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A. GOTZMANN, M. BREDEHÖFT, M. THEVIS, W. SCHÄNZER:
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Detection of Prednisolone, Prednisone and 20-Dihydroprednisolone in Human and Equine Urine by Means of LC/MS/MS

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ABSTRACT

Ten horses (geldings) were treated with a single dose (0.5 mg/kg bodyweight, i.m.) of prednisolone-21-acetate. One male volunteer was treated with prednisolone (10 mg, oral). Urine samples were collected 3 days before and 30 days after administration in the animal experiment, one urine sample before administration and 10 urine samples up to 40 h after in the human excretion study. Prednisolone, prednisone, 20β-dihydroprednisolone [20β-DHP] and hydrocortisone were quantified by means of liquid chromatography / tandem mass spectrometry [LC/MS/MS]. Quantification was conducted via calibration curves ranging from 2 ng/ml to 1000 ng/ml for prednisolone and 20β-DHP, and from 2 ng/ml to 500 ng/ml for prednisone and hydrocortisone. Urine samples were spiked with the respective substances containing fluoxymesterone as internal standard. 20β-DHP, prednisone and hydrocortisone showed an acceptable linearity within the working range. The limit of detection for 20β-DHP was 1 ng/ml and 2 ng/ml for prednisolone and prednisone. Thirty days after administration the prednisolone metabolite 20β-DHP was still detectable in all equine samples.

INTRODUCTION

In routine screening analysis for endogenous and synthetic corticosteroids in human and equine urine by LC/MS/MS, problems occur regarding the detection of prednisolone as parent compound due to interfering biological background. Thus, better conditions for the analysis of this corticosteroid are needful.

Other techniques, such as enzyme linked immuno sorbent assay [ELISA], gas chromatography / mass spectrometry [GC/MS] or high performance liquid chromatography / ultra violet detection [HPLC/UV] are available for the detection of corticosteroids in human and equine urine. There are several disadvantages regarding sensitivity, specifity or handling of a large number of samples. ELISA-techniques may improve the sensitivity (Schulz et al., 1994), but cross-reactions between the synthetic corticosteroids and other substances have been frequently observed (Zhu et al., 1992). In addition, several commercially available ELISA-kits can detect only one single corticosteroid. The ELISA technique has to be combined with other methods such as HPLC or LC/MS to confirm or quantify a screening result (Creaser et al., 1998; Chui et al., 1992).

The detection of corticosteroids by GC/MS causes problems, requiring numerous different derivatizations to improve the gas chromatographic properties of the analytes as a result of their heterogenous chemical structures (Schänzer et al., 1994).

For routine screening and also confirmation, LC/MS/MS analysis is more suitable due to fast sample preparation and higher sensitivity. An appropriate sample-throughput is possible and a great number of synthetic corticosteroids as well as hydrocortisone can be detected within one analytical run (Tang et al., 2001; Fluri et al., 2001).

MATERIALS AND METHODS

Animal and human experiment

A single dose (0.5 mg/kg bodyweight, i.m.) of prednisolone-21-acetate (Prednisolon-ratiopharm® 25 Kristallsuspension, Ratiopharm GmbH, Ulm, D) was administered to each of the ten horses (all geldings, aged 3 years). Urine samples were collected 3 days before and 30 days after administration of the corticosteroid. All animal experiments were performed at the Tierärztliche Hochschule Hannover (D). One single dose (0.1 mg/kg bodyweight, oral) of prednisolone (Decaprednil® 2 x 5 mg, Dorsch GmbH, Germany) was administered to a male volunteer (33 years, bodyweight 99 kg). In the human experiment urine samples were collected before and 40 h after administration of the glucocorticosteroid. All human and equine urine samples were stored at –20°C until analysis.

Chemicals

20β-Dihydroprednisolone [11β,17,20β,21-Tetrahydroxypregna-1,4-dien-3-one] and 20α-dihydroprednisolone [11β,17,20α,21-Tetrahydroxypregna-1,4-dien-3-one] were obtained from Steraloids (London, GB), hydrocortisone [11β,17,21-Trihydroxypregn-4-ene-3,20-dione] from Serva (Heidelberg, D), prednisolone [11β,17,21-Trihydroxypregna-1,4-diene-3,20-dione], prednisone [17,21-Dihydroxypregna-1,4-diene-3,11,20-trione] and fluoxymesterone [11β,17β-Dihydroxy-9α-fluoro-17α-methylandrost-4-en-3-one] from Sigma (Taufkirchen, D). 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).

