Research Paper

Analysis of glucocorticosteroids by atmospheric pressure chemical ionization-liquid chromatography mass spectrometry (APCI-LC/MS/MS)

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ABSTRACT

Objective: To develop a rapid liquid chromatography / mass spectrometry / mass spectrometry (LC/MS/MS) method for testing of glucocorticosteroids, which are banned in sports by the World Anti-doping Agency from January 1st 2004.

Materials and Methods: A total of 14 glucocorticosteroids were analyzed on LC/MS/MS using an Inertsil® ODS-3 (3.0 µm, 50 mm × 4.6 mm i.d.) C-18 column in atmospheric pressure chemical ionization mode (positive ionization) with a mobile phase consisting of ammonium acetate and acetonitrile. The analytical equipment used was Aglient 1100 HPLC and API-3200 Triple quadrupole mass spectrometer.

Results: All glucocorticosteroids could be detected within 8 minutes. The limit of detection of all glucocorticosteroids by this screening method was 1 ng/ml. The recovery percentage at 25 and 50 ng/ml concentrations ranged from 54% (Prednisone) to 144% (Methylprednisolone).The validated method has been used successfully for testing of 500 in-competition samples. Excretion study samples of budesonide, methyl prednisolone and prednisone were analyzed by this method and the parent drugs as well as metabolites could be detected. However, further work is in progress to combine this procedure with another LC/MS/MS procedure in the ESI mode (positive ionization) being used for few anabolic steroids and heat-labile stimulants. This would help in screening of all corticosteroids, few anabolic agents and stimulants with just one injection, thus saving time and effort.

KEY WORDS: Glucocorticosteroids, detection limit, liquid chromatography / mass spectrometry / mass spectrometry

Synthetic glucocorticosteroids are a subclass of steroids, which are analogs of 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 the regulation of inflammation, blood electrolyte levels and behavior.[1] Due to their regulation of inflammation, there is a widespread perception among sportsmen that these drugs have performance-enhancing effects in sports.[2] In light of these performance-enhancing and deleterious side effects of glucocorticosteroids, the World Anti-doping Agency (WADA) banned these drugs in sports from January 1st 2004[3] except to use them via the therapeutic use exemption.[4] These glucocorticosteroids come in a variety of forms-inhalant corticosteroids are used to prevent asthma attacks, while ointments, creams and gels are used to treat skin problems. Although these are powerful drugs in curing diseases, they may cause serious side effects when incorrectly taken. A literature survey revealed that gas chromatography / mass spectrometry (GC/MS) is not the method of choice for detection of glucocorticosteroids.[6] There are however, a number of reported methods using liquid chromatography / mass spectrometry / mass spectrometry (LC/MS/MS) for the detection of glucocorticosteroids in doping labs[7-14] and also in clinical practice.[15] But the sample extraction procedure involved in each of these methods varies depending on the extraction protocol of respective labs. The objective of the present study was to develop a rapid method by extending the existing extraction protocol in our lab so that no additional sample preparation costs are involved.

Materials and Methods

Reference standards

The reference standards of glucocorticosteroids were purchased from Sigma (St. Louis, MO, USA). The organic solvents and reagents were of high performance liquid chromatography (HPLC) grade. Acetonitrile and ethyl acetate were obtained from

Qualigens (Worli, Mumbai, India), methanol from J. T. Baker (Phillipburg, USA), *tert*-Butyl Methyl Ether (TBME) from Acros Organics (New Jersey, USA), formic acid from Merck (Worli, Mumbai, India). Deionised water was obtained from a Milli Q laboratory plant (Millipore, Bedford, USA).

