20	10.8
		365-329.3	43	28	
		365-147.1	43	45	
10.	5-α- 20-β –tetrahydroprednisone / 5- β-20-β–tetrahydroprednisone) [M-9]	363-297.3	42	30	9.5
		363-345.4	42	30	
		363-327	42	25	
11.	(5-α-20-α–tetrahydroxyprednisone / 5-β-20-α–tetrahydroprednisone) [M-10]	363-297.3	42	30	10.9
		363-345.4	42	30	
		363-327	42	25	

centrifugation for 10 min at 1118 g, the organic layer was separated. The pH of the aqueous layer was adjusted to 2–3 by 6 N HCL and a second extraction was done using 4 ml ethyl acetate. After mixing for 15 min and centrifugation for 10 min 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 (HPLC-ESI (+) MS/MS)

All HPLC-MS/MS experiments were performed with Agilent 1100 series, high-pressure gradient pumping system and autosampler (Agilent Technologies, Waldbronn, Germany) 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 Intersil® C-18 ODS-3 (3.0 µm, 50 mm x 4.6 mm). The column temperature was set at 40 °C. The mobile phase, delivered at a flow rate of 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 – 85% B; 30 min – 15% B. The injection volume was 10 µl.

The mass spectrometer operating conditions consisted of a source heater probe of 550 °C, with a Turbolonspray voltage of 5500 V, entrance potential of 10, curtain gas setting of 10 and CAD setting of 4. Collision energies were different for different analytes and are listed in Table 1. The ions for all the analytes were generated in the positive-ion mode. Data acquisition and quantitative processing were accomplished using PE Sciex Analyst™ 1.4.1 software.

Drug administration and excretion study

Three healthy male volunteers (25 ± 2 years, 70 ± 5 kg) gave their informed consent to participate in the study. The study protocol was reviewed and approved by the ethical committee of NDTL, India. Blank urine was collected before the administration of drug. Prednisolone (Omnacortil, Sun Pharma, India) was administered in three different dosage viz. 10 mg, 20 mg, and 40 mg and urine samples collected up to 72 h and immediately frozen at −20 °C.

Method validation

The analytical method was validated as per the requirement of WADA ISL (version 6.0).[28] Method validation was performed under the following headings: sensitivity, recovery, accuracy, precision, linearity, specificity, reproducibility, and repeatability. Quantitation of prednisolone, prednisone and 20-β-OH-prednisolone was done to prove the authenticity of the method for the new metabolites.

The four-level calibration curve was made by using defined volumes of ethanolic solution of the reference standards of prednisolone, prednisone and 20-β-OH-prednisolone. Quality control samples (spiked) were prepared in four 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 the mean value had to be within ± 15% of the actual value for accuracy. 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. Extraction efficiencies were determined by comparing a 10 µl injection of unextracted calibrator vs a 10 µl injection of extracted calibrator in a range of 15 ng/ml, 30 ng/ml, 60 ng/ml, and 120 ng/ml. Acceptable specificity was defined as area of possible interferences in blank urine samples. Blank urine samples had to be below one-third of the area of calibration standard of 1 ng/ml or not detectable. The specificity of the method was determined by analyzing five sample pairs at each concentration. Repeatability and reproducibility was determined in multiple measurements of the samples under the same analytical conditions.

