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Journal of Chromatography B, 702 (1997) 33–39

JOURNAL OF CHROMATOGRAPHY B

Improved determination of urinary cortisol and cortisone, or corticosterone and 11-dehydrocorticosterone by high-performance liquid chromatography with ultraviolet absorbance detection

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Received 25 March 1997; received in revised form 26 May 1997; accepted 4 July 1997

Abstract

A sensitive assay was developed for the determination of low levels of free (unconjugated) glucocorticoids in human, swine (cortisol and cortisone) and rat urine (corticosterone and 11-dehydrocorticosterone), using solid-phase extraction and HPLC with UV absorbance detection (254 nm). Precise quantitation is allowed by the use of internal standards (dexamethasone for swine urine and Reichstein's substance S for rat urine). This simple method allows the use of small urine samples (less than 2 ml), and is suitable for a wide range of applications in human and animal clinical and physiological studies. © 1997 Elsevier Science B.V.

Keywords: Cortisol; Cortisone; Corticosterone; 11-Dehydrocorticosterone

1. Introduction

Since the end of the 1950s, urinary free (i.e., unconjugated) cortisol level is considered as a good biochemical marker of the hypothalamic–pituitary–adrenocortical axis (HPA axis) activity. It is indeed correlated with the free (active) fraction of plasma cortisol [1–3] and the cortisol production rate [4]. For these reasons, the measurement of urinary cortisol is widely used for the assessment of Cushing's syndrome disease [5,6] and, since recently and coupled with a dexamethasone suppression test, for the assessment of HPA axis function in major depression [7,8].

Radioimmunoassay (RIA) and competitive protein-binding assay are often used as methods of choice for the dosage of cortisol. However, it is also well known that these methods, though very sensitive, suffer from lack of specificity, which may become crucial in such a complex biological matrix as urine [9–11]. This led to the use of preliminary chromatographic or high-performance liquid chromatography (HPLC) separation before RIA quantitation [9,12], which increases analysis time and cost.

HPLC separation of steroid hormones with ultraviolet (UV) absorbance and fluorescence detection have also been widely used [13]. These methods allow a very specific determination of urinary cortisol, provided a good sample preparation method and chromatography procedure are used [14]. However, they suffer from a lack of sensitivity compared to RIA techniques [13], which leads to the use of

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0378-4347/97/$17.00
PII S0378-4347(97)00361-7

large (2–20 ml) volumes of urine for the assays [14–17]. Other procedures including liquid chromatography–mass spectrometry (LC–MS) [18,19] or gas chromatography–mass spectrometry (GC–MS) [15] have also be developed but these techniques still remain very expensive for routine use.

This paper describes an optimized method for the simultaneous UV detection of urinary cortisol and cortisone or of corticosterone and 11-dehydrocorticosterone according to the species under study. An efficient and simple solid-phase extraction (SPE) procedure, was developed and allowed a sensitive determination of glucocorticoids in small urine samples. This method was first developed using swine urine, where it was expected to find less glucocorticoids than in human urine, since plasma concentrations of cortisol are much lower in pigs than in humans: 20–40 vs. about 100 ng/ml [20–23]. It was further used for rat urine, for which we also expected levels lower than those measured in humans, since mean plasma concentration of corticosterone, the natural endogenous equivalent of cortisol in rodents, is about 40–50 ng/ml [24].

2. Experimental

2.1. Chemicals and reagents

Cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione or F), cortisone (17α,21-dihydroxy-4-pregnene-3,11,20-trione or E), corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione or B), 11-dehydrocorticosterone (4-pregnen-21-ol-3,11,20-trione or A), dexamethasone (9α-fluoro-16α-methyl-prednisolone or D), Reichstein's "Substance S" (11-desoxy-17-hydroxycorticosterone or S) and acetonitrile HPLC-grade were obtained from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Methanol for HPLC was obtained from BDH Laboratory Supplies (Poole, UK). HPLC grade water was produced by a Milli-Q plus system (Millipore, Saint-Quentin-Yvelines, France).

