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Determination of endogenous and synthetic glucocorticoids in human urine by gas chromatography–mass spectrometry following microwave-assisted derivatization

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Abstract

A complete screening and confirmation analytical method for the direct determination of six endogenous (cortisol, cortisone, deoxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, tetrahydro-*S*) and 17 synthetic (amcinonide, betamethasone, desoximethasone, dexamethasone, fludrocortisone, flumethasone, flunisolide, flucinolone acetonide, flucinonide, fluprednisolone, flurandrenolide, fluorometholone, 6-methylprednisolone, prednisolone, prednisone, triamcinolone, triamcinolone acetonide) glucocorticoids in human urine by gas chromatography with mass spectrometric detection (GC–MS) is presented.

The analytical technique comprises a pre-treatment procedure and the instrumental analysis of the trimethylsilyl (TMS) derivatives, performed by GC–MS (quadrupole) with electron impact (EI) ionization. The derivatization yields obtained by two different derivatizing mixtures, namely *N*-methyl-*N*(trimethylsilyl)trifluoroacetamide (MTSFA):NH_4I:dithioerythritol (DTE) 1000:2:4 (usually indicated as TMSiodine); and *N*-trimethylsilylimidazole (TMSim):*N*,*O*-bis(trimethylsilyl)acetamide (BSA):trimethylchlorosilane (TMCS) 3:3:2, both under direct thermal heating and with microwave (MW) irradiation, were evaluated, also as a function of the temperature, of the MW power and of the incubation time.

The highest yields of the derivatization process were obtained, for most of the compounds here considered, by a two-step procedure: a microwave-assisted derivatization stage (40 min in a microwave oven at 900 W emitted power), followed by a traditional heat transfer derivatization (1.5 h in a thermostated bath at 70 °C) with the derivatization mixture TMSim:BSA:TMCS 3:3:2. In these operating conditions, diagnostic EI–MS spectra of all considered glucocorticoids were obtained. Limits of detection (LOD) of synthetic glucocorticoids in urine ranged from 3 to 25 μg/l. The effectiveness of the method for the determination of glucocorticoids in urine was evaluated on spiked urine samples and on real samples obtained from patients under pharmacological treatment with synthetic glucocorticoids.

Apart from the clinical monitoring of glucocorticoids in urine, the method can be applied as a complete screening + confirmation analytical protocol in antidoping tests for the detection of illicit administration of glucocorticoids by the athletes.
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Keywords: Glucocorticoids; Pharmacological treatment; Antidoping; Microwave irradiation; Gas chromatography–mass spectrometry

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1. Introduction

Glucocorticoids are widely used to treat various inflammatory and immunological diseases; they are also frequently employed as growth promoters [1,2]. In sport medicine, glucocorticoids are used because of their anti-inflammatory and analgesic properties. For both the potential positive effects on sport performance and the associated toxicological risks, the systemic administration of glucocorticoids has been forbidden by the International Olympic Committee (IOC) and by the World Antidoping Agency (WADA), except for topical use (i.e. by inhalation and by intra-articular or local injection), so that synthetic glucocorticoids are presently included in the list of doping substances and methods, among the "Classes of prohibited substances in certain sports" [3].

Detection and quantitative determination of glucocorticoids in routine analysis of biological matrices, including human urine, is presently being accomplished by a variety of methods, including immunological, electrochemical and chromatographic–spectrometric (HPLC–UV, HPLC–MS, GC–MS) techniques [4–12]. In clinical chemistry and forensic toxicology applied to sport medicine, and especially in the search for glucocorticoids in the urine of athletes by the antidoping laboratories, there is not yet a reference method followed by the antidoping laboratories accreditated by the IOC and/or approved by the World Antidoping Agency for the screening and confirmation analysis of glucocorticoids.

This work describes an alternative approach for the screening and confirmation analysis of corticosteroids, and particularly of glucocorticoids, in human urine. The study was carried out on six endogenous and 17 synthetic glucocorticoids. Native glucocorticoids (see Fig. 1 for the generic chemical structure) were derivatized to form the corresponding trimethylsilyl (TMS) derivatives and then analyzed by GC–MS with electron impact (EI) ionization, thus obtaining a reference MS spectrum for each compound. TMS derivatives were obtaining by a specific derivatization mixture, constituted by *N*-trimethylsilylimidazole (TMSim): bis(trimethylsilyl)acetamide (BSA):trimethylchlorosilane (TMCS), 3:3:2. The use of microwave (MW) irradiation, in addition to direct heating, for energy transfer, drastically accelerated the rate of the derivatization reaction, allowing the formation of the TMS derivatives of all glucocorticoids under investigation in less than 3 h.