Sample preparation

To a volume of 5 ml of urine 50 ng/ml fluoxymesterone (25 µl of a 10 µg/ml solution in methanol) was added as internal standard for quantification. The urine samples were 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 was extracted with 5 ml of *tert.*-butyl methyl ether and centrifuged at 750 *g* for 10 min. The organic layer was transferred to a new tube and evaporated to dryness *in vacuo*. The residue was dissolved in 100 µl of methanol and injected into the LC/MS/MS system. For calibration purposes, blank equine urine samples were spiked with 2-1000 ng/ml 20β-DHP, prednisolone and hydrocortisone, respectively (prednisone and hydrocortisone 2-500 ng/ml each).

Analytical parameters

The analyses were performed on a Hewlett Packard HP1100 liquid chromatograph coupled to a PE Sciex API 2000 triple quadrupole mass spectrometer. The column used was a Purospher Star RP-18e, 55 x 4 mm i.d., 3 µm particle from Merck (Darmstadt, D). The LC and MS conditions were as follows: mobile phase, A = ammonium acetate buffer (pH = 3.5, 5 mmol ammonium acetate, 1‰ glacial acetic acid in distilled water) / B = acetonitrile, gradient: 10% B to 90% B in 9 min, flow rate: 0.5 ml/min (splitless), injection volume 20 µl, interface and temperature: APCI 475°C, ionization mode: positive, multiple reaction monitoring of quasi-

molecular ions $(M+H)^+$ and specific product ions after collision-induced dissociation (see Table 1), dwell time: 100 msec, pause time 5 msec.

TABLE 1: Compound specific parameters of mass spectrometry, retention times and limits of detection (LOD).

Compound	Quasi-molecular ion $(M+H)^+$ *m/z*	Selected ion *m/z*	R.T. [min]	LOD [ng/ml]
20α-Dihydroprednisolone	363	267	5.4	-*
20β- Dihydroprednisolone	363	267	5.5	1
Prednisolone	361	343	5.9	2
Hydrocortisone	363	121	5.9	5
Prednisone	359	341	6.0	2
Fluoxymesterone, IS	337	317	6.7	-*
Dexamethasone	393	373	6.5	0.5
Triamcinolone acetonide	435	315	6.8	0.5
Prednylidene	373	121	5.8	10
Methylprednisolone	375	161	6.4	1
Triamcinolone	395	375	6.0	2
Fludrocortisone	381	181	6.0	2.5
Flumethasone	411	253	6.8	1
Beclomethasone	409	391	6.7	1
Isoflupredone	379	359	5.9	1

* not determined

RESULTS

In urine samples of ten horses, 20β-DHP, prednisolone and prednisone were detected and quantified by means of LC/MS/MS after application of a single dose (0.5 mg/kg bodyweight, i.m.) of prednisolone-21-acetate. The urine samples of the human volunteer (10 mg prednisolone, oral) were evaluated the same way. For 20β-DHP and prednisone the calibration curves showed an acceptable linearity within the working range. An example for 20β-DHP is depicted in Figure 1.

Human experiment

The estimated concentrations of 20β-DHP, prednisolone and prednisone after adminstration of one single dose of 10 mg prednisolone to a human volunteer are shown in Table 2. An example for LC/MS/MS analysis, 31.6 h after administration of the glucocorticosteroid, is shown in Figure 2.

TABLE 2: Urinary excretion of 20β-DHP, prednisolone and prednisone after one single oral administration of 10 mg prednisolone to a male volunteer.