2.2. Chromatography

The mobile phase [water–acetonitrile (76:24, v/v) for F and E assay and (73.5:26.5, v/v) for B and A assay] was delivered at 0.5 ml/min by a pump (Model LC-10AS, Schimadzu, Kyoto, Japan) to an automatic sampling auto-injector (Model 231 XL Gilson, Villiers-Le-Bel, France). The analytical column (Nucleosil 120-C_{18}, 5 μm, 100×3.2 mm I.D.) was furnished by Cluzeau-Info-Labo (Sainte-Foix-La-Grande, France). The column effluent was monitored at 254 nm with a Shimadzu UV spectrophometric detector (Model SPD-6A). Detector output was recorded by a data processor for chromatographs (Chromatopac C-R6A, Shimadzu). Attenuation was set at 3 (2^3).

2.3. Sample collection

Swine urine was collected from fifteen lactating multiparous Large White sows housed in stalls. Spontaneously voided urine was collected in a flask. It was then acidified using 6 *M* HCl (1% of urine volume) and frozen at −80°C.

Rat urine was collected during three consecutive days from four male Brown Norway (BN) and four male Fischer 344 (F344) rats placed in metabolic cages, after one week of acclimatization to this housing system [25]. A flask containing 0.2 ml 6 *M* HCl (i.e., about 1 to 2% of 24 h urine volume) was used to collect the urine during 24 h. Urine samples were then frozen at −80°C.

2.4. Urine analysis

Creatinine levels in swine urine were determined using a colorimetric quantitative reaction (Procedure 500, Sigma Diagnostics, Saint-Quentin-Fallavier, France). This method is based on the destruction of the color derived from the reaction between creatinine and alkaline picrate (Jaffe's reaction) when the mixture is acidified. Thus, the difference in color intensity measured at 500 nm before and after acidification of the mixture is proportional to creatinine concentration.

2.4.1. Extraction procedure

Urine was centrifuged for 30 min at 4000 *g*. It was then filtered using a single use filter unit (0.22 μm). The urine volume for each assay was adjusted according to its dilution, i.e., according to creatinine concentration for swine urine and to diuresis for rat

urine. Thus, between 0.75 and 3 ml of filtered swine urine was used per assay whereas for rat urine, the volume was set between 0.5 and 1.5 ml. The volume was then adjusted to 4 ml with water. Dexamethasone (30 ng) or substance S (40 ng) was added to each tube as internal standards (I.S.s) for swine and rat urine, respectively. Indeed, dexamethasone was inappropriate as I.S. for rat urine since its retention time was similar to that of A.

Two more tubes ("tests") containing 4 ml water received 30 μl of a pool standard containing F, E and D (swine urine assay) or 40 μl of a pool standard containing A, B and S (rat urine assay), each standard concentration being at 1 μg/ml in the pool.

For the SPE procedure, Isolute cartridges (Monofunctional silane MFC18 200 mg/10 ml IST, Mid Glamorgan, UK) were mounted on a VacMaster-20 sample processing station (IST). The vacuum applied to the cartridge outlet was adjusted to obtain a flow-rate of about 20 drops/min. The extraction procedure consisted of the following steps: (i) solvation of the columns: 2×3 ml absolute ethanol; (ii) column preequilibration: 2×3 ml water, (iii) transfer of the content of the tubes to the cartridges, (iv) washing with 3×3 ml water, then 3 ml of 0.01 *M* NaOH–methanol [(70:30, v/v) for swine urine, (65:35, v/v) for rat urine], 2×3 ml water and 3 ml of 0.01 *M* HCl–methanol [(70:30, v/v) for swine urine, (65:35, v/v) for rat urine], (v) elution from the cartridges with 3 ml absolute ethanol into 5 ml glass tubes.

