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Method for confirmation of synthetic corticosteroids in doping urine samples by liquid chromatography–electrospray ionisation mass spectrometry

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Abstract

In this study, we report on the development of a method to confirm simultaneously nine of the most commonly abused synthetic corticosteroids in urine based on liquid chromatography–electrospray ionisation mass spectrometry. A considerable simplified sample preparation procedure, including liquid–liquid phase extraction with Extrelut-NT3 columns, provided both excellent sample purification and high overall recoveries. Complete HPLC separations were obtained on a reversed-phase column with 1 m*M* ammonium acetate–acetonitrile (60:40, v/v) as mobile phase. Mass spectral acquisition was done in the negative ion, and selected ion monitoring modes to identify the drugs with at least three characteristic ions. Detection limits were determined at ≤1 ng/ml and the confirmation limits at 1 to 5 ng/ml. © 2001 Published by Elsevier Science B.V.

Keywords: Doping analysis; Corticosteroids; Steroids

1. Introduction

Corticosteroids are widely used to treat various inflammatory and immunologically mediated diseases both in human and veterinary medicine. Based on their pharmacological characteristics, natural and synthetic corticosteroids can be classified into two main groups: glucocorticosteroids and mineral corticosteroids. The first and most important group in sports doping predominantly affects protein and carbohydrate metabolism and produces the potent anti-inflammatory effects of these drugs. The second group influences the electrolyte–water balance [1]. Not only for therapeutic purposes to reduce pain and inflammation, corticosteroids are frequently abused by athletes in sports such as cycling and horse racing to improve the performance. For this reason corticosteroids are considered as doping agents. The situation is complicated by the fact that applications of corticosteroids are permitted by the International Olympic Committee (IOC) only under medical supervision [2]. The lack of an adequate chromatographic method of screening (low sensitivity, bad coverage of the targeted compounds, time consuming, poor information content and too complex procedures) leads to the fact that these drugs are not systematically controlled in the IOC accredited laboratories.

Corticosteroid determinations in doping samples present some difficulties. In most instances, their concentrations in urine at the time of control is very

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low because they present a short half-life in plasma and urine. However, the effects on the target tissues are generally very long. Finally, for doping control, the presence of endogenous corticosteroids requires special analytical methods to reject any false positive results. For this reason, fluorimetric, UV absorbance and fluorescence as detection methods are not suitable [3–6].

Gas chromatography–mass spectrometry (GC–MS) provides good sensitivity and selectivity [7–9]. Unfortunately, most of the corticosteroids are thermally labile, and their volatility is too low for direct GC analysis.

In addition to the long separation time, the required derivatization of the analytes only complicates sample preparation. Modifying the chemical structure of the molecules reduces quality of information and selectivity.

High-performance liquid chromatography (HPLC)–MS offers the advantage to analyze corticosteroids without thermal degradation and derivatization at a comparable sensitivity and selectivity. Different interfaces and ionisation modes have been introduced in the past [10–17]. Moreover, specific but exclusive sample preparations, as immunoaffinity chromatography, have been proposed to increase sensitivity [12]. Other authors have used LC–MS–MS to confirm the presence of drugs at low concentration in biological samples [18–20], but failed to fully characterise all of them [18].

The aim of this study was to develop a multi-drug analysis method to confirm simultaneously nine of the most commonly abused synthetic corticosteroids (Fig. 1) [2] in human urine by liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS). The limits of detection and confirmation were significantly improved by a simplified sample preparation procedure, which provides both excellent sample purities as well as high overall recoveries.

Mass spectra obtained in the negative ionization mode enables identification and confirmation of the drugs in urine. This way, we assure the exclusion of false positive results given by enzyme-linked immunosorbent assay (ELISA), resulting from cross-reaction with endogenous corticosteroids and other hydrophilic substances.