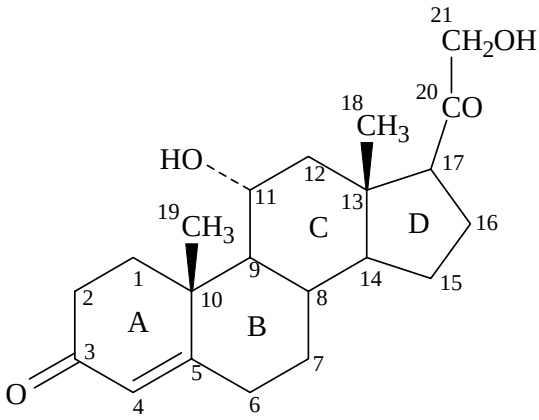

Fig. 1. Generic chemical structure of the glucocorticoids is considered in the present study. The position of the carbon atoms (1–21) and the arrangement of the four rings (A–D) are indicated according to the international steroid nomenclature.

2. Materials and methods

2.1. Reagents and glucocorticoids reference standards

All reagents (analytical grade) were supplied by Carlo Erba (Milan, Italy). β-Glucuronidase from *Escherichia coli* was supplied by Boehringer Mannheim (Mannheim, Germany). *N*-Methyl-*N*(trimethylsilyl)-trifluoroacetamide (MTSFA) was supplied by Macherey-Nachel (Düren, Germany). Ammonium iodide, dithioerythritol (DTE), trimethylchlorosilane and *N*-trimethylsilylimidazole were supplied by Sigma Aldrich (St. Louis, MO, USA); *N*,*O*-bis(trimethylsilyl)acetamide was supplied by Fluka (Milan, Italy); methyltestosterone (used as internal standard, ISTD) and the 23 glucocorticoids considered in the present study were supplied by Sigma Aldrich (St. Louis, MO, USA), apart from fluocinonide and flurandrenolide that were supplied by Steraloids-Chebios Italia (Rome, Italy). Stock standard solutions were prepared dissolving the reference standard in methanol (1 mg/ml); all stock solutions were darkly stored in screwed cap vials at −20 °C. Working standard solutions were prepared weekly, at the appropriate

dilution, from the corresponding stock solution, and stored at 4 °C. Spiked urine samples were prepared by diluting the corresponding methanol working standard solution with blank reference urine to the final desired concentration. Betamethasone and prednisolone positive reference urine samples were obtained by excretion studies performed on patient volunteers (caucasian males, age 32 and 40 years, respectively, normal body mass index). C_{18} cartridges (Sep-Pak) were supplied by Waters (Milan, Italy).

2.2. Urine pre-treatment

The pre-treatment of urine samples is analogous to the one generally followed by the antidoping laboratories for the screening analysis by GC–MS of the TMS derivatives of endogenous and synthetic anabolic steroids [13]: 3 ml of sample/urine, added with 50 μl of ISTD (methyltestosterone 200 μg/l in methanol) are passed across a C_{18} Sep-Pak (previously activated by 3 ml of MeOH and washed twice with 3 ml water and 3 ml MeOH) and then eluted with 3 ml of methanol; the eluate is evaporated to dryness under N_2 stream at 40 °C; the residue is taken up in 2 ml of phosphate buffer (0.2 M, pH 7.4) to be incubated with 50 μl β-glucuronidase from *E. coli* at 50 °C for 1 h; after correction of pH to 9.2 with 2 ml carbonate buffer, the organic fraction is extracted by 10 ml of *tert*-butyl methyl ether and again brought to dryness; the dried extracts are derivatized with 50 μl of the selected derivatizing reagent in different operating conditions (direct heating and/or microwave-assisted energy transfer). Derivatization has been carried out for every glucocorticoid in the following conditions:

(i) MTSFA:NH_4I:DTE 1000:2:4, incubation at room temperature overnight;
(ii) TMSim:BSA:TMCS 3:3:2, incubation at room temperature overnight;
(iii) MTSFA:NH_4I:DTE 1000:2:4, incubation at 70 °C for 30, 60, 90, and 180 min;
(iv) TMSim:BSA:TMCS 3:3:2, incubation at 70 °C for 30, 60, 90, and 180 min;
(v) MTSFA:NH_4I:DTE 1000:2:4 under MW irradiation (emitted power: 900 W) for 10–40 min;
(vi) TMSim:BSA:TMCS 3:3:2 under MW irradiation (emitted power: 900 W) for 10–60 min;
(vii) TMSim:BSA:TMCS 3:3:2 under MW irradiation (emitted power: 900 W) for 40 min followed by incubation at 70 °C for 30, 60, and 90 min;
(viii) TMSim:BSA:TMCS 3:3:2 incubation at 100 °C for 40 min followed by incubation at 70 °C for 30, 60, and 90 min.

In the case of MW-assisted derivatization, all materials were preliminarily tested to verify their resistance to the operating conditions. The reaction tubes were stoppered by silicon septa and placed in a water bath to maintain the temperature $\leq$100 °C during the MW irradiation. Maximum continuous irradiation time was 10 min. Longer periods of incubation were fractioned into consecutive 10 min steps, after replacing the water in the outer bath at the end of each step.

2.3. Instrumentation and GC–MS parameters

The microwave oven used for the MW-assisted derivatization of glucocorticoids was a Whirlpool MWO105 (Whirlpool Italia).