Eluates were then evaporated in a concentrator–evaporator (Model RC 1010, Jouan, Saint-Herblain, France) connected to a vacuum pump (RCT 90, Jouan). After complete evaporation, dried residues were dissolved in 150 μl of mobile phase, 100 μl of which were injected into the HPLC system. This procedure allowed the reutilization of the Isolute cartridges at least five times without any diminution of the recovery, provided an additional rinsing with absolute ethanol was carried out between two series.

2.5. Quantitation

For each test and sample assay, the ratio $R=H_X/H_{I.S.}$ was calculated, were H_X is the height of the peak of cortisol, cortisone, corticosterone or 11-dehydrocorticosterone and $H_{I.S.}$ is the height of the peak of the respective internal standard. Concentrations of each compound $[X]$ in urine were then calculated from the following equation:

$$[X] = Q_{I.S.} \times \frac{(R_{assay})}{(R_{test})} \times \frac{1}{V}$$

where $[X]$ is the concentration of the compound in the sample (ng/ml), $Q_{I.S.}$ is the quantity of internal standard put in each sample (30 ng or 40 ng), R_{test} is the ratio $H_X/H_{I.S.}$ in the test assays (mean of the two assays), R_{assay} is the ratio $H_X/H_{I.S.}$ in the sample assay and V is the volume of urine used in the assay (ml).

This calculation method allows to take into account differences in the recovery of the different compounds. Each sample was assayed in duplicate or in triplicate. The mean of these replicates was taken as the concentration in the urine sample.

3. Results

Typical chromatograms of standards, pig, human and rat urine are shown in Figs. 1 and 2.

3.1. Recovery

The recovery of each compound is listed in Table 1.

3.2. Precision of the method

The intra-assay and inter-assay coefficients of variation, determined in twelve replicates of the same pooled urine sample (1.2 ml), for F, E (swine urine), A and B (rat urine) were 7.4% and 10.6%, 5.5% and 10.9%, 7.1% and 11.3%, 7.9% and 12.1%, respectively.

3.3. Sensitivity

Limits of detection, defined by the capacity of the computing integrator to discriminate between the baseline noise and peaks, were approximately 1 ng in the 100 μl injected in the system for each compound, i.e., 1.5 ng in the 150 μl of re-dissolved dried residues. According to the losses (about 25%) during

