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LC-MS/MS Analysis of Glucocorticosteroids: First Experiences with Therapeutic Use Exemption in Routine Doping Analysis

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Summary

According to the regulations of the World Anti-Doping Agency (WADA) glucocorticosteroids belong to the 'Prohibited List of Substances 2004' for in-competition-testing [1]. Due to the therapeutic value of this group of remedies, exemptions concerning the means of administration are provided. Since January 1st 2004, the administration of glucocorticosteroids by non-systemic-routes (inhalation, intra-articular, topic, etc.) requires a therapeutic use exemption, abbreviated process (TUE2), as defined by the 'International Standard for Therapeutic Use Exemptions' [2]. The implementation of sample preparation for glucocorticosteroids into screening procedure 4, detection of anabolic androgenic substances (combined fraction), followed by LC-MS/MS analysis will be presented. First experiences including communication with the national and international federations as well as the National Anti-Doping Agencies (NADAs) concerning TUE2 with our laboratory are reported. Frequently asked questions have arisen by medical team physicians, athletes and others regarding the time span of analytical traceability of glucocorticosteroids administered in training by systemic or non-systemic routes. Examples of routine work and follow-up studies will be discussed.

Chemicals and glucocorticosteroids

Budesonide, desonide, 20β-dihydroprednisolone and fludrocortisone were obtained from Steraloids (London, GB), hydrocortisone from Serva (Heidelberg, D), beclomethasone, dexamethasone, flumethasone, flunisolide, fluoxymesterone, methylprednisolone, 17α-methyltestosterone, prednisolone prednisone, triamcinolone and triamcinolone acetonide from Sigma-Aldrich (Taufkirchen, D). 16α-Hydroxyprednisolone from AstraZeneca (Lund, S) was a gift from the Gent laboratory (B), 16-methyleneprednisolone a gift from Hoechst (Frankfurt, D) and fluocortolone a gift from Schering (Berlin, D). Isoflupredone was kindly

donated from the anti-doping laboratory Rome (I). All corresponding chemical names are listed in Table 2.

Ammonium acetate, potassium carbonate, sodium hydrogen carbonate, *tert.*-butyl methyl ether, methanol and glacial acetic acid (all p.a.) were purchased from Merck (Darmstadt, D) and acetonitrile (ultra gradient HPLC grade) from Baker (Deventer, NL) and β-glucuronidase from E. Coli from Roche Diagnostics (Mannheim, D).

Sample preparation

The analysis of glucocorticosteroids is implemented in the existing screening procedure 4, detection of anabolic androgenic substances (combined fraction). Depending on the specific gravity 3 ml ($d > 1.01$ g/cm^3) or 6 ml ($d < 1.01$ g/cm^3) of urine is transferred into a glass tube. Different internal standards are added as described elsewhere [3]. For the analysis and detection of glucocorticosteroids 1500 ng of 17α-methyltestosterone, (60 µl of a 25 µg/ml solution in methanol) as internal standard, 1 ml phosphate buffer 0.8 m, pH 7.0, and 25 µl β-glucuronidase from E. Coli are added before heating at 50°C for 1 hour. The hydrolysis is stopped by adding 750 µl of a 20% aqueous solution of potassium hydrogen carbonate and potassium carbonate (1:1; w:w). Six ml of *tert.*-butyl methyl ether is added, and the glass tube is shaken mechanically for 5 minutes followed by centrifugation, 10 min at 750 *g*. One ml of the organic layer is transferred into a new tube and evaporated to dryness *in vacuo*. The dried residue is dissolved in 60 µl of a solution of methanol / ammonium acetate buffer, 5 mmol ammonium acetate, 1‰ glacial acetic acid in distilled water, pH = 3.5, (1:1, v:v) prior LC-MS/MS analysis. The remaining ether layer is decanted to a new tube, evaporated to dryness *in vacuo* and derivatised with 100 µl MSTFA/NH4I/ethan-thiol (1000:2:3; v:w:v) at 60°C for 20 minutes followed by GC-MS analysis [3].