The GC–MS system was a HP5890–5970 (Agilent Technologies Italia, Milan, Italy) equipped with a phenyl-methylsilicone column, length 30 m × 0.2 mm internal diameter, 0.11 μm film (HP5, Agilent Technologies Italia). Helium for spectrometry was used as carrier gas at constant flow of 0.8 ml/min. Injection mode: split 1:10; injection volume: 2 μl; injector temperature: 280 °C; thermal program: 200 °C, 2 min, 15 °C/min; final temperature: 300 °C, 40 min, 20 °C/min; final temperature: 320 °C, 6 min; transfer line: 290 °C.

3. Results

Table 1 reports the relative derivatization yields of all endogenous and synthetic glucocorticoids considered in this study, indicated by common and IUPAC name, obtained by using the derivatizing mixture TMSim:BSA:TMCS 3:3:2 in different operating conditions.

Relative retention times (RRTs), base peaks, and other diagnostic *m/z* fragments, including those, indicated in boldface, suitable for the GC–MS analysis in the selected ion monitoring (SIM) mode, are

Table 1
Relative derivatization yields (as (yield/yield$_{max}$) × 100), in different operating conditions, of all the endogenous (in italics) and synthetic glucocorticoids considered in the present study

Compounds (common name/IUPAC name)	Heating (70 °C)	Heating (100 + 70 °C)	MW incubation (900 W)				MW incubation (900 W) + heating at T = 70 °C			MW incubation (900 w)	
	180 min	40 + 90 min	10 min	20 min	30 min	40 min	40 + 60 min	40 + 90 min[a]	40 + 180 min	50 min	60 min
Amcinonide 11β,16α-21-(Acetyloxy)-16,17-[cyclopentylidenebis(oxy)]-9-fluoro-11-hydroxypregna-1,4-diene-3,20-dione	80	85	10	15	30	40	70	100	90	35	30
Betamethasone 11β,16β-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	55	55	15	30	35	50	70	100	90	35	35
Cortisol 11β-11,17,21-Trihydroxypregn-4-ene-3,20-dione	25	25	10	10	20	25	100	100	70	20	20
Cortisone 17,21-Dihydroxypregn-4-ene-3,11,20-trione	30	40	25	25	35	40	70	100	80	30	30
Desoximetasone 11β,16α-9-Fluoro-11,21-dihydroxy-16-methylpregna- 1,4-diene-3,20-dione	65	60	15	20	30	45	60	100	100	30	30
Dexamethasone 11β,16α-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	25	25	0	5	15	35	50	90	100	45	45
Fludrocortisone 11β-9-Fluoro-11,17,21-trihydroxypregn-4-ene-3,20-dione	40	45	10	15	30	40	60	100	80	30	35
Flumethasone 6α,11β,16α-6,9-Difluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	20	20	0	4	12	30	55	85	100	40	40
Flunisolide 6α,11β,16α-6-Fluoro-11,21-dihydroxy-16,17-[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3,20-dione	60	60	25	25	40	40	80	100	80	30	30
Fluocinolone acetonide 6α,11β,16α-6,9-Difluoro-11,21-dihydroxy-16,17-[(1-methylethylidene)bis(oxy)]-pregna-1,4-diene-3,20-dione	60	60	10	15	25	40	70	100	100	30	30
Fluocinonide 6α,11β,16α-21-(Acetyloxy)-6,9-difluoro-11-hydroxy-16,17-[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3,20-dione	45	45	25	30	35	45	70	100	85	35	30
Fluorometholone 6α,11β-9-Fluoro-11,17-dihydroxy-6-methylpregna-1,4-diene-3,20-dione	55	50	15	25	30	45	65	100	95	35	35
Fluprednisolone 6α,11β-6-Fluoro-11,17,21-trihydroxypregna-1,4-diene-3,20-dione	65	60	30	30	35	40	70	100	80	30	30
Flurandrenolide 6α,11β,16α-6-Fluoro-11,21-dihydroxy-16,17-[(1-methylethylidene)bis(oxy)]pregn-4-ene-3,20-dione	100	100	25	25	35	40	90	100	80	40	40

Methylprednisolone 6α-11β-11,17,21-Trihydroxy-6-methylpregna-1,4-diene-3,20-dione	60	60	30	25	35	45	75	100	95	30	30
Prednisolone 11β-11,17,21-Trihydroxypregna-1,4-diene-3,20-dione	60	60	25	25	30	40	70	100	80	30	30
Prednisone 17,21-Dihydroxypregna-1,4-diene-3,11,20-trione	100	75	40	40	50	50	80	100	85	30	25
Tetrahydrocortisol 5β-Pregnane-3α,11β,17α,21-tetrol-20-one	34	43	35	35	45	55	70	100	100	50	50
Tetrahydrocortisone 3α,5β-3,17,21-Trihydroxypregnane-11,20-dione	17	33	35	35	45	50	70	100	90	40	40
Tetrahydrodeoxicorticosterone 5β-Pregnane-3α,21-diol-20-one	40	48	40	40	50	50	80	100	85	40	40
Tetrahydro-S 5β-Pregnane-3α,17α,21-triol-20-one	33	43	35	35	45	50	75	100	100	45	45
Triamcinolone 11β,16α-9-Fluoro-11,16,17,21-tetrahydroxypregna-1,4-diene-3,20-dione	65	60	5	15	20	40	50	100	95	30	15
Triamcinolone acetonide 11β,16α-9-Fluoro-11,21-dihydroxy-16,17-[1-methylethylidenebis(oxy)]pregna-1,4-diene-3,20-dione	68	65	10	15	25	40	65	100	90	30	30

All data are obtained by the derivatization mixture TMSim:BSA:TMCS 3:3:2 (see text for further details).