Sample extraction procedure

The sample extraction procedure used in this study is the same as the one being used for anabolic steroids.[5] A urine sample (3 or 6 ml based on specific gravity) was passed through a prepared XAD_2® column. Elution was performed with 2.5 ml of methanol after rinsing the column with 1 ml of deionised water. The extract was hydrolyzed using β-glucuronidase enzyme (*E. coli*). Liquid-liquid extraction was done by the addition of 6 ml of TBME; 5 mL of this layer was taken in a separate tube, dried under nitrogen at 60˚C, derivatized and injected into the GC/MS for analysis of anabolic steroids. The remaining 1 ml of the organic layer was separated and evaporated to dryness. The dried extract was reconstituted in 60 µl of ammonium acetate / acetonitrile (70:30, v/v) and 10 µL of this reconstituted sample was injected into the LC/MS/MS for the detection of glucocorticosteroids.

Instrumentation and conditions

The chromatographic system consisted of an Aglient 1100 series (Aglient Technologies, Waldron, Germany) equipped with a G1311A high-pressure gradient pumping system, G1329A autosampler, G1379A degasser and G1316A column compartment. An Inertsil® C-18 column octadecyl silyl (ODS)-3 (3.0 µm, 50 mm × 4.6 mm internal diameter) (GL Sciences Inc, Tokyo, Japan) was used. The mobile phase composition was a mixture of ammonium acetate buffer / Acetonitrile (pH adjusted to 3.5 with acetic acid). It was pumped at a flow rate of 0.8 ml/min with the proportion of acetonitrile increasing from 10 to 100% in 8 minutes. Mass spectrometric analyses were conducted using an API 3200 Triple quadrupole instrument (Applied-Biosystem-Sciex, Concord, Canada) equipped with a pneumatically assisted APCI (heated nebulizer) ionization source. The main working parameters of the mass spectrometer are summarized in Table 1. The whole system was controlled using Analyst 1.4® software (Applied-Biosystem-Sciex, Concord, Canada).

Method development

Ionization in the heated nebulizer source was performed in positive mode, scanning masses from m/z 100 to 500 with a 0.2 µ step size. The development of the analytical method was initiated with different mobile phases to achieve better separation, short run time and maximum resolution with a low flow rate. The elution of all glucocorticosteroids was between 4.98 to 6.84 minutes and of the internal standard was at 7.01 minutes. Mobile phase was introduced into the mass spectrometer via the APCI source operating in the positive ion mode under multiple reaction monitoring conditions (MRM). Nitrogen was used as a nebulizing and curtain gas to achieve fragmentation. The dwell time for each transition was 60 msec and the interchannel delay was 5 msec. For maximum sensitivity, the mass spectrometer parameters such as nebulising gas, curtain gas and collision gas were optimized. The temperature was 550˚C and resolution was set at the unit.

Results

Method validation

Limit of detection

To measure the limit of detection for glucocorticosteroids, a negative urine sample was spiked with 1 ng/mL of the glucocorticosteroid. Six aliquots of the control and negative urine were extracted and analyzed for a time period that covered different degrees of performance for the instrumentation. Three times the standard deviation of noise in the negative urine was used to estimate the limit of detection. Table 2 shows ions with positive ionization, collision energy and relative retention time of different glucocorticosteroids. The total ion chromatogram of glucocorticosteroids is presented in Figure 1.

Calibration and quality control samples

Working solutions for calibration and quality control samples were prepared from the stock solution by dilution using methanol. The internal standard (17α-Methyl testosterone) solution (500 ng/mL) was prepared by diluting its stock solution with methanol. Calibration standards were prepared by addition of ten micolitres of working solution to drug free urine (DFU) to obtain concentration levels of 10, 25, 50, 100 and 300 ng/mL. Quality control samples were prepared at concentrations of 30 ng/mL for determination of minimum required performance limit (MRPL) required by WADA, 1 ng/mL for Limit of detection (LOD) and 10 ng/mL for lower limit of quantitation (LLOQ).

Calibration curve

A calibration curve was constructed with five concentrations ranging between 10-300 ng/mL (10, 25, 50, 100, 300 ng/mL).