For confirmation and follow-up studies of known administrations of glucocorticosteroids the sample preparation is performed as follows: to a volume of 5 ml of urine 50 ng/ml fluoxymesterone (25 µl of a 10 µg/ml solution in methanol) is added as internal standard. The urine samples are adjusted to pH 9.6 by adding 0.5 g of a mixture of sodium hydrogen carbonate and potassium carbonate (2:1, w:w). The aqueous layer is extracted with 7 ml *tert.*-butyl methyl ether and centrifuged at 750 *g* for 10 min. The organic layer is transferred to a

new tube and evaporated to dryness *in vacuo*. The residue is dissolved in 100 µl of methanol and injected into the LC-MS/MS system.

The analyses for glucocorticosteroids, screening and confirmation, are performed on a Hewlett Packard HP1100 liquid chromatograph coupled to a PE Sciex API 2000 triple quadrupole mass spectrometer. The column used is a Purospher Star RP-18e, 55 x 4 mm i.d., 3 µm particle from Merck (Darmstadt, D). For screening purposes the LC and MS conditions are as listed in Table 1. The respective protonated molecules $(M+H)^+$, as well as the selected ions of the different compounds are listed in Table 2.

TABLE 1: Analytical parameters.

Flow:	0.3 ml/min (splitless)
Solvents:	A: Ammonium acetate buffer (pH = 3.5, 5 mmol ammonium acetate,1‰ glacial acetic acid) B: Acetonitrile
Gradient: Injection volume:	10% Acetonitrile to 100% in 9 min 10 µl
Run Time / Post Time:	11 min / 3.5 min
Ion source: Interface Temperature: Ionisation mode:	APCI 400°C Positive, multiple reaction monitoring (MRM)
Dwell time:	40 msec

This screening method for determination of glucocorticosteroids is validated regarding specifity, recovery, and intermediate precision at the minimum required performance limit of 30 ng/ml. The linearity is estimated according to DIN 38402, part 51)* at 99% at a working range of 2-60 ng/ml. It is proof exemplarity for the substances prednisone, 6α-methylprednisolone, dexamethasone and triamcinolone acetonide. In addition the limit of detection is appraised at a signal to noise ratio of 3:1 for all substances (Table 2).

)* DIN 38402: Deutsche Einheitsverfahren zur Wasser- Abwasser und Schlammuntersuchung. Allgemeine Angaben (Gruppe A) Kalibrierung von Analysenverfahren, Auswertung von Analysenergebnissen und lineare Kalibrierfunktion für die Bestimmung von Verfahrenskenngrößen, Beuth Verlag, Berlin 1986

TABLE 2: Screening and confirmation of glucocorticosteroids, compound specific parameters of mass spectrometry, retention times (R.T.) and limits of detection (LOD).

Compound	Chemical name	Precursor ion $(M+H)^+$ *m/z*	Product ion screening (confirmation) *m/z*	R.T. [min]	LOD [ng/ml]	Recovery [%]	Linearity test 2-60 ng/ml
16α-Hydroxyprednisolone	pregna-1,4-diene-11β,16α,17,21-tetrol-3,20-dione	377	359 (323, 341, 305)	4.5	5		
Triamcinolone	9α-fluoropregna-1,4-diene-11β,16α,17,21-tetrol-3,20-dione	395	375 (357, 225)	4.6	2		
20β-Dihydroprednisolone	pregna-1,4-diene-11β,17,20β,21-tetrol-3-one	363	267 (345, 171)	4.6	2		
16-Methyleneprednisolone	16-methylenepregna-1,4-diene-11β,17,21-triol-3,20-dione	373	337 (355, 147, 171)	5.0	10		
Prednisolone	pregna-1,4-diene-11β,17,21-triol-3,20-dione	361	343 (147, 307)	5.1	5		
Hydrocortisone	pregn-4-ene-11β,17,21-triol-3,20-dione	363	121	5.1	n/a		
Prednisone	pregna-1,4-diene-17,21-diol-3,11,20-trione	359	147 (341, 171)	5.1	3	32	linear
Isoflupredone	9α-fluoropregna-1,4-diene-11β,17,21-triol-3,20-dione	379	359 (91, 77, 121)	5.1	4		
Fludrocortisone	9α-fluoropregn-4-ene-11β,17,21-triol-3,20-dione	381	91, 181 (105, 115)	5.2	20		
6α-Methylprednisolone	6α-methylpregna-1,4-diene-11β,17α,21-triol-3,20-dione	375	161 (357, 339)	5.5	1	33	linear
Dexamethasone	9α-fluoro-16α-methylpregna-1,4-diene-11β,17,21-triol-3,20-dione	393	373 (355, 147)	5.6	2	38	linear
Betamethasone	9α-fluoro-16β-methylpregna-1,4-diene-11β,17,21-triol-3,20-dione	393	373 (355, 147)	5.6	2		
Flumethasone	6α,9α-difluoro-16α-methylpregna-1,4-diene-11β,17,21-triol-3,20-dione	411	253 (121, 235, 335)	5.7	2		
Beclomethasone	9α-chloro-16β-methylpregna-1,4-diene-11β,17,21-triol-3,20-dione	409	391 (147, 279, 373)	5.7	1		
Triamcinolone acetonide	9α-fluoropregna-1,4-diene-11β,16α,17,21-tetrol-3,20-dione 16α,17-acetonide	435	415 (397, 121)	5.8	2	36	linear
Desonide	pregna-1,4-diene-11β,16α,17,21-tetrol-3,20-dion 16α,17-acetonide	417	147 (399, 341, 323)	5.8	2		
Flunisolide	6α-fluoropregna-1,4-diene-11β,16α,17,21-tetrol-3,20-dion 16α,17-acetonide	435	121 (339, 321, 171)	5.8	4		
Fluocortolone	6α-fluoro-16α-methylpregna-1,4-diene-11β,21-diol-3,20-dione	377	171 (303, 321, 339)	6.0	1		
Budesonide	16α,17-butylidenedioxypregna-1,4-diene-11β,21-diol-3,20-dione	431	323 (413, 147, 173)	6.6	3		
17α-Methyltestosterone, IS	17α-methylandrosta-4-en-17β-ol-3-one	303	109	6.9	n/a	29	