[a] The column indicates the optimal derivatization conditions, corresponding to the two-stage process combining MW irradiation at 900 W emitted power for 40 min, followed by thermal incubation at 70 °C for 90 min.

Table 2
GC–EI–MS data of the TMS derivatives of all the endogenous and synthetic glucocorticoids considered in the present study

N	Compounds	RRT	Molecular weight	100% ion (*m*/*z*)	Other diagnostic ions (*m*/*z*)
1	Amcinonide-TMS	2.98	574	**473**	**574**, 554, 545, 389, 263
2	Betamethasone-4TMS	1.79	680	**387**	608, **477**, 457, 367, 297
3	Cortisol-4TMS	1.55	650	339	**650**, **519**, 446, 429, 404
4	Cortisone-3TMS	1.63	576	331	**561**, 486, **471**, 372, 305
5	Desoximetasone-2TMS	1.12	520	157	520, **500**, **299**, 279, 193
6	Dexamethasone-4TMS	2.31	680	305	**680**, 590, **345**, 332, 305
7	Fludrocortisone-4TMS	1.63	668	**537**	**668**, 464, 447, 357, 337
8	Flumethasone-4TMS	2.35	698	305	**698**, 608, **345**, 332, 305
9	Flunisolide-2TMS	2.00	578	**447**	578, 505, 447, 299, **279**
10	Fluocinolone acetonide-2TMS	2.12	596	**465**	407, 387, 297, 235, 207
11	Fluocinonide-2TMS	2.47	638	**505**	638, 565, **523**, 477, 281
12	Fluorometholone-3TMS	1.46	592	207	**477**, **387**, 367, 297, 277
13	Fluprednisolone-4TMS	1.80	666	**463**	489, 373, **353**, 283, 263
14	Flurandrenolide-2TMS	1.93	580	**449**	580, 507, **429**, 391, 301
15	Methylprednisolone-3TMS	1.77	590	**279**	**459**, 395, 369, 264
16	Prednisolone-3TMS	1.70	576	**265**	455, **355**, 250
17	Prednisone-2TMS	1.62	502	**371**	397, 295, 281, 263
18	Tetrahydrocortisol-4TMS	1.21	564	253	549, **523**, 459, **433**, 343
19	Tetrahydrocortisone-4TMS	1.30	652	**331**	**652**, 562, 405, 305, 281
20	Tetrahydrodeoxicorticosterone-3TMS	1.14	550	**257**	550, **463**, 375, 359, 281
21	Tetrahydro-*S*-4TMS	1.55	638	255	**435**, **345**, 279, 215
22	Triamcinolone-4TMS	2.03	682	**461**	551, 441, **371**, 281, 193
23	Triamcinolone acetonide-TMS	2.08	578	**447**	578, 389, 369, 351, 279

Ion fragments used for the screening analysis in SIM are indicated in boldface All data refer to TMS derivatives obtained following the two-stage process combining MW irradiation at 900 W emitted power for 40 min, followed by thermal incubation at 70 °C for 90 min.

summarized in Table 2. All data refer to TMS derivatives obtained following the optimal operating conditions for derivatization of Table 1 (40 min of microwave irradiation at 900 W emitted power followed by incubation at 70 °C for 1.5 h).

Table 3 reports the limits of detection (LODs) in human urine of all synthetic glucocorticoids, obtained by GC–EI–MS experiments performed following the guidelines of the International Olympic Committee for the confirmation analysis in the SIM mode. Data refer to concentration values, obtained by progressively diluting a reference positive urine containing a known concentration of the glucocorticoid under investigation with blank urine, giving a value of the signal-to-noise ratio ≥ 3 for at least three diagnostic ions chosen among those reported in boldface in Table 2. LODs—in water—were also obtained for endogenous glucocorticoids and they were markedly lower than their baseline concentration in urine in physiological conditions.