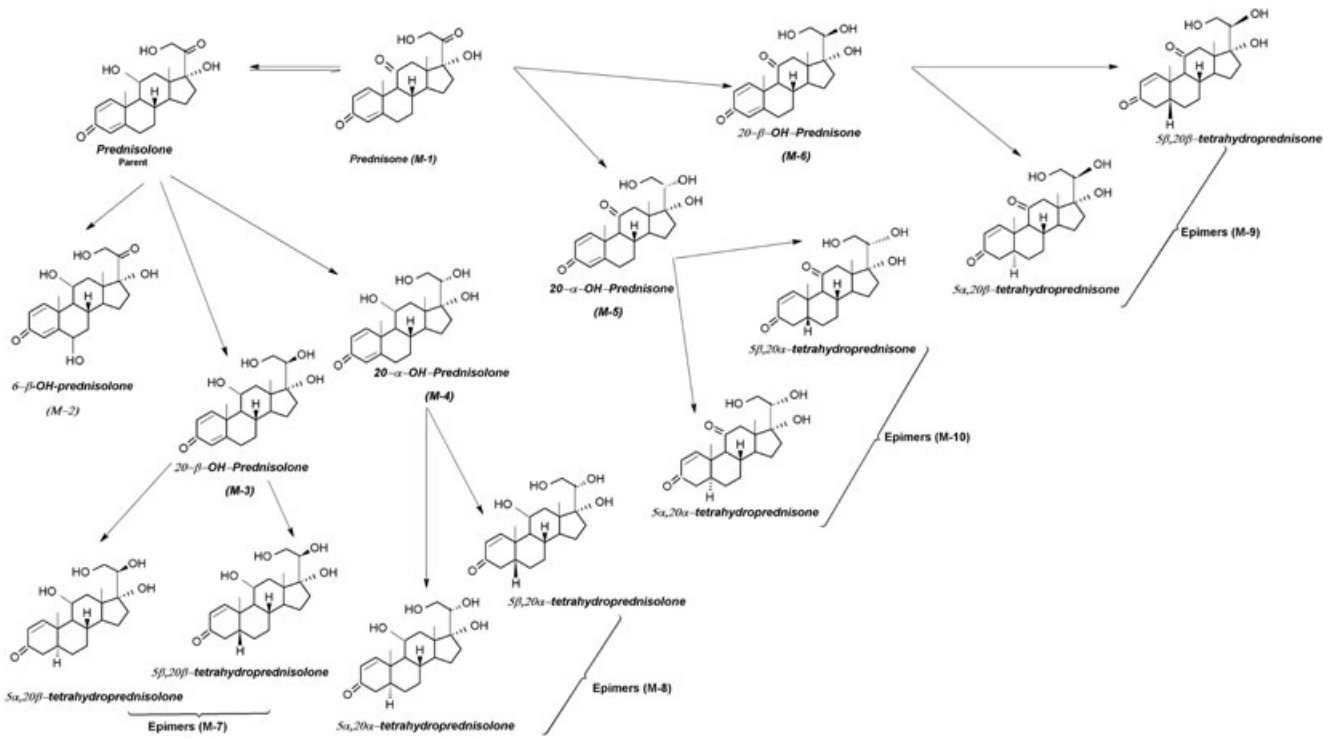

Figure 1. Proposed metabolic fate of prednisolone.

Result and discussion

Detailed information about the molecular structure of the possible metabolites was investigated. The full scan mass spectrum of extracted urine samples after ingesting of 10 mg of prednisolone was compared with that of blank urine sample to find out the probable metabolites. The final MRM transitions for the metabolites resulted after performing full scan parent