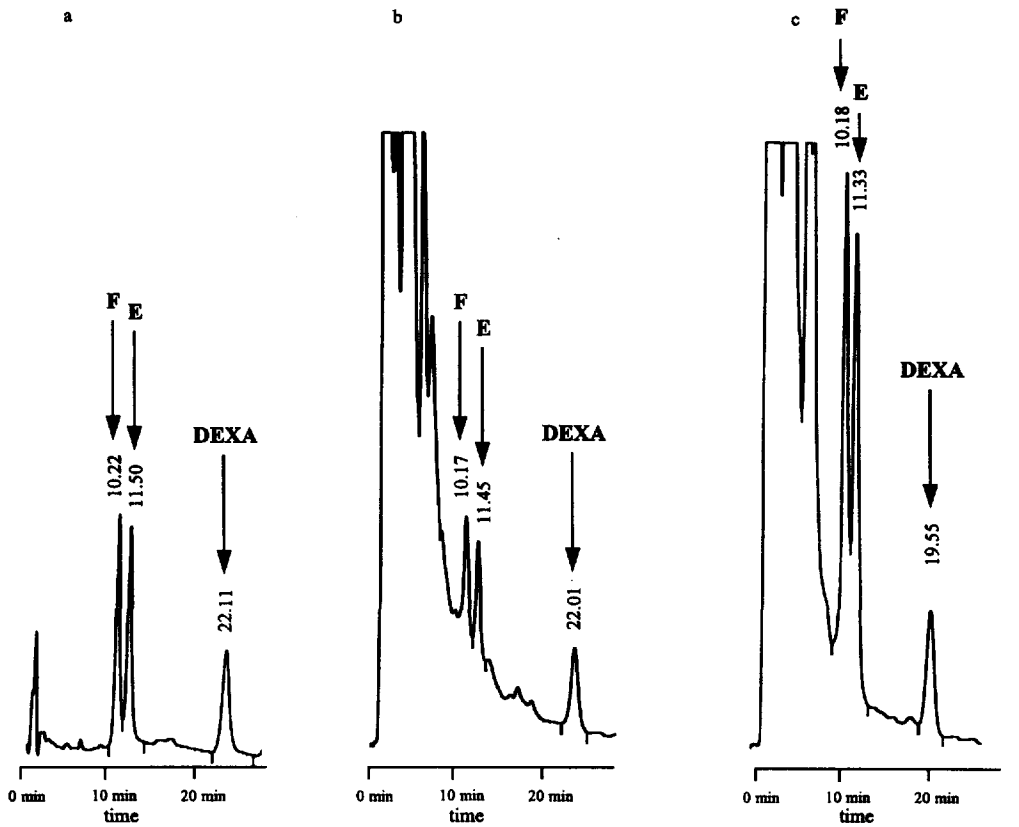

Fig. 1. Chromatogram of a standard pool (a) containing 20 ng of cortisol (F), cortisone (E) and dexamethasone (DEXA), with their respective retention times (top of the peaks). Typical chromatogram of a swine urine (b) containing 11.7 ng/ml of F and 12.0 ng/ml of E (determined from 1.2 ml urine sample added with 20 ng dexamethasone). Typical chromatogram of a human urine (c) containing 75.7 ng/ml of F and 94.7 ng/ml of E (determined from 0.75 ml urine sample added with 30 ng dexamethasone).

the extraction this corresponds to 2 ng present in the urine sample. The smallest concentrations we found for cortisol and cortisone were 1.7 and 3.8 ng/ml, respectively, using 2 or 2.5 ml of swine urine for the assay. In rat urine, the smallest concentrations were lower than 4 ng/ml for A and B, using urine volumes of less than 1.2 ml. For more diluted urine it remains still possible to use larger volumes.

3.4. Linearity

Linearity of the method was tested by adding known amounts of E and F to 0.5 ml swine urine and of A and B to 0.6 ml rat urine. Each point was done in duplicate. Figs. 3 and 4 show the recovery for E and F and for A and B, respectively, after correction with the internal standard recovery. It is clear from the Figures that the recovery and detection were linear over the range studied. Linear correlation coefficients (r^2) between added and recovered compounds were 0.998, 0.998, 0.998 and 0.997 for F, E, A and B, respectively.

3.5. Urine samples analysis

Forty swine urine samples were analysed as in Section 2.4. Mean and standard error of the mean (S.E.M.) cortisol and cortisone concentrations were 10.99 (1.34) and 11.06 (0.92) ng/ml, respectively. Minimum and maximum values were 2.57 and 22.71 ng/ml for cortisol and 3.09 and 18.64 ng/ml for cortisone. When expressed as a function of creatinine