Finally, Figs. 2 and 3 show the GC–EI–MS chromatograms (SIM mode) recorded in the screening

Table 3
Limits of detection in human urine of all synthetic glucocorticoids considered in the present study

Compounds	LOD (ng/ml)
Amcinonide	15
Betamethasone	12
Desoximethasone	5
Dexamethasone	25
Fludrocortisone	5
Flumethasone	15
Flunisolide	6
Fluocinolone acetonide	3
Fluocinonide	15
Fluoromethenolone	6
Fluprednisolone	10
Flurandrenolide	5
Methylprednisolone	3
Prednisolone	4
Prednisone	6
Triamcinolone	25
Triamcinolone acetonide	3

Data refer to GC–EI–MS experiments performed in SIM mode on positive reference urines. The reported values refer to the concentration of glucocorticoid giving a signal-to-noise ratio ≥ 3 for at least three diagnostic ions chosen among those reported in Table 2.

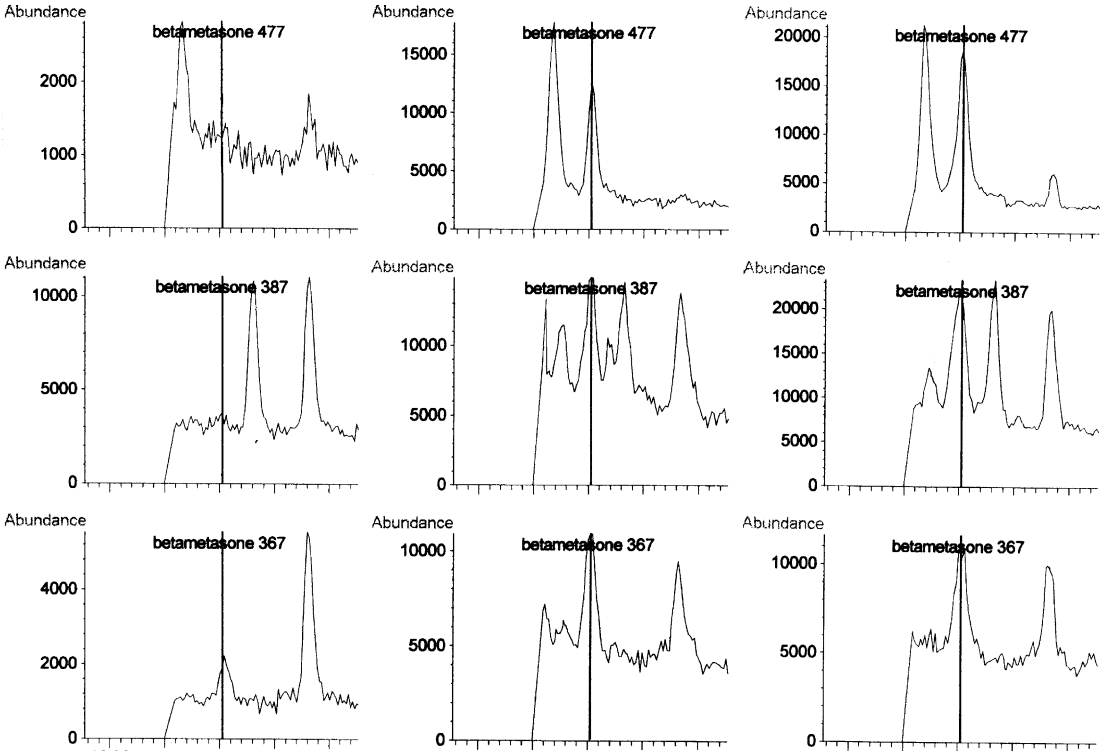

Fig. 2. GC–EI–MS screening analysis (SIM mode) for betamethasone. Plots refer to the screening windows of betamethasone of: (left) a blank urine; (center) a urine sample obtained 33 h after the administration of 1 mg of betamethasone per os; and (right) a positive reference urine at 12 ng/ml, i.e. the same negative urine of the blank plot, but spiked with betamethasone 12 ng/ml.

analysis of urine samples collected from patients after oral administration of a single dose of betamethasone (1 mg) or prednisone (5 mg), respectively. Data refer to the GC–EI–MS–SIM chromatograms of a blank urine, of a urine sample collected after administration of the drug and of positive reference urines.

4. Discussion

Gas chromatography with mass spectrometric detection is among the most widely employed techniques for the screening and confirmation analysis of drug residues in biological matrices. The main advantages of this technique are due to an ideal combination of sensitivity, selectivity and capability to screen for multiple analytes/classes of analytes by a unique preanalytical/instrumental protocol. From a general point of view, the gas-chromatographic analysis of corticosteroids (including glucocorticoids) is very problematic due to both their extremely low volatility and to the thermal instability of the hydroxyacetone side chain in position C17 [14]. These basic obstacles can in principle be overcome by modifying the native structure to form suitable derivatives. Many strategies have therefore been proposed to obtain significant yields of various derivatives of 17-OH corticosteroids [14,15]. Several drawbacks were however reported, and primarily among them:

(i) the rate of the derivatization reaction on the –OH residues in position C11 and C17 is very slow;
(ii) the thermolable side chain on C17 needs to be made more stable, possibly by preliminary derivatization of the C20 keto residue;
(iii) in the case of synthetic glucocorticoids, the presence of additional residues, such as the methyl or