2. Experimental

2.1. Reagents

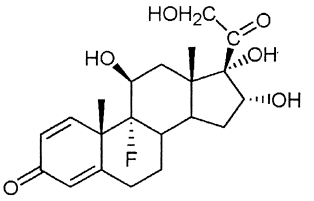
Cortisone, hydrocortisone, triamcinolone, triamcinolone-acetonide, prednisolone, prednisone, 6α-methylpredinsolone, betamethasone, dexamethasone, flumethasone, flunisolide, fluoxymesterone were obtained from Sigma (Buchs, Switzerland). All chemicals were of analytical grade purity. Acetonitrile (G Chromasolv for HPLC, supergradient grade, min. 99.9%) was purchased from Riedel-de Haën (Seelze, Germany), *n*-heptane (analytical-reagent grade) and dichloromethane (SupraSolv) from Merck (Darmstadt, Germany), and methanol (HPLC grade, 99.9%) from Biosolv (Valkenswaard, The Netherlands). Toluene, diethyl ether (AnalaR) and *tert*.-butyl methyl ether (HiPerSolv for HPLC) were used from BDH (Poole, UK). Ammonium acetate (MicroSelect), ascorbic acid, ammonium hydroxide solution (25%), chloroform, isopropanol, ethyl acetate and formic acid (all puriss. analytical-reagent grade) were purchased from Fluka (Buchs, Switzerland). Purified water for HPLC was obtained with a ''Milli-Q plus'' purification system (Millipore, Molsheim, France).

For screening, corticosteroid group ELISA kits from Neogen (Lexington, USA) were used on urine samples without any preparation.

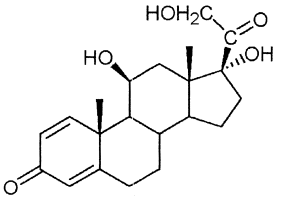
The following solid-phase extraction (SPE) cartridges were used: Oasis HLB 6 ml, Oasis MCX 6 ml (Waters, Milford, MA, USA), Bakerbond SPE C_{18} 6 ml (J.T. Baker, Phillipsburg, NJ, USA), Bond Elut C_{18} 6 ml, and Abselut Nexus LRC 60 mg (Varian, Harbor City, CA, USA). Non-ionic polymeric absorbent XAD-7 resin was obtained from Fluka (250–850 wet μm size, 450 m^2/g surface area), prepared for 60–140 μm particle size [21]. The resin was filtered with Baker disposable filtration columns (J.T. Baker). For liquid–liquid extraction (LLE), Extrelut-NT3 extraction columns (Merck) were used.

2.2. Instrumentation

The LC–MS system (PE Applied Biosystems Europe, Rotkreuz, Switzerland) consisting of two



Triamcinolone
$C_{21}H_{27}FO_6$ M_r=394



Prednisolone
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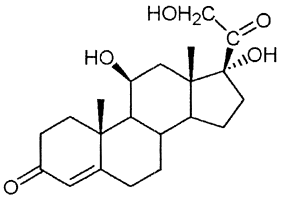
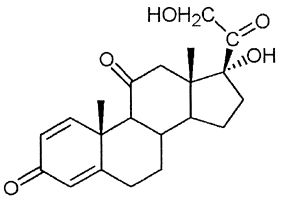


Hydrocortisone
$C_{21}H_{30}O_5$ M_r=362



Prednisone
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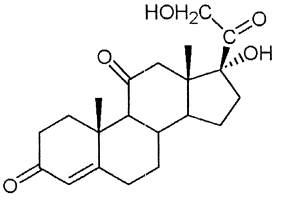
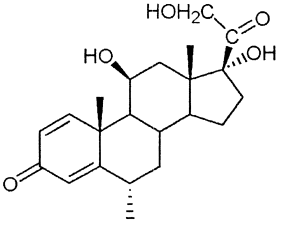
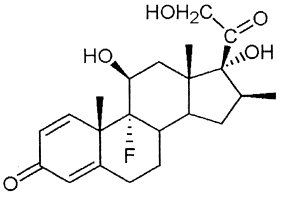