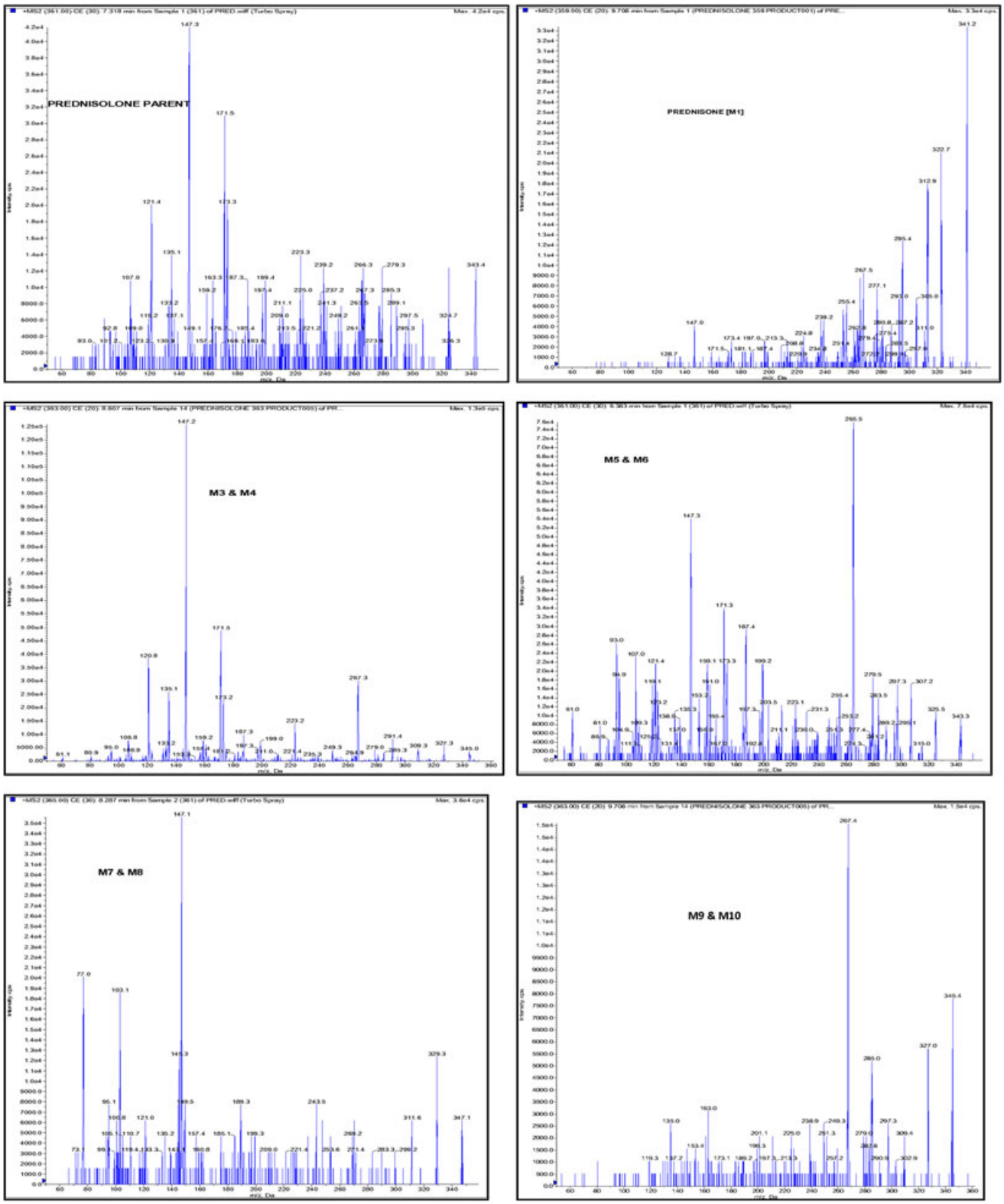

Figure 2. Product ion scan of prednisolone and its ten metabolites.