Table 1

Tandem mass spectrometer working parameters

Parameter	*Value*
Scan type	Multiple reaction monitoring
Polarity	Positive
Ion source	Heated nebulizer
Nebulising gas	3
Curtain gas	10
Collision gas	3
Source temperature	550°C
Dwell time	60 msec

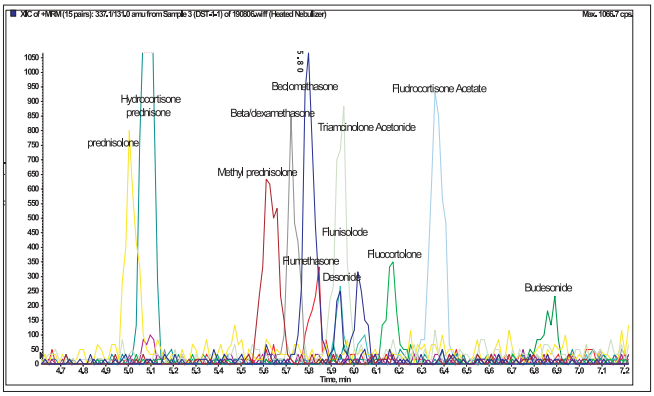

Figure 1: Total ion chromatogram of glucocorticosteroids

Each concentration consisted of five replicates along with an internal standard and it was injected for three consecutive days. Linearity was assessed by a weighted (1/x) least squares regression analysis method. The calibration curve had a correlation coefficient (r^2) of 0.9999 [Figure 2]. The acceptance criterion for each calculated standard concentration was 15% deviation from the nominal value. Table 3 summarizes accuracy and precision of calculated concentrations of spiked samples of glucocorticosteroids in human urine.

Specificity and matrix effect

The specificity and matrix effect of the method was examined by analyzing the drug-free urine extract and drug-free urine spiked with the internal standard. Endogenous corticosteroid, hydrocortisone was observed with a specific peak at 5.03 min retention time. Ion suppression effect was observed, at the specific retention times of the analytes. In order to remove this effect, the flow rate was optimized where the retention time of the particular eluting analyte was changed. No other significant interferences were observed with the MRM channels of the analyte.

Precision and accuracy

The within-batch precision and accuracy were determined by analyzing six sets of quality control samples in a batch. The between-batch precision and accuracy were determined by analyzing six sets of quality control samples in three different batches. The quality control samples were randomized daily, processed and analyzed in position at the end of the batch. The acceptance criteria of within and between-batch precision were 20% and 15% respectively or better for the rest of concentrations. The accuracy value is within 15% of the actual value except at LLOQ, where it is not deviated by more than 20%. The precision of the method was expressed as relative standard deviation and accuracy of the method was expressed in terms of bias (percentage deviation from true value).

Recovery

Recovery of glucocorticosteroids and internal standard was evaluated by comparing the mean peak areas of three processed samples (spiked) with the mean peak areas of unprocessed direct reference standard solutions of the same concentrations. Recovery of all compounds was within the limits. Internal

Table 2

Ions with positive ionization, collision energy and relative retention times of different glucocorticosteroids

Compounds	*Molecular weight*	*Ions with +ve ionization (m/z)*	*Collision energy*	*Relative retention time (min)*
Prednisolone	361	361, 343, 147, 307	30	0.710
Hydrocortisone	363	363, 121	31	0.717
Prednisone	359	359, 147, 341, 171	25	0.716
Methyl prednisolone	375	375, 161, 357, 339	30	0.774
Beta / dexamethasone	393	393, 147, 355, 373	30	0.820
Flumethasone	411	411, 253, 121, 235, 335	26	0.810
Beclomethasone	409	409, 147, 279, 391, 373	30	0.823
Triamcinolone acetonide	435	435, 213, 397, 415, 121	27	0.835
Desonide	417	417, 399, 147, 341, 323	27	0.835
Flunisolide	435	435, 321, 121, 339, 171	25	0.850
Flucortolone	377	377, 171, 303, 339	22	0.868
Fludrocortisone acetate	423	423, 239, 181, 105	45	0.894
Budesonide	431	431, 173, 323, 413	33	0.975