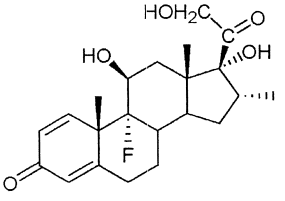
Cortisone
$C_{21}H_{28}O_5$ M_r=360



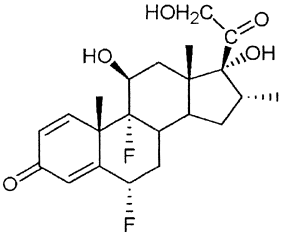
6α-Methylprednisolone
$C_{22}H_{30}O_5$ M_r=374



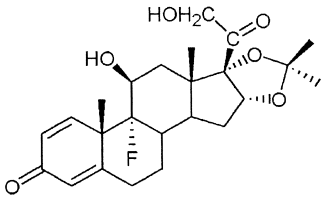
Betamethasone
$C_{22}H_{29}FO_5$ M_r=392



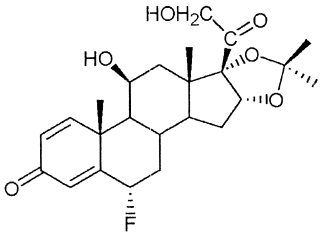
Dexamethasone
$C_{22}H_{29}FO_5$ M_r=392



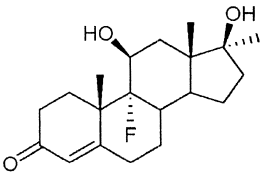
Flumethasone
$C_{22}H_{28}F_2O_5$ M_r=410



Triamcinolone-acetonide
$C_{24}H_{31}FO_6$ M_r=434



Flunisolide
$C_{24}H_{31}FO_6$ M_r=434



Fluoxymesterone (IS)
$C_{20}H_{29}FO_3$ M_r=336

Fig. 1. Chemical formulae of selected corticosteroids.

high-pressure pumps (PE, Series 200 Micro Pump) and an autosampler (PE, Series 200), was connected to a PE Sciex API 150EX single quadrupole instrument with Turbo-Ionspray interface, used as electrospray ionization source.

2.3. Sample preparation

2.3.1. Extractions with XAD-7 resin

A 5-ml volume of urine containing 100 ng/ml fluoxymesterone as internal standard (I.S.) were centrifuged and mixed with 5 ml of 10 m*M* ascorbic acid–ammonia buffer (pH 9.5). The pH was adjusted to 9.5 with ammonium hydroxide solution (25%). After addition of 0.2 g XAD-7 resin, the mixture was shaken on a rotary shaker for 30 min. Then the urine was aspirated off and the XAD-7 resin was mixed with 5 ml of 1 m*M* ascorbic acid–ammonia buffer (pH 9.5). After 2 min shaking, the mixture was centrifuged and the liquid was discarded. The washing step was repeated with a further 5 ml of buffer. The mixture was poured into a J.T. Baker disposable filtration column fitted with 20 μm frits. The buffer was discarded and the resin dried for 15 min in vacuum. The corticosteroids were eluted with 2×3 ml of dichloromethane–ethyl acetate–0.1 *M* formic acid (60:40:0.5, v/v), dried under a stream of nitrogen and reconstituted in 100 μl, 1 m*M* ammonium acetate solution (pH 6.8). A 10-μl voluem was injected to the LC–MS system.

2.3.2. Liquid–liquid extractions with Extrelut-NT3 columns

A solution consisting of 2.5 ml urine, and 100 ng/ml fluoxymesterone as I.S. were adjusted with ammonium hydroxide solution to pH 9.5. The liquid was transferred to the Extrelut-NT3 extraction columns. After 10–15 min of impregnation, the components were eluted with 12 ml of a mixture of toluene–diethyl ether (50:50, v/v). The sample solution evaporated, and 10 μl of the 50 μl reconstituted sample solution (1 m*M* ammonium acetate solution, pH 6.8) was injected.