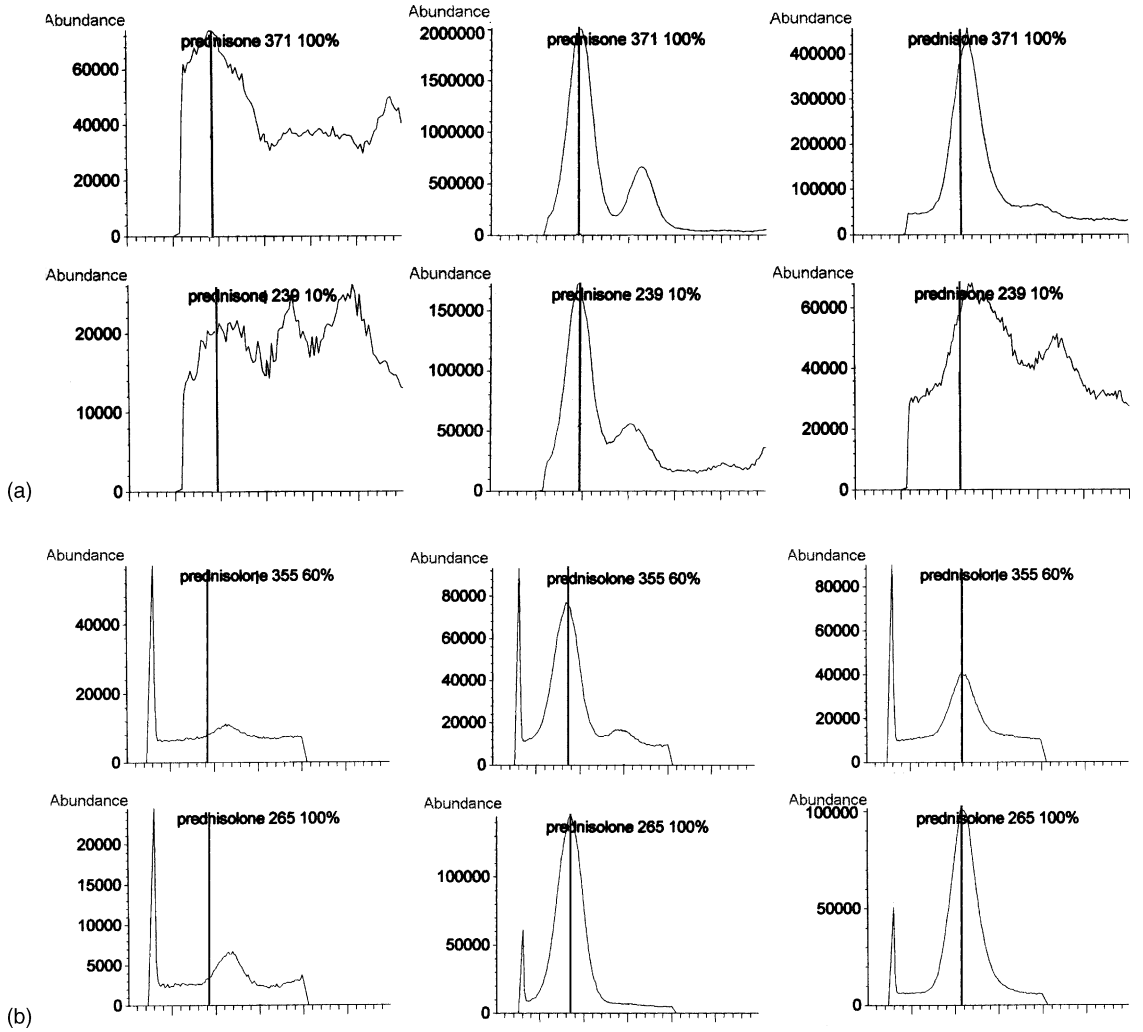

Fig. 3. GC–EI–MS screening analysis (SIM mode) for prednisone/prednisolone. Plots refer to the screening windows of prednisone (a) and of its active metabolite prednisolone (b) of: (left) a blank urine; (center) a urine sample obtained 10 h after the administration of 5 mg of prednisone per os; and (right) a positive reference urine at 100 ng/ml, i.e. the same negative urine of the blank plot, but spiked with prednisone and prednisolone both 100 ng/ml.

hydroxy group on C16, can hinder the C17 hydroxy group, further reducing its reactivity;

(iv) the use of aggressive silanyzing reagents, under alkaline catalysis, to overcome the above-mentioned inconvenients, although allowing an appreciable yields of the TMS derivative on unhindered hydroxy groups, leads to the formation of a series of enol–TMS–ethers with dispersion of the overall yields and consequent reduction of sensitivity.

It is therefore necessary to follow a selective derivatization process in order to retain all significant structural information; but since most of the active residues that can be chosen as targets of derivatization reactions are active oxygens, the use of strong oxidating agents is not recommended whenever it is necessary to carry out screening and confirmation analyses for multiple analytes with spectrometric identification of the specific glucocorticoid. The ideal derivatization technique should indeed ensure a sufficient degree of derivatiza-

tion, but allowing at the same time the preservation of all key structural information. These objectives can be achieved by the use of a suitable derivatization mixture in the most appropriate derivatization conditions.