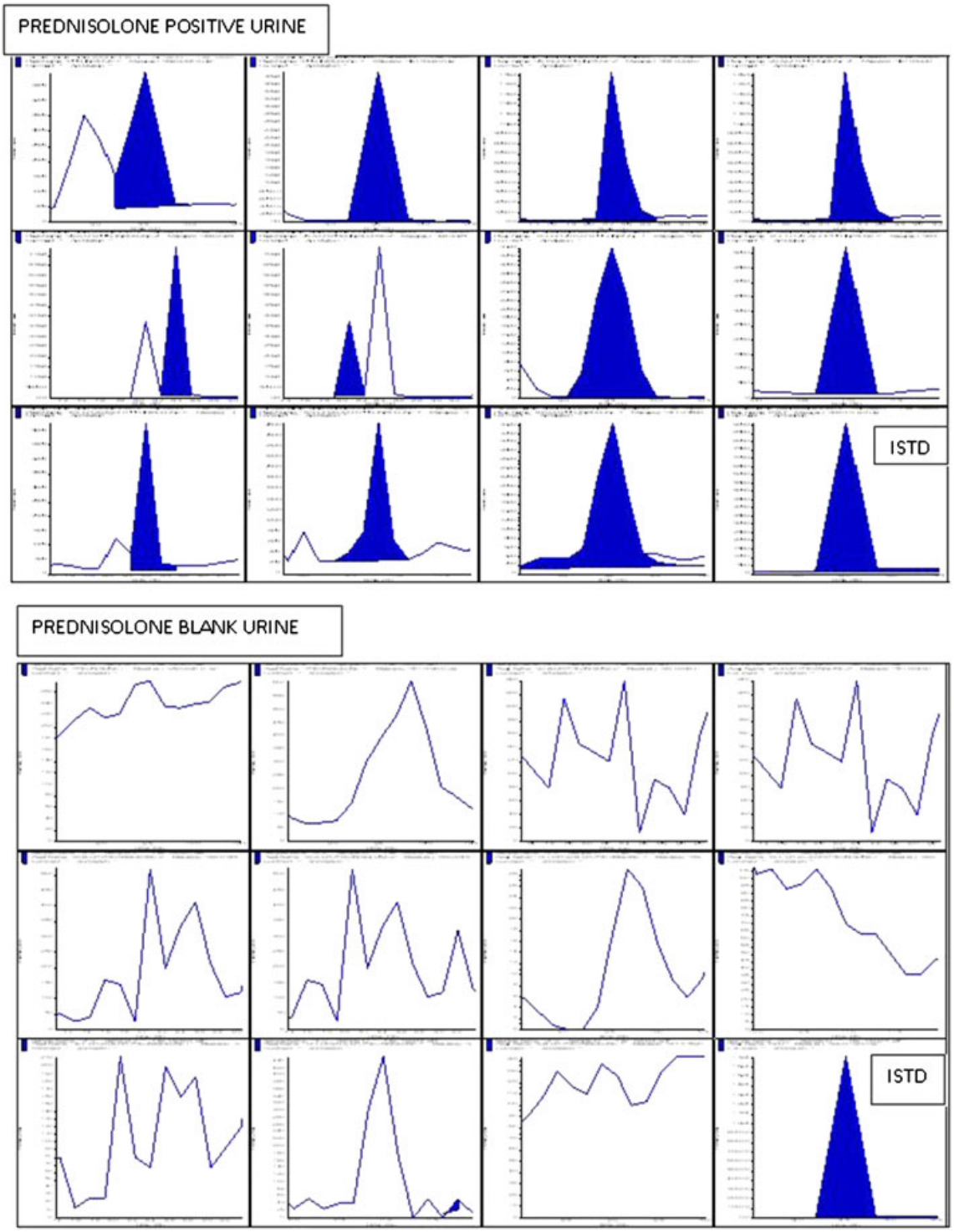

Figure 3. Drug free urine showing no matrix interference for prednisolone and its ten metabolites when compared to prednisolone positive urine.

ion scan followed by full scan product ion scan. Structural assignment of metabolites was based on changes in molecular masses, their characteristic fragmentation pattern and retention times.[21–26]

It was possible to identify ten urinary metabolites of prednisolone with the retention time varying between 8.2 to 10.9 min. The various metabolites detected were prednisone (11-oxo metabolite) [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], two tetrahydro epimers of 20-β-OH-prednisolone (5-α-20-β–tetrahydroprednisolone & 5-β-20-β–tetrahydroprednisolone) [M-7], two tetrahydro epimers of 20-α-OH-prednisolone (5-α-20-α–tetrahydroprednisolone & 5-β-20-α–tetrahydroprednisolone) [M-8], two tetrahydro epimers of 20-β-OH-prednisone (5-α- 20-β –tetrahydroprednisone & 5- β- 20-β–tetrahydroprednisone) [M-9], two tetrahydro epimers of 20-α–OH-prednisone (5-α-20-α–tetrahydroxyprednisone & 5-β-20-α–tetrahydroprednisone) [M-10] (Figure 1).

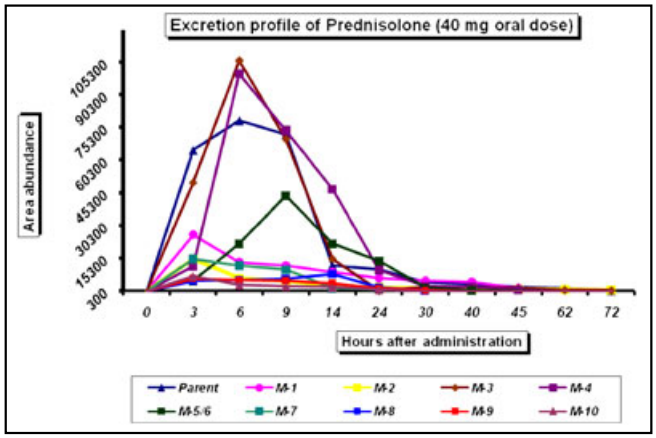

Figure 4. Urinary excretion profile of prednisolone and ten metabolites in 72 hours.