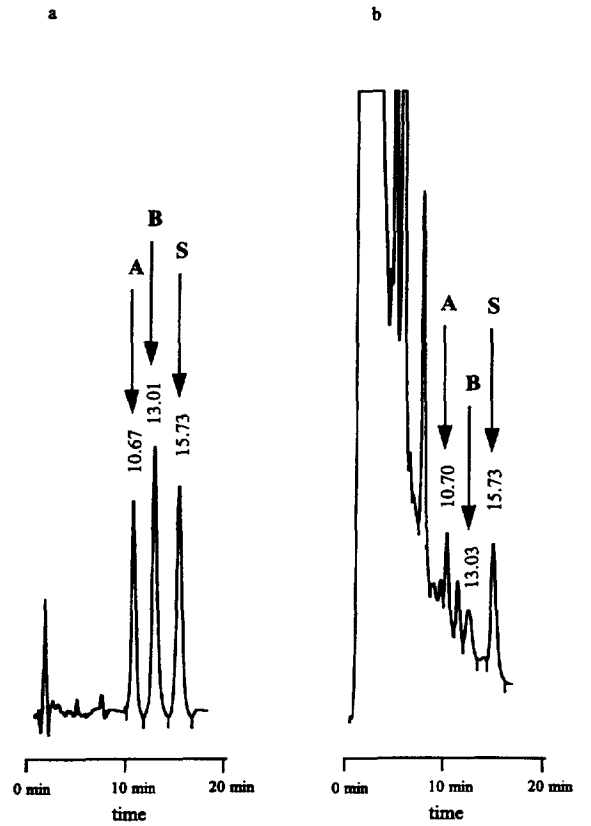

Fig. 2. Chromatogram of a standard pool (a) containing 40 ng of 11-dehydrocorticosterone (A), corticosterone (B) and substance S, with their respective retention times (top of the peaks). Typical chromatogram of a rat urine (b) containing 12.2 ng/ml of A and 5.5 ng/ml of B (determined from 1.1 ml urine sample added with 40 ng S substance).

excretion, mean (S.E.M.) cortisol and cortisone concentrations were 6.21 (1.07) and 7.11 (1.09) pg/μg creatinine, respectively. Minimum and maximum values were 20.68 and 41.93 pg/μg creatinine for cortisol and 8.30 and 18.80 pg/μg creatinine for cortisone.

Table 1
Mean recovery (%) of cortisol (F), cortisone (E), dexamethasone (DEXA), 11-dehydrocorticosterone (A), corticosterone (B) and substance S (S) after extraction procedures

$n=17$	F	E	DEXA	A	B	S
Mean recovery	83.0	79.3	83.5	64.3	75.3	71.9
S.E.M.	1.5	1.3	2.2	1.6	1.9	1.7

S.E.M.: standard error of the mean.

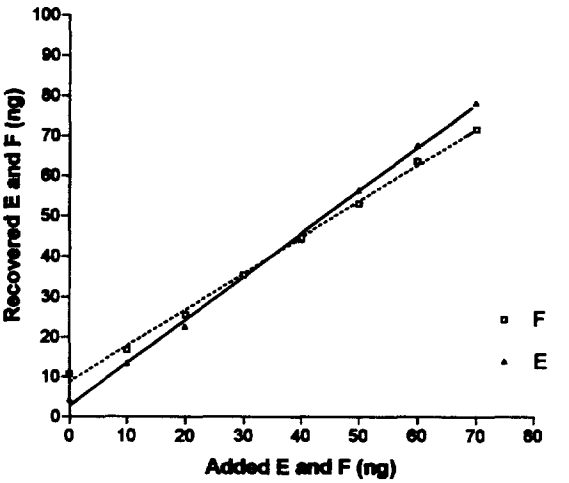

Fig. 3. Linearity of the extraction and detection procedure for cortisol (F) and cortisone (E). To 0.5 ml of swine urine were added 0 to 70 ng of E and F (duplicates). Recovery results are obtained after correction by internal standard recovery. For F regression curve, slope = 0.902 (±0.018); y-intercept = 8.79 (±0.76), $r^2 = 0.998$. For E regression curve, slope = 1.077 (±0.018); y-intercept = 2.66 (±0.76), $r^2 = 0.998$.