Table 3

Precision and accuracy data of calculated concentrations of spiked samples of glucocorticosteroids in human urine

Compounds	*Concentration added (ng/mL)*	*Concentration found (ng/mL) Mean ± SE*	*CV %*	*Accuracy*
Prednisolone	100	100 ± 5.7	7.5	100
Prednisone	100	105 ± 5.7	7.2	105
Methylprednisolone	100	91 ± 3.8	7.1	91
Betamethasone	100	99 ± 5.5	9.5	99
Flumethasone	100	83 ± 1.8	3.7	83
Beclomethasone	100	90 ± 1.7	3.2	90
Triamcinolone acetonide	100	87 ± 1.2	2.4	87
Desonide	100	93 ± 4.2	7.8	93
Flucortolone	100	100 ± 7.6	13	100
Fludrocortisone acetate	100	96 ± 0.7	2.6	96
Budesonide	100	90 ± 0.8	1.4	90

Figure 2: Typical calibration curve of one drug

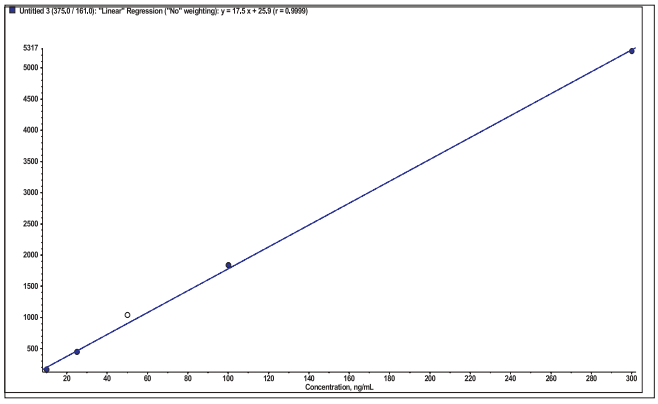

Figure 3: Mass spectrum of methylprednisolone from human urine excretion study sample

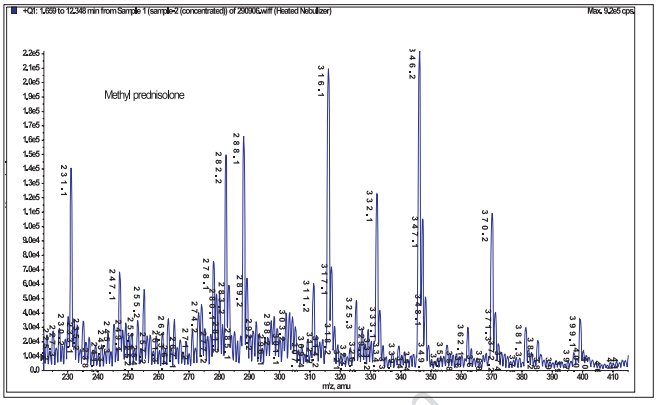

Figure 4: Mass spectrum of prednisone from human urine excretion study sample

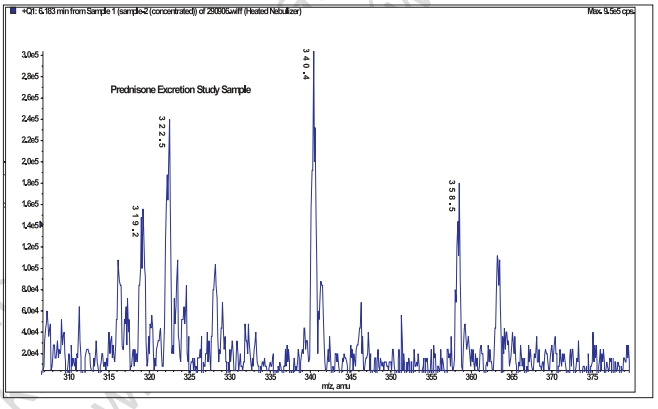

Figure 5: Mass spectrum of methylprednisone from human urine excretion study sample