Time [h]	20β- Dihydroprednisolone [ng/ml]	Prednisolone [ng/ml]	Prednisone [ng/ml]
0	0	0	0
0.3	415	801	709
3.8	402	124	314
6.8	203	36	56
12.3	257	46	55
18.0	92	17	32
24.6	32	8	7
28.6	17	5	3
31.6	7	2	2
36.2	3	< 2	n.d.*
40.2	1	n.d.*	n.d.*

*not detectable

Equine experiment

In case of 20β-DHP, urinary peak concentrations were estimated between 560 ng/ml and 6180 ng/ml (mean: 2330 ng/ml ±1540 ng/ml; n=10). They were reached within 2 to 30 hours after administration of the glucocorticosteroid. In all horses, 20β-DHP was still detectable 732 hours after administration with a mean concentration of 16 ng/ml ±9 ng/ml (Figure 3). Both isomers, 20β-DHP and 20α-DHP, are excreted in equine urine, whereby the β-isomer prevailed. Both isomers are separated by the employed high speed reversed phase column.

For prednisolone, peak concentrations of approximately 2300 ng/ml were calculated. These maximal values were reached after 0.6 to 28 hours. Here, an unknown peak originating from biological background made an evaluation difficult or even impossible in nearly 50% of the samples. Therefore, the data of urinary prednisolone concentrations are incomplete.

Prednisone showed peak concentrations between 220 ng/ml to 475 ng/ml (mean 326 ng/ml ±105 ng/ml; n=9), which were reached similar to prednisolone from 0.6 hours up to 28 hours after application. Prednisone was detected from 108 hours (horse 5) to 732 hours (horse 2 and 6).

DISCUSSION

The direct detection and quantification of prednisolone in human and equine urine is often difficult due to interfering biological background. The results of the present study suggest that the determination of prednisolone and its main metabolites 20β-DHP and prednisone remain in urine for a longer time. Furthermore, the described combination of sample preparation and LC/MS/MS detection is fast and sensitive. Compared to immunoassay screening, a great number of synthetic glucocorticosteroids (e.g. dexamethasone, betamethasone, isoflupredone, prednylidene, methylprednisolone, flumethasone, beclomethasone, fludrocortisone, triamcinolone, triamcinolone acetonide) is detectable within one analytical run (Figure 4). This procedure enables a high sample-throughput and is suitable for routine screening analysis. The detection of 20β-DHP (in addition to prednisolone) solves the problem of interfering biological background in equine and human urine, which often co-elutes with prednisolone. This may also improve the retrospective, assuming that this metabolite is excreted for a longer time period into human and equine urine as the administered substance prednisolone. Further investigations in this area are warranted.

ACKNOWLEDGEMENTS

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FIGURES

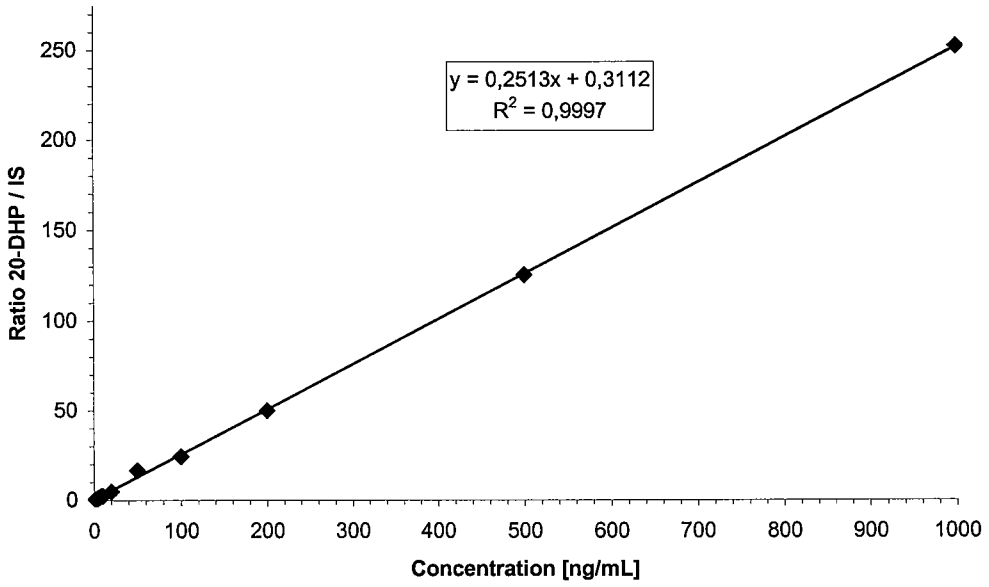

Figure 1: Calibration curve for 20β-dihydroprednisolone in spiked equine urine by LC/MS/MS. Added amounts of 20β-dihydroprednisolone: 2, 4, 6, 8,10, 20, 50, 100, 200, 500 and 1000 ng/ml; internal standard: fluoxymesterone 50 ng/ml