Results and discussion

From January until March 2004 1810 samples were analysed with the routine screening procedure. A total of 81% (=1470) were competition-samples and evaluated for the presence of glucocorticosteroids. Glucocorticosteroids were detected in 9 samples, and a request for a therapeutic use exemption was sent to the respective national/international federation. A special questionnaire was designed to minimize the administrative workload between the laboratory and the respective federation (Annex 1). According to WADA regulations only samples without TUE should enter a confirmatory analysis and be termed positive if the screening result is confirmed. The administration of different synthetic glucocorticosteroids was detected in several sports in broadly differing concentrations (Table 3).

TABLE 3: Samples tested positive with different synthetic glucocorticosteroids from January until March 2004 at laboratory Cologne.

Laboratory Number	Sport	Substance	Urinary concentration [ng/ml]
0154	Soccer	Methylprednisolone	1.5
0318	Track and field	Triamcinolone acetonide	1.2
0712	Weightlifting	Betamethasone	0.5
0783	Squash	Betamethasone	6
0882	Cycling	Betamethasone	2
0916	Ski	Methylprednisolone	2.2
1099	Soccer	Betamethasone	2
1123	Basketball	Dexamethasone	2
1436	Soccer	Betamethasone	28

Several factors are affecting the duration of detectability of synthetic glucocorticosteroids in man: the receptor affinity and plasma half-life period of the administered glucocorticoid, the formulation of medication and the means of administration. According to plasma half-life it is distinguished between short-, medium- and long-acting substances. Short acting substances are the naturally occurring glucocorticosteroids hydrocortisone and cortisone with a plasma half-life after oral administration of 0.5-1 hour and a relative receptor affinity of 0.23 (relative to dexamethasone = 1). Medium-acting substances are, amongst others, prednisolone, prednisone and methylprednisolone, their plasma half-life after oral administration is about 2-3 hours, the relative receptor affinity is 0.5-1.3. Long-acting substances are dexamethasone and betamethasone with a plasma half-life period after oral administration of 3-7 hours and a receptor affinity = 1 [4].

For methylprednisolone, 3 different formulations are available: the free alcohol, hydroxy group C-21 is the tablet style formulation for oral application. This form is sparingly soluble in water. Highly soluble in water is the methylprednisolone sodium succinate form, for *i.m.* and *i.v.* parenterals, whereas the methylprednisolone acetate is virtually insoluble in water and used for sustained release depot parenterals. Due to their long time of adsorption from sight as result of the very low water solubility, these esters have advantages in treatment. This formulation can be injected directly into the affected joints and a continuing therapeutic effect over weeks is provided. Therefore negative side effects compared to an oral application over time are reduced [4,5].