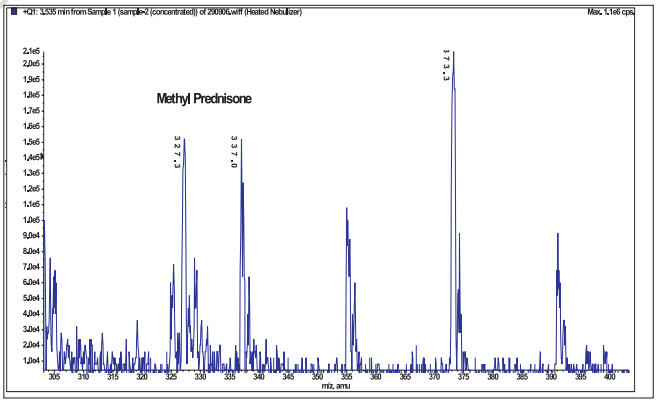

Table 4

Percent recovery of glucocorticosteroids in spiked samples of human urine

	% Recovery	
Compounds	***25 ng/ml***	***50 ng/ml***
Prednisolone	137	90
Prednisone	NA	54
Methylprednisolone	61	144
Betamethasone / dexamethasone	112	101
Flumethasone	94	NA
Fluoxymestrone	122	125
Beclomethasone	59	NA
Triamcinolone acetonide	139	NA
Desonide	83	130
Flucortolone	NA	81
Fludrocortisone acetate	122	112
Budesonide	112	141

standard was acceptable as it was consistent, precise and reproducible. The range of extraction recovery varied from 54 to 144% [Table 4].

Excretion study sample

Excretion study samples of methylprednisolone, prednisone and methylprednisone were analyzed by this method and parent drug with metabolites could be detected [Figures 3-5].

Discussion

The objective of detecting and analyzing glucocorticosteroids by the existing screening protocol was achieved. A total of 14 glucocorticosteroids could be detected at the minimum required performance limit of 30 ng/ml laid down by WADA. The recovery experiments demonstrated that the analytes spiked into urine at 25 and 50 ng/ml were recovered with efficiencies ranging from 54 to 144%. However, percentage recovery of glucocorticosteroids in urine has been reported in the range of 82 to 138.[15] Extraction recovery percentages in the range of 80-120% are considered to be acceptable by international validation protocols.[16] The protocol for bioanalytical method validation explains that the recovery of an analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise and reproducible.[17]

The separation of dexamethasone and betamethasone which are isomers could not be achieved by this method because both have similar MS/MS spectrum and identical retention time on a C-18 column. Another method has been developed to separate these two compounds. The validated method has been successfully used for screening and confirmation of analyte concentrations in human urine samples. However, positive

excretion study samples of budesonide, methylprednisolone and prednisone were analyzed using this method. We could detect all the parent drugs and their metabolites by this method. The mass spectra of budesonide, methyl prednisolone and prednisone were confirmed with reference standards as per the ion match criteria of WADA. The confirmation of metabolites (16-α hydroxyl prednisolone, methylprednisone, prednisolone, 20β-dihydroprednisolone) was done by comparing with the reference mass spectra of metabolites as acceptable criteria laid down in WADA International Standard of Laboratories.

Conclusion

The present method could successfully detect corticosteroids as specified by WADA using the existing protocol with a detection level lower than 30 ng/mL. The method can also be used successfully in clinical conditions to evaluate effects of topical and systemic synthetic corticosteroids. Further work is in progress to detect glucocorticosteroids in urine after different routes of administration.

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