A wide variety of derivatizing agents in different experimental conditions have been described in the literature, the most common being TMSimidazole, MTSFA:NH_4I:DTE (1000:2:4), usually indicated as TMSiodine (TMSI) under acidic catalysis, and a mixture of TMSim, BSTFA and TMCS [16–21]. The efficacy of these reagents has been evaluated in different experimental conditions, but the energy required to activate and support the derivatization reaction has always been supplied by traditional heat tranfer equipments (water and/or oil thermostated baths, heating jackets, thermostated incubators and so on). These studies have led to the following fundamental evidence:

(i) TMSim has been proven to be the most powerful silanyzing agent, since it can react—although partially—with the sterically hindered hydroxyl groups on C11 even in the absence of a catalyzer (usually TMCS). Significant yields are however obtained only after prolonged incubation times. Complete derivatization of all hydroxyl groups can be obtained by TMSim-BSA-TMCS after 60 h of incubation at 60 °C. The addition of an acid catalyzer increases the reaction rate on unhindered –OH groups and somehow activates the attack of TMS on hindered –OH groups (C17). Derivatization by TMSim:BSA:TMCS 2:2:1 overnight led to formation of trimethylsilyl ethers from any carbonyl and/or hydroxyl group for many different corticosteroids, apart from triamcinolone acetonide, possibly as a consequence of the steric hindrance on the carbonyl group on C20 [18,19]. In terms of relative reactivity, as far as the –OH group on C17 is concerned, betamethasone is more reactive than dexamethasone since the latter is hindered by the *cis*-methyl group on C16.

(ii) Another derivatization strategy requires the use of a basic catalyzer (like potassium acetate) in synergy with BSTFA or MTSFA:NH_4I:DTE [17,21]. Addition of a nucleophilic agent to a silanyzing reagent promotes the formation of trimethylsilyl derivatives, with the peculiar difference, with respect to acid catalysis, that hydroxyl and/or carbonyl groups in C11 and/or C17 are not completely silylated: this suggests that in these conditions a preferential effect of the base on the keto-enolic equilibrium occurs, rather than on the promotion of the rate of the silanization reaction. In these conditions the side chain on C17 is easily converted to TMS–enol–TMS.

(iii) An alternative strategy goes through the formation of methoximes (MO) from carbonyl groups by a specific derivatization mixture (methoxyamine hydrochloride + pyridine). The reaction is carried out at 60 °C for 15 min; the methoxime derivative on the –OH groups can then be converted to the corresponding TMS derivative by incubation with TMSim at $T = 100$ °C for 2 h [4,21]. In general, the rate determining step is always the silylation of the –OH group on C17: 3–13 h are usually necessary to obtain MO–TMS derivatives of synthetic glucocorticoids with substituents on C16 [22]. Also in this case the reaction does not occur on particularly hindered residues (e.g. the CO group on C11 with methyl groups on C18 or C19).

(iv) A further strategy comprises the oxidation by a mixture of pyridinium chlorochromate:sodium acetate (2:1), followed by heating at 92 °C for 3 h [5,16]. The drawback of this approach is that the native structure of the glucocorticoid is drastically modified: the side chain on C17, as well as the C17 –OH group (if present) are lost, while the –OH on C11 is oxidized to ketone. The result is a thermally stable derivative, but lacking any original structural information as far as substitution on C3, C10 and C17 is concerned.

We are here proposing a specifically developed derivatization procedure, based on the use of a highly reactive derivatization mixture (constituted by TMSim:BSA:TMCS 3:3:2) and using a combination of microwave irradiation and direct thermal heating to supply the energy required to activate and support the derivatization reaction, to obtain significant yields of TMS derivatives of endogenous and synthetic glucocorticoids that are suitable for GC–MS analysis.

The use of microwaves as a non-conventional energy source to supply the energy transfer in organic

reaction has been widely used for synthetic purposes (reviewed in [23,24]) and also to specifically assist derivatization reactions of drugs/metabolites in toxicological analysis [25–27]. In principle, microwave irradiation can provide an effective tool to achieve appreciable yields of specific derivatives on poorly reactive residues, at the same time increasing the rate of the derivatization reaction on more reactive sites. Regardless of the exact mechanism by which microwave irradiation can promote an increase of the reaction rate, the general effect is a more rapid and uniform energy transfer that appears to take place independently from the possibility to excite rotational transitions [24].

The experimental strategy here presented allowed to preliminarily evaluate the effectiveness of various derivatization reagents to obtain TMS derivatives in different operating conditions. Derivatization has been carried out for every glucocorticoids using two different derivatization agents (MTSFA:NH_4I:DTE 1000:2:4 or TMSim:BSA:TMCS 3:3:2) and in different incubation conditions (at room temperature for times up to 24 h; at 70 °C for times up to 3 h; under microwave irradiation at 900 W emitted power for times up to 1 h; and using a combination of the last two procedures).