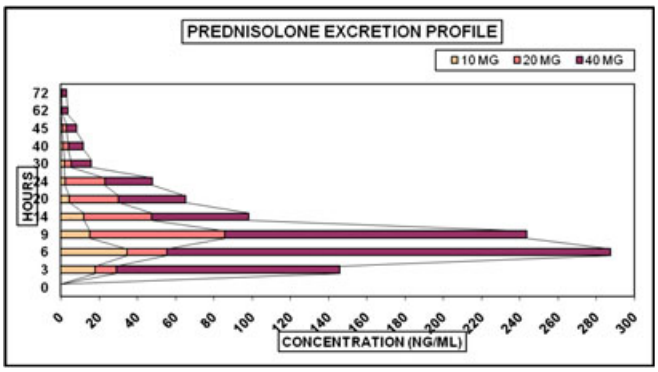

Figure 5. Excretion profile of prednisolone at different dosage in human.

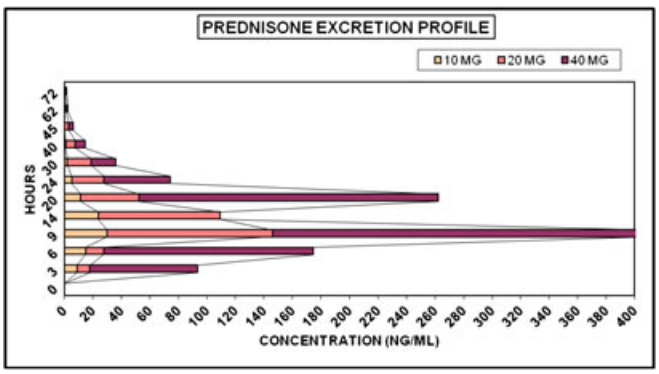

Figure 6. Excretion profile of prednisone at different dosage in human.

The chromatographic conditions (column and mobile phase) were chosen in an appropriate way and were found to be compatible with the API source. HPLC conditions were optimized to allow baseline resolution of the parent drug and all ten metabolites. Gradient studies were performed to achieve a fast elution ramp without losing too much of sensitivity and resolution. The separation of the analytes was carried out under acidic conditions (pH-5) in order to limit secondary interaction on the free silanol groups of the C-18 column. Ionization of mobile phase components (1% formic acid, acetonitrile) and endogenous compounds were the main source of background noise from which the compounds of interest were successfully differentiated.

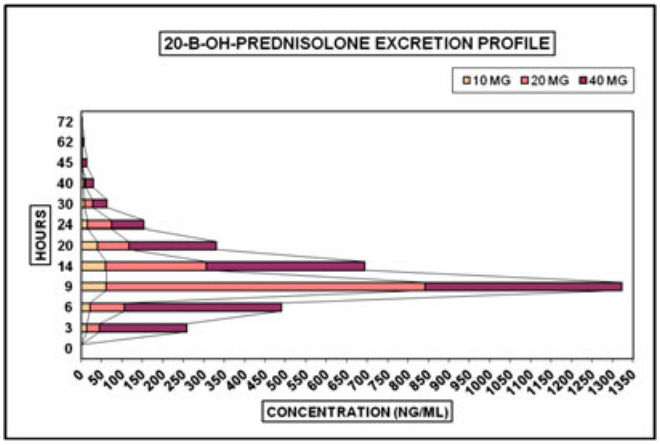

Figure 7. Excretion profile of 20-B-OH-prednisolone at different dosage in human.

Table 2. Calibration data for prednisolone, prednisone and 20-β-OH-prednisolone.

Metabolite	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

Table 3. Recovery percentage of prednisolone, prednisone and 20-β-OH-prednisolone in urine.