2.4. Liquid chromatography

The chromatographic separation was performed on an Inertsil 3 ODS-3 column (150×3 mm I.D., 3 μm particle size; Chrompack, The Netherlands). The mobile phase consisted of a mixture of 1 m*M* ammonium acetate solution (pH 6.8)–acetonitrile (60:40, v/v). The flow-rate was kept constant at 0.4 ml/min. The following step-wise gradient elution program was used: The acetonitrile concentration was maintained at 40% (v/v) for 10 min, then, within 2 min increased to 100% and kept constant for the last 5 min. These conditions were applied for all of the described LC–MS measurements in scanning and selected ion monitoring (SIM) modes.

2.5. LC–MS analysis

As preliminary tests indicated that atmospheric pressure chemical ionsation (APCI) modes gave poor sensitivity, a PE Sciex API 150EX mass spectrometer equipped with Turbo-Ionspray interface suitable for electrospray ionisation, is used in the negative ion mode. Conditions for the Turbo-Ionspray: temperature 400°C, heater gas flow 7 l/min, ionspray voltage −4500 V, nebuliser gas position 11 corresponding to 120 ml/min nitrogen, approximately, and curtain gas position 10 corresponding to 80 ml/min nitrogen. The voltages for the curtain plate orifice and the ring electrodes during SIM are presented in the Table 1.

3. Results and discussion

3.1. Mass spectra

Both negative and positive ionization modes were examined. Due to less fragmentation and a better signal-to-noise ratio (>4:1), sensitivity increases for measurements in the negative ionization mode. Fragmentation of corticosteroids in the ESI negative mode is simple as few ions are produced: for most tested compounds, the M−31 anion (probable loss of CH_2OH moiety) is the base peak (see Table 2). For triamcinolone and flunisolide, additional loss of their respective substituent (loss of 49 and 59 u, respectively) at carbon number 13 is observed. The obtained mass spectra correlate well with published data [18]. The fragmentation patterns have not been described in full but we found them to be reproducible between runs when using standards' solutions.

Table 1
Molecular mass, relative intensities, base peak and confirmation ions with ESI parameters for SIM of the targeted corticosteroids

Analyte	M_r	m/z (% intensity)	OR (V)	RNG (V)	Analyte	M_r	m/z (% intensity)	OR (V)	RNG (V)
Fluoxymesterone (I.S.)	336	315.2 (100)	−40	−240	6α-Methylprednisolone	374	343.2 (100)	−50	−260
Beta/Dexamethasone	392	361.2 (100)	−50	−240			309.2 (50)	−50	−260
		307.2 (80/50)	−50	−240			294.2 (30)	−50	−260
		325.2 (40/40)	−50	−240	Prednisolone	360	329.2 (100)	−40	−240
Cortisone	360	329.2 (100)	−40	−240			295.2 (20)	−40	−240
		301.2 (20)	−40	−240			280.2 (10)	−40	−240
		311.2 (10)	−40	−240	Prednisone	358	327.2 (100)	−40	−240
Flumethasone	410	379.2 (100)	−50	−240			299.2 (20)	−40	−240
		325.2 (30)	−50	−240			285.2 (10)	−40	−240
		305.2 (30)	−50	−240	Triamcinolone	394	345.2 (100)	−50	−260
Flunisolide	434	375.2 (100)	−40	−240			325.2 (50)	−50	−240
		357.2 (60)	−40	−240			393.2 (10)	−50	−260
		433.2 (30)	−40	−240	Triamcinolone-acetonide	434	413.2 (100)	−40	−240
Hydrocortisone	362	331.2 (100)	−50	−260			337.2 (20)	−40	−240
		297.2 (40)	−50	−260			375.2 (10)	−40	−240
		282.2 (10)	−50	−260			469.2 (5)	−40	−240

The orifice potential (OR) significantly influences corticosteroid fragmentation. For molecular ions, only a weak signal was observed at OR=−30 V. To obtain several characteristic diagnostic ions showing significant intensity, the MS parameters need to be adjusted carefully. Therefore, voltages of OR/RNG/Q0=−50 V/−260 V/−5 V were determined from measurements in the scan mode (m/z 100–500) by injecting 2 μl standard solutions (5 μg/ml) under the given HPLC conditions. All the characteristic ions are listed in Table 1. In order to increase sensitivity, the SIM mode was used. For corticosteroid identification, the base peak and two confirmation ions were selected for each substance.