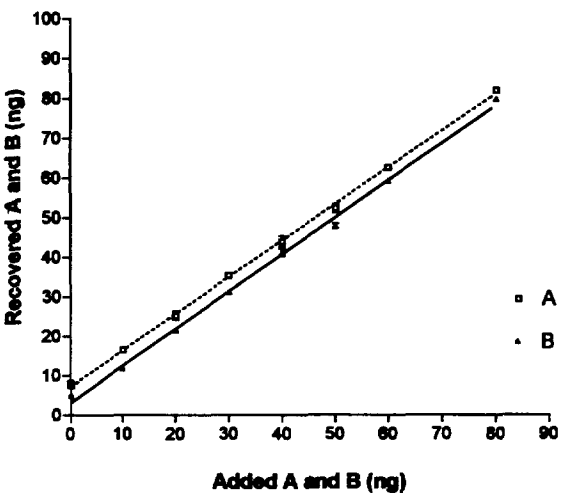

Fig. 4. Linearity of the extraction and detection procedure for corticosterone (B) and 11-dehydrocorticosterone (A). To 0.6 ml of rat urine were added 0 to 80 ng of E and F (duplicates). Recovery results are obtained after correction by internal standard recovery. For B regression curve, slope = 0.942 (±0.014); y-intercept = 3.185 (±0.605), $r^2 = 0.997$. For A regression curve, slope = 0.923 (±0.011); y-intercept = 7.18 (±0.48), $r^2 = 0.998$.

Table 2
Mean urinary concentrations (ng/ml) and mean 24-h urinary excretion (ng/24 h) of 11-dehydrocorticosterone (A) and corticosterone (B) in Brown Norway (BN) and Fischer (F344) rats during three consecutive days ($n=4$)

	BN			F344		
	Mean	S.E.M.	Range (min.–max.)	Mean	S.E.M.	Range (min.-max.)
A (ng/24 h)	91.72	10.94	43.17–190.64	162.95	23.32	54.69–350.81
B (ng/24 h)	67.02	4.26	42.42–92.82	35.01	4.43	17.78–64.77
A (ng/ml)	10.36	1.96	4.80–27.38	36.88	4.79	10.72–71.54
B (ng/ml)	7.55	1.24	4.37–19.41	8.07	1.20	4.19–20.20

S.E.M.: standard error of the mean.

Mean concentrations (ng/ml) and mean 24 h excretion of A and B in rat urine are shown in Table 2.

4. Discussion and conclusions

The procedure described above provides a simple, sensitive and specific determination of urinary free glucocorticoids. Thus very low concentrations can be determined (below 1 ng/ml), which competes with most RIA procedures [26–29] and allows the use of small urine volumes for the assay. Other advantages over RIA procedures include the lack of crossreactivity and the suppression of costs associated with the elimination of radioactive material.

Moreover, this procedure allows the simultaneous determination of both cortisol and cortisone, or corticosterone and 11-dehydrocorticosterone, which makes it suitable for a variety of clinical applications like the assessment of adrenocortical status in hypercorticism [5,30], depression [31] and other psychiatric disorders [32], or the diagnosis of the apparent mineralocorticoid excess syndrome [33]. For these applications, it compares favourably with GC–MS procedures [33], offering a simpler and less expensive alternative.

It is also suitable for the study of HPA axis activity under basal and stress conditions in a variety of species including laboratory, wild and farm animals, [25,27,34–37], since urine, as opposed to plasma, can be collected without any disturbance of the animals and artefactual activation of HPA axis activity.