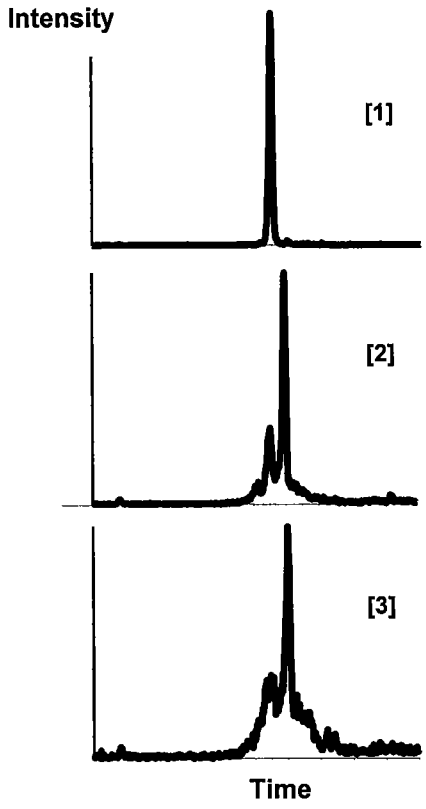

Figure 2: LC/MS/MS chromatogram of a human urine sample 31.6 h after oral administration of 10 mg prednisolone (sample preparation and analytical conditions see text).

[1] 20β-DHP, ion transition: 363-267; R.T. 5.5 min; area 12342; 7 ng/ml

[2] Prednisolone, ion transition: 361-343; R.T. 5.9 min; area 5044; 2 ng/ml

[3] Prednisone, ion transition: 359-341; R.T. 6.0 min; area 1616; 2 ng/ml

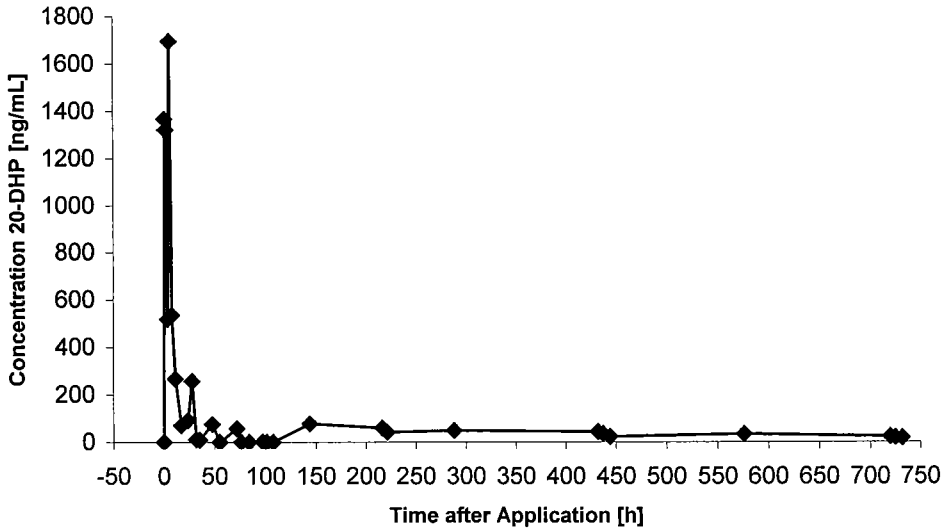

Figure 3: Mean values of urinary 20β-dihydroprednisolone concentration from 0-732 hours after application of one single dose of prednisolone-21-acetate (0.5 mg/kg bodyweight, i.m.) for 10 horses.