All kinds of systemic treatment with glucocorticosteroids of any formulation are allowed during out-of-competition periods. However, it is possible that those compounds are detected weeks later in competition-samples owing to the sensitive nature of the applied analytical technique. A follow-up study of an athlete who was treated in an emergency case with Volon® A40 (Bristol-Myers Squibb, München, D), 40 mg triamcinolone acetonide, *i.m.* confirmed this assumption. The urinary elimination of triamcinolone acetonide is depicted in Figure 1. With the high sensitivity of the detection method of LC-MS/MS, the detection limit for triamcinolone acetonide is 0.5 ng/ml in the confirmation procedure, a positive result in competition-samples may be possible long after the treatment.

In a cyclists doping control sample 2 ng/ml of betamethasone were detected (Table 3, sample 0882). In the accompanying doping control form (health booklet) an intra-articular injection of betamethasone into the right knee 19 days before the competition was declared.

Another unexpected source of glucocorticosteroids became apparent by an athlete who underwent a dental treatment. This athlete was tested positive for triamcinolone acetonide, but could only specify a visit to his dentist over the past week before the respective in-competition-test. Afterwards his dentist declared the local administration of Ledermix® paste (Riemser, Greifswald, D). One gram of this paste contains a combination of 10 mg triamcinolone acetonide and 30.2 mg of the antibiotic demeclocycline. It is used to treat endodontitis and pulp inflammation in gangrene therapy. Traces of triamcinolone acetonide, about 1 ng/ml, could be detected in a volunteer's urine 2 days after an accordant dental treatment.

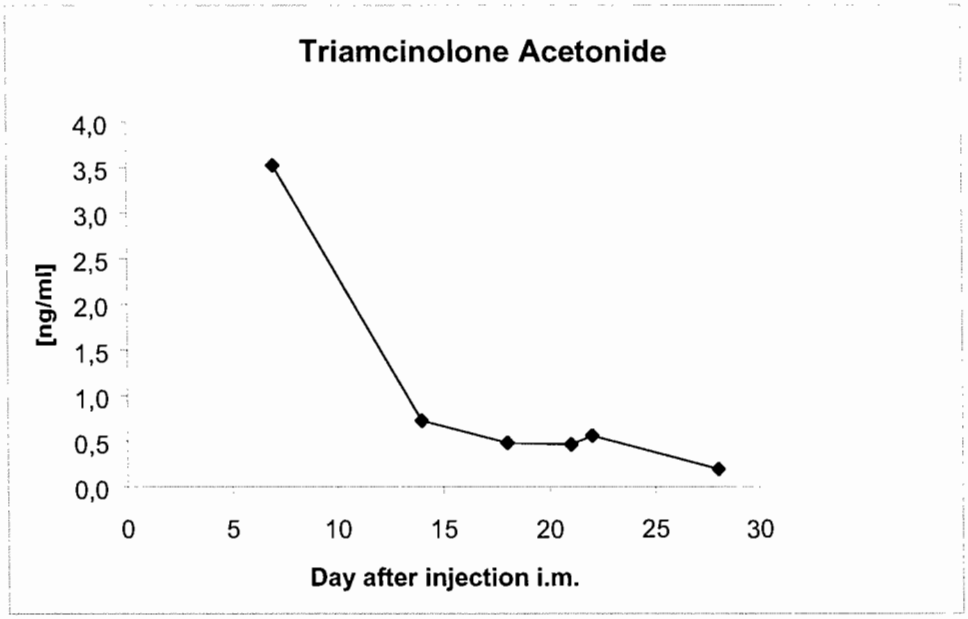

FIGURE 1: Urinary concentration of triamcinolone acetonide after parenteral injection (*i.m.*) of Volon® A40, containing 40 mg triamcinolone acetonide.

LC-MS/MS analysis is the method of choice to detect glucocorticosteroids in low concentrations in human urine in doping analysis [6,7,8]. In the present study it is shown that the sample preparation can be implemented into other screening procedures, e.g. detection of anabolic androgenic substances, combined fraction. The advantage is to minimize the workload of sample preparation at sufficient sensitivity. In addition the total volume of invested urine for screening purposes can be reduced. This becomes more and more important as new classes of forbidden substances that need a new and additional screening procedure, are rising continuously. Due to the experience made within the first 3 month in routine analysis with in-competition-samples and results of some follow-up studies the following conclusions can be made:

- In all cases of non-systemic-routes of administration the treatment with glucocorticosteroids as depot formulations should be always declared by TUE2 in a time frame up to 6 weeks prior competition.
- Physicians should be very careful using depot formulations by systemic administration (*i.v., i.m.*). Their time of duration, elimination and detectability in doping analysis is difficult or even impossible to control. A therapeutic use exemption as standard application form may be requested prior to any treatment.
- Short acting glucocorticosteroid formulations should always be favored.