References

[1] C.L. Cope, E.G. Black, Br. Med. J. 2 (1959) 1117.
[2] W.R. Beisel, J.J. Cos, R. Horton, P.Y. Chao, P.H. Forsham, J. Clin. Endocrinol. 24 (1964) 887.
[3] C.W. Burke, C.G. Beardwell, Q. J. Med. XLII (1973) 175.
[4] R.E. Juselius, F.M. Kenny, Metabolism 23 (1974) 847.
[5] H. Fujita, K. Hata, E. Ogata, I. Kojima, Endocrinol. Jpn. 38 (1991) 441.
[6] J. Köbberling, A. Von zur Mühlen, J. Clin. Endocrinol. Metab. 38 (1974) 313.
[7] S.I. Tomitaka, K. Sakamoto, A. Tamura, I. Kojima, H. Fujita, Biol. Psychiatry 39 (1996) 220.
[8] M. Maes, C. Vandervorst, E. Suy, B. Minner, J. Raus, Acta Psychiatr. Scand. 83 (1991) 480.
[9] C. Franck, M.C. Patricot, B. Mathian, A. Revol, Ann. Biol. Clin. 42 (1984) 221.
[10] A.W. Meikle, H. Takiguchi, S. Mizutani, F.H. Tyler, C.D. West, J. Lab. Clin. Med. 74 (1969) 803.
[11] B.E.P. Murphy, L.M. Okouneff, G.P. Klein, J. Clin. Endocrinol. Metab. 53 (1981) 91.
[12] M. Schöneshöfer, A. Fenner, G. Altinok, H.J. Dulce, Clin. Chim. Acta 106 (1980) 63.
[13] P. Volin, J. Chromatogr. B 671 (1995) 319.
[14] D.A. Jenner, J. Richards, J. Pharm. Biomed. Anal. 3 (1985) 251.
[15] C.H.L. Shackleton, J.O. Whitney, Clin. Chim. Acta 107 (1980) 231.
[16] M.C. Patricot, H. Fraass, B. Mathian, A. Revol, Ann. Biol. Clin. 46 (1988) 177.
[17] B. Dingeon, M.T. Quenard, H. Tome, Ann. Biol. Clin. 46 (1988) 263.
[18] S.-J. Park, Y.-J. Kim, H.-S. Pyo, J. Park, J. Anal. Toxicol. 14 (1990) 102.
[19] A.L. Yergey, Y. Teffera, N.V. Esteban, F.P. Abramson, Steroids 60 (1995) 295.
[20] J.L. Barnett, P.H. Hemsworth, G.M. Cronin, Gen. Comp. Endocrinol. 47 (1982) 516.
[21] B.A. Becker, R.K. Christenson, J.J. Ford, J.A. Nienaber, J.A. DeShazer, G.L. Hahn, Physiol. Behav. 45 (1989) 1171.
[22] M. Follenius, G. Brandenberger, B. Hietter, J. Clin. Endocrinol. Metab. 55 (1982) 757.

[23] K. Tanaka, N. Shimizu, H. Imura, J. Fukata, I. Hibi, T. Tanaka, S. Nakagawa, K. Fujieda, K. Takebe, Endocrinol. J. 40 (1993) 571.
[24] M.F. Dallman, S.F. Akana, K.A. Scribner, M.J. Bradbury, C.D. Walker, A.M. Strack, C.S. Cascio, J. Neuroendocrinol. 4 (1992) 517.
[25] E.P. Gomez-Sanchez, C.E. Gomez-Sanchez, Steroids 56 (1991) 451.
[26] L.H. Graham, J.L. Brown, Zoo Biol. 15 (1996) 71.
[27] K. Carlstead, J.L. Brown, S.L. Monfort, R. Killens, D. Wildt, Zoo Biol. 11 (1992) 165.
[28] H.J. Ruder, R.L. Guy, M.B. Lipsett, J. Clin. Endocrinol. Metab. 35 (1972) 219.
[29] K. Nahoul, M.C. Patricot, J.-P. Moatti, A. Revol, J. Steroid Biochem. Mol. Biol. 43 (1992) 573.
[30] R.E. Allin, J.H. McBride, D.O. Rodgerson, Clin. Chim. Acta 143 (1984) 17.
[31] A.P. Zis, R.A. Remick, B.E.K. Grany, M. Bernstein, A. Grant, Arch. Gen. Psychiat. 44 (1987) 919.
[32] S.I. Tomitaka, K. Sakamoto, I. Kojima, H. Fujita, Biol. Psychiatry 38 (1995) 128.
[33] M. Palermo, C.H.L. Shackleton, F. Mantero, P.M. Stewart, Clin. Endocrinol. 45 (1996) 605.
[34] H.K. Kley, L. Herberg, H.L. Krüskemper, J. Steroid Biochem. 7 (1976) 381.
[35] J.W. Mason, C.T. Harwood, N.R. Rosenthal, Am. J. Physiol. 190 (1957) 429.
[36] B. Beerda, M.B.H. Schilder, N.S.C.R.M. Janssen, J.A. Mol, Hormone Behav. 30 (1996) 272.
[37] D. Saltz, G.C. White, R.M. Bartmann, J. Wildlife Manage. 56 (1992) 640.