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

With the MS/MS method, the signal intensities for all metabolites were found better in positive mode as compared to the negative mode because of better electrospray ionization of positively charged steroids. The LC-MS/MS method was optimized to detect these steroids, using the multiple reaction monitoring (MRM) pair comprising the precursor and product ions. The daughter ions (Q3) of all the steroids were obtained during the collision of the precursor ions (Q1) in tandem MS. The corresponding retention

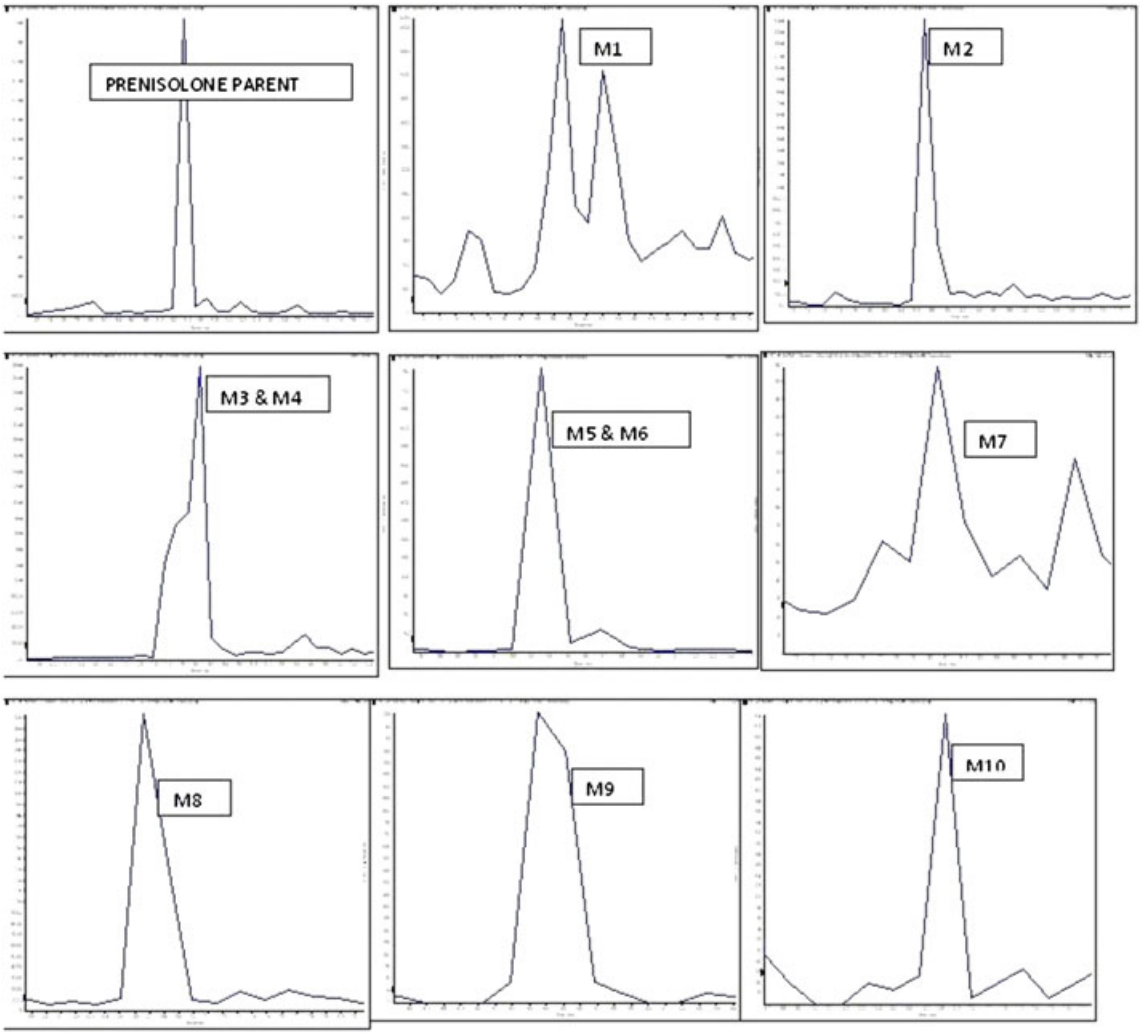

Figure 8. Identification of prednisolone (parent) and its ten metabolites in prednisolone positive doping control urine sample.

times, MS and MS/MS spectra were then used to obtain structural information (Figure 2). The three diagnostic MRM ion transitions for prednisolone and all the ten metabolites are listed in Table 1. All the metabolites and the parent compound were clearly distinguishable from the physiologic population of endogenous glucocorticosteroids. The specificity of the new method was found to be satisfactory as no interference from endogenous glucocorticosteroids was observed in ten blank urine samples of different origins (Figure 3).