- All treatments with glucocorticosteroids in emergency cases should be indicated fastest.
- The designed questionnaire is very helpful in terms of communication with the federations and unnecessary paperwork is reduced to a minimum.

Detailed investigations concerning analytical traceability of glucocorticosteroids in human urine after different non-systemic and systemic routes of administration, as well as different pharmacological substances are not accessible. This scientific work should be done soon in order to help physicians and athletes to avoid accidental positive cases with gluco-corticosteroids.

References

[1] World Anti-Doping Agency. The 2004 Prohibited List. Montreal 2004

[2] World Anti-Doping Agency. International Standard for Therapeutic Use Exemptions. Montreal 2004

[3] Mareck, U., Thevis, M., Gotzmann, A., Bredehöft, M., Geyer, H., Schänzer, W. Comprehensive sample preparation for anabolic steroids, glucocorticosteroids, beta-receptor blocking agents, selected anabolic androgenic steroids and buprenorphine in human urine. In: Schänzer, W., Geyer, H., Gotzmann, A., Mareck, U. (eds.) *Recent Advances in Doping Analysis (12)*. Sport und Buch Strauß, Köln 2004, 65-68

[4] Hatz, H.J. Glucocorticoide, immunologische Grundlagen, Pharmakologie und Therapierichtlinien. Wissenschaftliche Verlagsgesellschaft, Stuttgart 1998

[5] Depot-medrol as methylprednisolone acetate *http://otc.isu.edu/~das/PRESENTATIONS%202003/Depot-Medrol_files/frame.htm* found December 2003

[6] Fluri K., Rivier L., Nagy A.D., Yoy C., Maître A., Schweizer C., Saugy M., Mangin P. Method for confirmation of synthetic corticosteroids in doping urine samples by liquid chromatography-electrospray ionisation mass spectrometry. *J Chromatogr B.* 2001, 926, 87-95

[7] Deventer K., Delbeke F.T. Validation of a qualitative screening method for corticosteroids by liquid chromatography tandem mass spectrometry. In: Schänzer, W., Geyer, H., Gotzmann, A., Mareck, U. (eds): *Recent Advances in Doping Analysis (11).* Sport und Buch Strauß, Köln 2003, 23-31

[8] Gotzmann, A., Bredehöft, M., Thevis, M., Schänzer, W. Detection of prednisolone, prednisone and 20-dihydroprednisolone in human and equine urine by means of LC/MS/MS. In: Schänzer, W., Geyer, H., Gotzmann, A., Mareck, U. (eds.) *Recent Advances in Doping Analysis (11).* Sport und Buch Strauß, Köln 2003, 33-41

Annex 1

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Carl-Diem-Weg 6
D50933 Köln
Fax: +49 221 497 3236

Köln, *date*

Federation: _____

Date of event: _____

Preliminary analysis report no.: S200400*xxxx*

During mass spectrometric screening indications for the presence of the

glucocorticosteroid *Name of glucocorticosteroid*

were detected in A-sample: *xxxxxx*

> *Glucocorticosteroids are prohibited when administered orally, rectally, or by intravenous or intramuscular administration. All other administration routes require a medical notification in accordance with section 8 of the International Standard for Therapeutic Use Exemptions.* (WADA: The 2004 Prohibited List, International Standard)

Only samples without a valid therapeutic use exemption (TUE) will enter a confirmation analysis. After confirmation the A-sample will be reported as adverse analytical finding (with copies to IF and WADA). Therefore we kindly ask you to return this questionnaire by fax **within 2 weeks** after reporting date to **Institute of Biochemistry Cologne, IOC/WADA accredited laboratory.**

Fax: +49 - 221 - 497 3236

_____
(Name, signature of representative of laboratory)

A therapeutic use exemption (TUE) for glucocorticosteroids for sample *xxxxxx*

is present ☐ is not present ☐

_____ _____
Place and date Name

_____
Signature