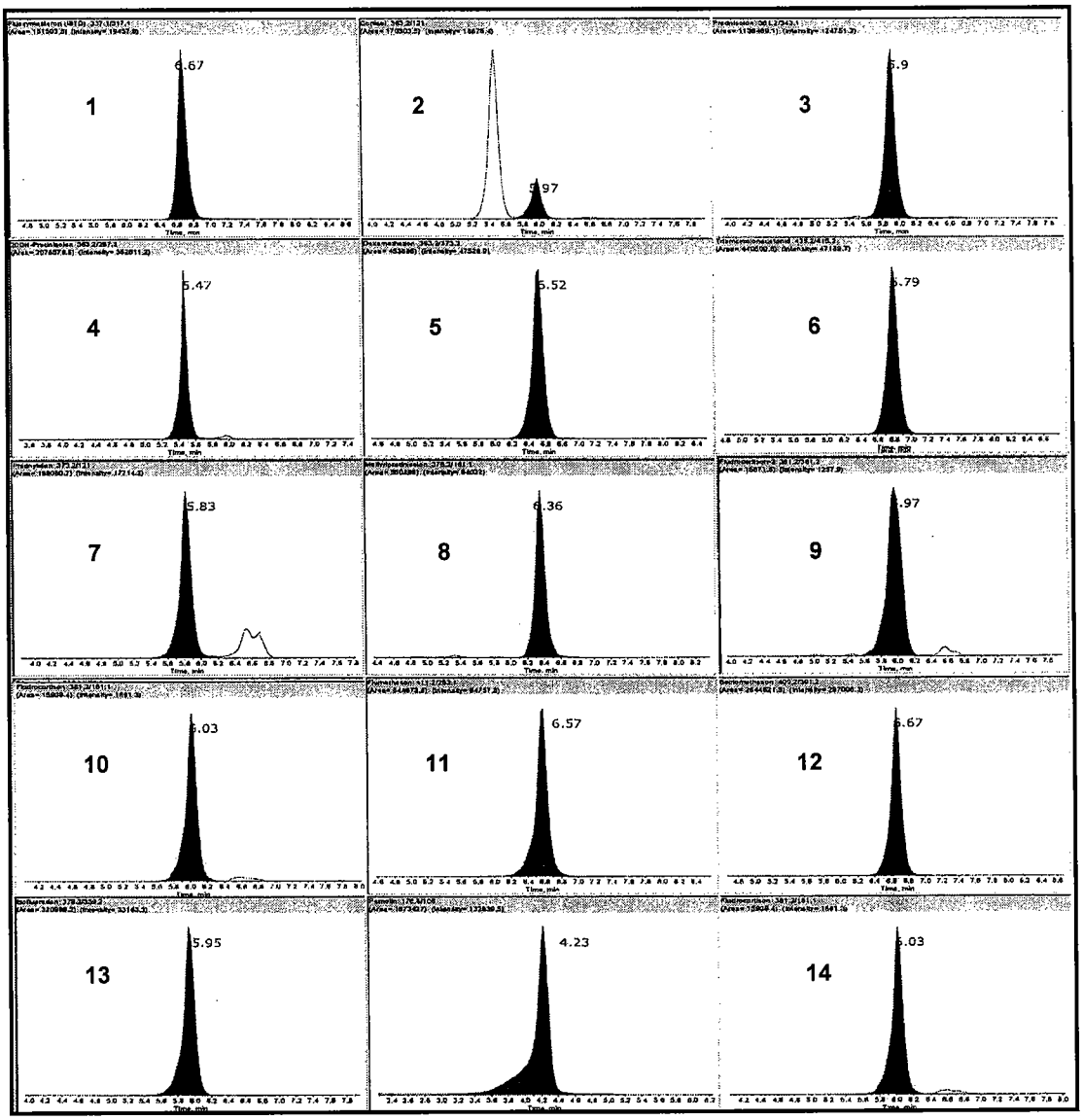

Figure 4: LC/MS/MS screening of 12 different synthetic glucocorticosteroids and endogenous hydrocortisone, 100 ng each, ion transition:
[1] fluoxymesterone (IS) 337-317; [2] hydrocortisone 363-121; [3] prednisolone 361-343; [4] 20β-dihydroprednisolone 363-267; [5] dexamethasone 393-373; [6] triamcinolone acetonide 435-315; [7] prednylidene 373-121; [8] methylprednisolone 375-161; [9] triamcinolone 395-375; [10] fludrocortisone (1) 381-181; [11] flumethasone 411-253; [12] beclomethasone 409-391; [13] isoflupredone 379-359; [14] fludrocortisone (2) 381-361. For analytical details see text.