3.2. Sample preparation

From a veterinary application, deconjugation of corticosteroids from their glucurono- and sulfo-esters was deemed necessary [18]. In our hands, however

Table 2
Validation data from extractions with Extrelut-NT3 columns

Substance	t_R (min)	LOD (ng/ml)	S/N	RSD (%)	LOC (ng/ml)	Correlation coefficient
Triamcinolone	3.0	5	>3	11	20	0.998
Prednisolone	4.2	1	>4	7	1–5	0.999
Hydrocortisone	4.4	n.d.	n.d.	n.d.	n.d.	n.d.
Prednisone	4.4	1	>8	6	5	0.992
Cortisone	4.8	n.d.	n.d.	n.d.	n.d.	n.d.
6α-Methylprednisolone	5.7	1	>9	4	1–5	0.998
Betamethasone	6.4	1	>9	5	1–5	0.999
Dexamethasone	6.6	1	>9	6	5	0.999
Flumethasone	7.1	1	>19	3	5	0.999
Fluoxymesterone (I.S.)	7.4	n.d.	n.d.	n.d.	n.d.	n.d.
Triamcinolone-acetonide	8.3	1	>9	6	1–5	1.000
Flunisolide	9.0	1	>3	8	5	0.998

Data were collected from six different fortified urine samples at the indicated concentrations. Limit of detection (LOD) with corresponding S/N ratio at measured concentration are given with the corresponding relative standard deviation (RSD) expressed in %. The limit of confirmation (LOC) was defined as the minimum concentration giving a S/N ratio of the second confirmation ion equal or higher than 3.

(data not shown), the amounts of corticosteroids extracted from a pool of several human positive urines with or without enzymatic hydrolysis (glucuronidase from *Escherichia coli* and both glucuronidase and sulfatase from *Helix pomatia* preparations) were similar, so it was decided for human urines to skip the time consuming enzymatic hydrolysis step. Several sample preparation methods including SPE and LLE were then investigated. The following cartridges for SPE were used: Oasis HLB, Oasis MCX, J.T.Baker C_{18}, Bond Elut C_{18} and Abselut Nexus. Among these cartridges Oasis HLB has shown the best results in terms of recovery (85%). Compared to this, the extraction of urine samples with XAD-7 resin [22] resulted in cleaner reconstituted samples. Although recovery was only between 40 and 67%, sensitivity was slightly improved.

A simplified sample preparation procedure was performed with Extrelut-NT3 columns including four extraction media: (a) toluene–diethyl ether (50:50, v/v), (b) chloroform–2-propanol–*n*-heptane (25:10:65, v/v), (c) dichloromethane–ethyl acetate–0.1 *M* formic acid (60:40:0.5, v/v), and (d) *tert*.-butyl methyl ether. For toluene–diethyl ether (50:50, v/v) the extracts exhibited a lower background and were therefore used for method validation. In comparison with all the described methods, extractions with Extrelut-NT3 columns not only provide excellent sample purities (clear and transparent, reconstituted samples) and high overall recoveries (>90%, compared with non-extracted, spiked buffer samples), the method is much simpler to apply, more robust and less time consuming.

3.3. Retention time

With the exception of prednisone and hydrocortisone, all tested corticosteroids were separated within a 10 min run under isocratic conditions (Fig. 2). The mentioned corticosteroids can easily be distinguished by their different fragments. This is not the case for the stereoisomers beta- and dexamethasone (Fig. 3). The difference in retention time however amounts to 20 s approximately (Fig. 2). Therefore, these two components may be distinguished and identified by the addition of a known amount of beta- or dexamethasone. The extracted