The analysis of prednisolone by LC-ESI (+) MS/MS has shown that beside the unchanged parent drug, ten metabolites were detected [M1–M10]. It also shows that there is no formation of adduct ions with formic acid from mobile phase and the compounds are characterized by $(M + H^+)$ ion. The unchanged drug (prednisolone) with parent ion 361 was eluted at 9.5 min. The M-1 formed due to the C-11 hydroxyl group oxidation of parent gives the parent ion 359 and was eluted at 9.5 min along with the parent but gives different spectrum when extracted. The M-2 is formed due to the hydroxylation of parent drug at C-6 position, giving 377 as the parent ion and elutes at 9.0 min. The epimeric pair of metabolites M-3 and M-4 with parent ion 363, formed due to reduction of C = O bond at C-20 position in the parent drug are eluted at 8.2 and 8.3 min, respectively. Similarly, the metabolites M-5 and M-6 which are epimers of M-1, with reduced C = O bond at C-20 position and were eluted at 8.2 minwith 360 as the parent ion. M-7 and M-8 are the metabolites formed due to the hydrogenation of C-5 atom of M-3 and M-4, respectively. Both M-7 and M-8 gives the parent ion 365 and elute at 8.3 and 10.8 min, respectively. Similarly, M-9 and M-10 are the metabolites formed due to the hydrogenation of C-5 atom of M-6 and M-5, respectively. Both M-9 and M-10 elutes at 9.5 and 10.9 min, respectively with 363 as their parent ion. The last four metabolites M-7, M-8, M-9, and M-10 are the epimeric pairs which could not be resolved in our chromatographic system.

The urine samples of volunteers after different dosage of drugs showed that the parent, M-1, M-2 and M-3 could be detected up to 72 h, while rest of the metabolites were detectable up to 24 h. However, of all ten detectable metabolites [M1-M10], M-1, M-3, M-4, M-5 and M-6 are the metabolites which show maximum abundance in 24 h (Figure 4). The quantitation of prednisolone, prednisone, and 20-β-OH-prednisolone was done and the peak levels were found between 6 and 10 h (Figures 5–7). Based on this, the abundance of the rest of the metabolites was also analyzed, and was shown to give the same pattern.

The calibration curve was linear in the range of 15 ng/ml to 120 ng/ml of prednisolone, prednisone and 20-β-OH-prednisolone in human urine as shown in Table 2. The recoveries of all the three at 15, 30, 60, and 120 ng/ml ranged from 90.0% to 97.9% which passes the acceptance criterion (Table 3).

The existing literature shows few methods developed and adopted by various WADA-accredited laboratories including ours.[5–8] The focus of these methods was on the detection of parent corticosteroids, which serves the purpose for the detection of corticosteroids excreted as parent only. However, prednisolone is metabolized extensively; hence the relevance of identification of multiple metabolites is obvious. The identification of 20-β-OH-prednisolone, 20-α-OH-prednisolone & 6-β-OH-prednisolone was performed by Garg *et al*. and Rocci *et al*.[16,17] whereas Kareim *et al*. developed a method for identification of seven metabolites of prednisolone using a PGC column.[21] The present study shows detection of ten metabolites which include additional three metabolites viz. 6-β-OH-prednisolone [M-2], two tetrahydro epimers of 20-β-OH-prednisolone [M-7], and two tetrahydro epimers of 20-α-OH-prednisolone [M-8]. So far only prednisone [M-1] and 20-β-OH-prednisolone [M-3] have been considered as the long-term marker metabolites for detection of prednisolone abuse in sports. However, the present study explores one newer long-term metabolite 6-β-OH-prednisolone [M-2] which would further aid in long-term detection of prednisolone abuse in sports and also in detecting their adulteration in ayurvedic and homeopathic preparations. Further work is in progress to perform route-of-administration specific study for the detection of the prednisolone and its ten identified metabolites in human biological samples.

The described method was applied to 05 doping control samples reported positive for misuse of prednisolone in National Dope Testing Laboratory (NDTL), India in the year 2010. All ten reported metabolites along with parent were detected in all 05 samples (Figure 8).

Conclusion

Ten urinary metabolites of prednisolone could be identified after oral administration of drug. The newer metabolite identified can be used for the confirmatory purposes and long-term detection of prednisolone abuse.

Acknowledgements

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