Preliminary experiments have shown that the mixture TMSim:BSA:TMCS 3:3:2 is more reactive than MTSFA:NH_4I:DTE 1000:2:4 in all operating conditions, especially as far as the derivatization yield on sterically hindered synthetic glucocorticoids is concerned. In addition to this, microwave-assisted derivatization drastically enhanced the reactivity of the derivatization mixture TMSim:BSA:TMCS 3:3:2, rather than of MTSFA:NH_4I:DTE 1000:2:4, on sterically hindered sites. (data not shown). The effectiveness of different combinations of thermal/microwave energy transfer has then been evaluated on all endogenous and synthetic glucocorticoids here considered using the mixture TMSim:BSA:TMCS 3:3:2 as the derivatizing reagent. The results are summarized in Table 1.

As it can be seen, the best compromise conditions for the derivatization of all compounds considered so far by the derivatization mixture TMSim:BSA:TMCS 3:3:2 comprise a microwave-assisted derivatization step (40 min at 900 W) followed by a “traditional” thermal derivatization step (90 min at 70 °C in a thermostated bath). This derivatization procedure allows the formation of derivatives suitable for GC analysis for all the considered glucocorticoids with high yields, including sterically hindered sites and retaining, at the same time, any information useful for structural analysis. In the selected operating conditions, considerable yields are obtained on all derivatizable sites apart from the keto group on C3 when it is conjugated to two double bonds.

Although our data are not conclusive, comparison of the derivatization yields given in Table 1 shows that the effect of microwaves appears to be specific and different from the one obtained by the classical thermal transfer: derivatization yields after thermal heating ($T = 70$ °C) for incubation times up to 3 h are generally lower than those obtained after MW irradiation for incubation times $\geq$40 min; furthermore, assuming that, during the 40 min of incubation under microwave irradiation, the temperature, because of the outer water bath, is always at or near 100 °C, we have undertaken an additional set of experiments to compare the yields obtained by a two-stage thermal incubation process (40 min at 100 °C + 90 min at 70 °C) with those of the combined MW-thermal process (40 min at 900 W followed by 90 min at 70 °C). As it can be seen, again from data in Table 1, the yields obtained by the combined process are higher than those of the two-step thermal process, the latter being comparable to those obtained by incubation for 180 min at 70 °C.

The GC–EI–MS spectra of the TMS derivatives of all glucocorticoids, obtained following derivatization in the above experimental conditions, are characteristic of the corresponding glucocorticoid and contain a sufficient number of diagnostic ions to allow the unambiguous identification of each compound. Table 2 reports, for each TMS derivative, the relative retention time, the molecular weight, the most abundant ion fragment (base peak) and the other diagnostic ion fragments, including those (in boldface) most suitable for the GC–MS screening analysis in SIM. It has to be noted that fragments of endogenous corticosteroids selected for SIM experiments are not always the most abundant, in order to avoid sudden increases of the signal with consequent switch-off of the filament, since endogenous glucocorticoids are usually much more concentrated than synthetic ones in real samples.

Table 3 reports the LODs of synthetic glucocorticoids in urine: it has to be stressed that these basic values can be easily improved by specific pre-treatment of urine samples (i.e. following immunoaffinity prepurification) for confirmation purposes, and/or by more advanced GC–MS equipment.

To test the effectiveness of the proposed method for the analysis of real samples in antidoping tests, we have analyzed samples of urine obtained after oral administration of two synthetic glucocorticoids, namely betamethasone (Fig. 2) and prednisone (Fig. 3). The GC–EI–MS–SIM chromatograms reported in the figures show that the screening procedure here proposed is able to clearly distinguish a negative from a positive urine, the latter showing in both cases the same ion fragments of the corresponding positive reference urine. In the case of prednisone administration, it is possible to detect the presence of both prednisone unchanged (Fig. 3a) and its active metabolite prednisolone (Fig. 3b).

In conclusion, the procedure here presented is suitable for the rapid screening and confirmation of urine samples collected in antidoping testing programs. The detection of forbidden drugs in doping control analysis is indeed based on the identification of key compounds in urine that can be considered reliable indexes of their administration. The analysis is focused on the identification of specific metabolites whenever it is not possible to confirm the presence of the unchanged drug. This is the case, for instance, of most anabolic androgenic steroids, that are rapidly converted into their metabolites and whose concentration in urine are however too low to allow their direct identification. The situation is different with glucocorticoids, mainly for two reasons: (i) the identification of the unchanged drug, if unambiguous, would make pleonastic the identification of its metabolite(s); and (ii) a long-term retrospective analysis for the administration of glucocorticoids is, at present, not required by the sport Authorities (the International Olympic Committee and the World Antidoping Agency), since their use is forbidden only for “in competition” testing [3].

Although the study was planned to draw practical indication for the activity of an antidoping laboratory, many consideration can be extended to other fields of analytical toxicology and clinical chemistry, including the analysis of glucocorticoids residues in food matrices and/or in bioptic materials.

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