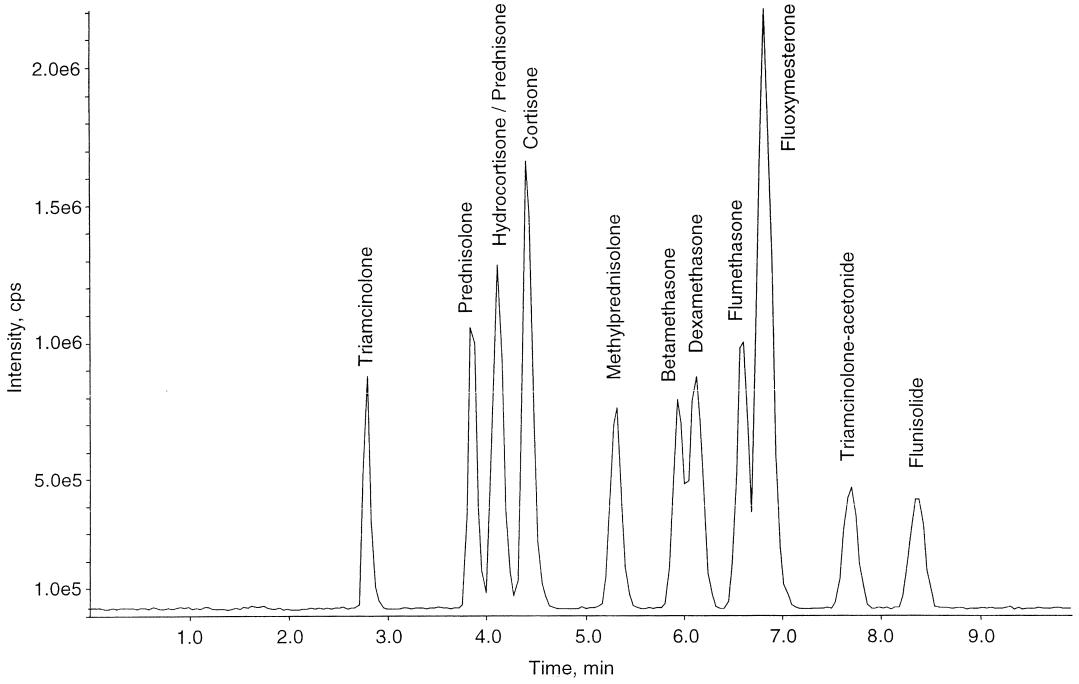


Fig. 2. Reconstructed base peak chromatogram of a mixture of 11 corticosteroid standards (1 μg/ml) with I.S. (5 μg/ml).

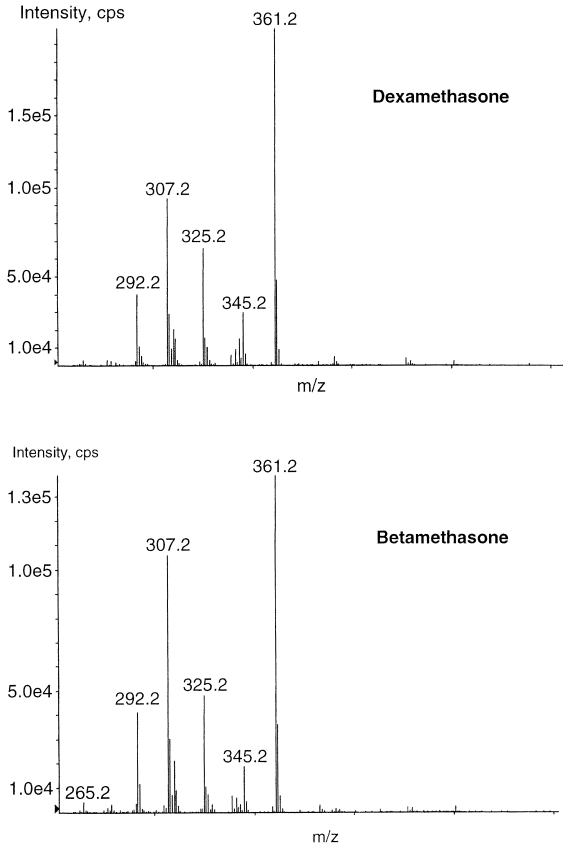


Fig. 3. ESI mass spectra of dexamethasone and betamethasone recorded in scan mode (m/z 100–500) by injecting 10 μl standard solutions (400 ng/ml) through the HPLC system.

blank urine contained the two endogenous corticosteroids, cortisone and hydrocortisone. As for most HPLC separations, absolute retention time changes from run to run, thus, relative retention times are used instead for comparison as additional confirmatory help.

3.4. Sensitivity and linearity

The limit of detection (LOD) was determined in the SIM mode and defined as the sample concentration which produces a peak with a height three times the level of the baseline noise. For confirmation of doping agents in urine, at least three characteristic ions are necessary. According to this, the limit of confirmation (LOC) was defined as the LOD of the second, less intensive confirmation ion. LOD was determined at ≤1 ng/ml and LOC at 1 to 5 ng/ml for 10 of the 11 corticosteroids. An exception was triamcinolone. The higher polarity decreased the recovery to approximately 40%. Values of LOD and LOC are shown in Table 2.

Fluoxymesterone has been chosen as best suited I.S. because of its similarity in chemical structure. In doping control, another procedure has first to make clear that this molecule is absent in the urines tested for corticosteroids.

For doping control, quantification of corticosteroids is not an issue, because the use without medical prescription is illegal at any dose. Nevertheless, for the present study the I.S. fluoxymesterone allows quantification of these drugs. Good linearity was found for all of the corticosteroids (Table 2). The calibration was made with spiked urine between 1 and 50 ng/ml (5 and 50 ng/ml for triamcinolone), including 100 ng/ml I.S.

3.5. Real case example

The present analytical protocol has been routinely applied in our laboratory for more than 9 months; in this time period 30 suspicious corticosteroid group immuno-positive urine samples have been confirmed with a 90% success rate. Immuno-negative urines were not checked by LC–MS for possible ‘‘false negative reaction’’. Further, confirming three immuno-positive urines was not successful and thus, were reported as negative (in forensic toxicology, these urines could have been possibly reported as ‘‘non confirmed immuno-positive cases’’). We postulated that either the unspecific immunological tests did indeed react with other corticoids, or gave a non-specific positive response due to the presence in these samples of several metabolites not covered by our confirmatory procedure. All other urine samples could be fully confirmed with the present protocol. In order to illustrate our approach, a urine sample received during the 2000 cycling tour, Tour de Suisse, previously tested positive by ELISA, gave typical results. For confirmation and identification, the sample was treated with two different extraction methods and analysed by LC–ESI-MS. A comparison of the performance with XAD-7 resin and Extrelut-NT3 columns is shown in Fig. 4. In addition to the clear detection of the base peak (m/z 413),

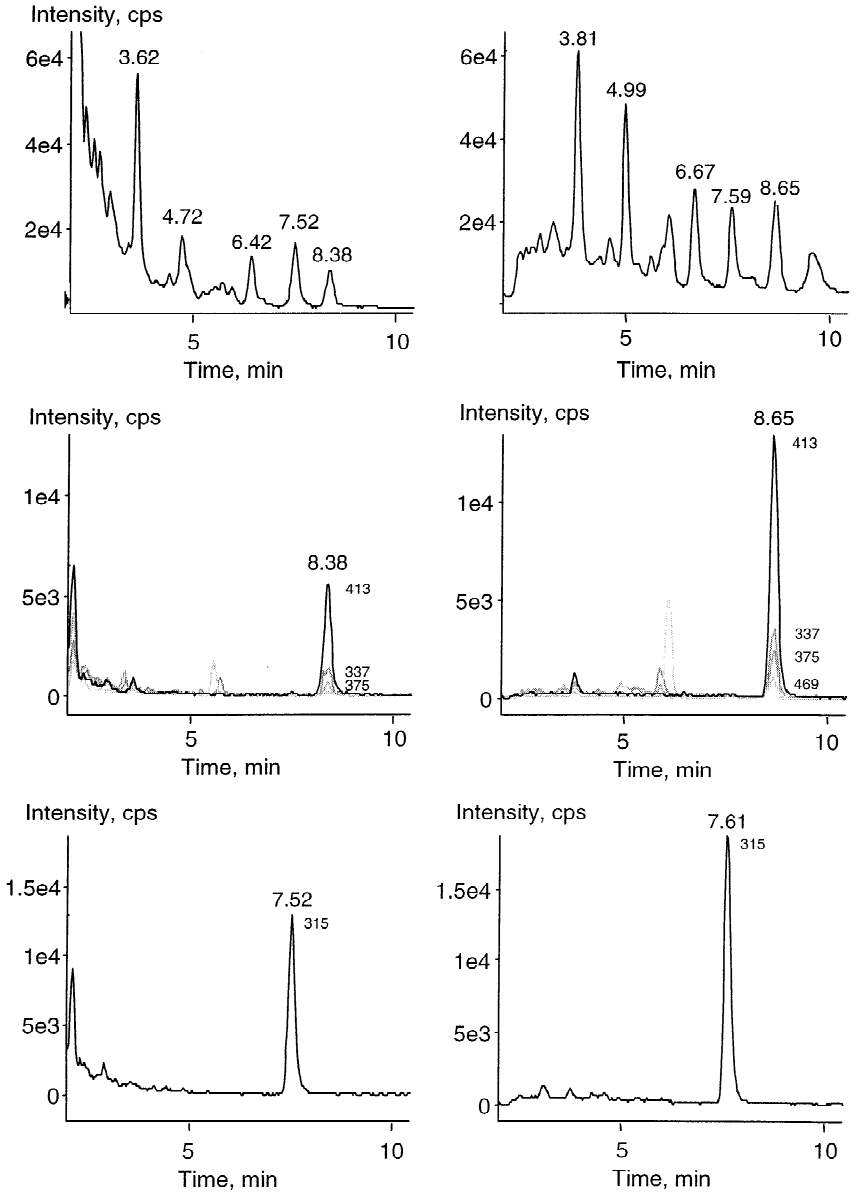


Fig. 4. Results from extractions of a real case doping sample with XAD-7 resin (on the left), and Extrelut-NT3 columns (on the right): total ion chromatograms (top) and extracted ion chromatograms showing a clear positive result (middle) for triamcinolone-acetonide corresponding to 23.0 ng/ml, and fluoxymesterone (bottom) used as internal standard (I.S. at 100.0 ng/ml).

two diagnostic ions for XAD-7 extraction [S/N= 20:1 (m/z 337) and 10:1 (m/z 375)], and three for Extrelut-NT3 [S/N=70:1 (m/z 337); 50:1 (m/z 375) and 30:1 (m/z 469)], are obtained to identify clearly the presence of triamcinolone-acetonide (23.0 ng/ml). Not only does the Extrelut-NT3 extraction procedure offer significantly better extraction yield, but also, the background noise is reduced, allowing more informative (three, instead of two additional ions) and clearer data (higher signal-to-noise ratios for all ions) to be collected.

Due to the current lack of a clear security limit for

the quantification of cortisone and hydrocortisone [18] to distinguish between high excretion of endogenously produced levels or abusive uses of external sources, it is not yet relevant in doping controls to report any positive finding for these two endogenous corticosteroids.

4. Conclusion

The present work shows that 11 corticosteroids in urine samples can be analysed in a single HPLC run, based on LC–ESI-MS. The selective and sensitive method for confirmation and identification of nine synthetic corticosteroids assures the exclusion of false positive results obtained by corticosteroid group ELISA screening tests. A very simple and time saving preparation procedure based on Extrelut-NT3 LLE leads to clean reconstituted samples. Detection limits were determined as ≤ 1 ng/ml, the limits of confirmation at 1 to 5 ng/ml. Only the more polar corticosteroid triamcinolone and prednisolone exhibited a lower sensitivity.

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