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ORIGINAL PAPER

Hair cortisol: a parameter of chronic stress? Insights from a radiometabolism study in guinea pigs

Karin Keckeis · Michael Lepschy · Hanna Schöpper · Lukas Moser · Josef Troxler · Rupert Palme

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Abstract Measurement of hair cortisol has become popular in the evaluation of chronic stress in various species. However, a sound validation is still missing. Therefore, deposition of radioactivity in hair and excretion into feces and urine after repeated injection of ^{3}H-cortisol was studied in guinea pigs ($n = 8$). Each animal was given intraperitoneally 243.6 kBq ^{3}H-cortisol/day on 3 successive days. After the first injection, all voided excreta were collected for 3 days. After the second injection, hair was shaved off the animals' back and newly grown hair was obtained on day 7. Following methanol extraction, radiolabeled and unlabeled glucocorticoid metabolites (GCM) in fecal and hair samples were characterized by high-performance liquid chromatography (HPLC) and enzyme immunoassays (EIA). In feces, maximum radioactivity was reached 8 h (median) post each injection, whereas maxima in urine were detected in the first samples (median 2.5 h). Metabolites excreted into feces (13.3 % ± 3.7) or urine (86.7 %) returned nearly to background levels. HPLC of fecal extracts showed minor variation between individuals and sexes. In hair, small amounts of radioactivity were present. However, two EIAs detected large amounts of unlabeled GCM, including high levels at the position of the cortisol standard; radioactivity was absent in this fraction, demonstrating that ^{3}H-cortisol was metabolized. Furthermore, large amounts of immunoreactivity coinciding with a radioactive peak at the elution position of cortisone were found. These results show for the first time that only small amounts of systemically administered radioactive glucocorticoids are deposited in hair of guinea pigs, while measurement of large amounts of unlabeled GCM strongly suggests local production of glucocorticoids in hair follicles.

Keywords Feces · Glucocorticoids · Guinea pig · Hair follicle · Non-invasive · Stress

Abbreviations

ACTH	Adrenocorticotropic hormone
CRH	Corticotropin releasing hormone
GC	Glucocorticoids
GCM	Glucocorticoid metabolites
EIA	Enzyme immunoassay
HPA axis	Hypothalamic–pituitary–adrenal axis
MSH	Melanocyte-stimulating hormone

K. Keckeis and M. Lepschy contributed equally to this work.

K. Keckeis (✉) · J. Troxler
Department for Farm Animals and Veterinary Public Health, Institute of Animal Husbandry and Animal Welfare, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria
e-mail: karin.keckeis@vetmeduni.ac.at; karin.keckeis@aon.at

J. Troxler
e-mail: josef.troxler@vetmeduni.ac.at

M. Lepschy · L. Moser · R. Palme
Department of Biomedical Sciences, Institute of Chemistry and Biochemistry, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria
e-mail: michael.lepschy@vetmeduni.ac.at

R. Palme
e-mail: rupert.palme@vetmeduni.ac.at

H. Schöpper
Department for Integrative Biology and Evolution, Research Institute of Wildlife Ecology, University of Veterinary Medicine Vienna, Savoyenstrasse 1, 1160 Vienna, Austria
e-mail: hanna.schoepper@vetmeduni.ac.at

H. Schöpper
Department of Pathobiology, Institute of Anatomy, Histology and Embryology, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

POMC	Proopiomelanocortin
RP-HPLC	Reversed-phase high-performance liquid chromatography

Introduction

Changes in the adrenocortical secretion of glucocorticoids (GC) upon activation of the hypothalamic–pituitary–adrenocortical (HPA) axis is a routinely used endocrine parameter of stress in mammals (Cook et al. 2000; Möstl and Palme 2002; Sapolsky et al. 2000; Wasser et al. 2000). Whereas the biological response to short-term stress features adaptational processes to reestablish homeostasis, the exposure to chronic stressors may elicit deleterious effects on an animal's health and well-being, including inhibition of reproduction and growth, immunosuppression and altered metabolism (Boonstra 2005; Elsasser et al. 2000; Moberg 2000; Wingfield et al. 1998).

The quantification of glucocorticoid metabolites (GCM) in feces has become widely accepted as a non-invasive method in a variety of disciplines (Lepschy et al. 2010; Millspaugh and Washburn 2004; Mormède et al. 2007; Sheriff et al. 2011; Touma and Palme 2005). Collection of fecal samples offers major advantages in terms of minimal disturbance of the animals during sampling and ease of collection. Moreover, results remain unaffected by GC secretion in response to acute stress induced by handling, capture and immobilization of the animals (Huber et al. 2003; Millspaugh et al. 2002; Möstl and Palme 2002; Wasser et al. 2000). Considering the multiple sources of variation in fecal GC measurement, including handling of samples, assay precision and biological effects along with interspecies differences in metabolism and excretion, the validation of this technique for any given species is crucial for reliable application and the interpretation of results (Millspaugh and Washburn 2004; Palme 2005; Sheriff et al. 2011; Touma and Palme 2005).

In the guinea pig, which frequently serves as model species in biomedicine, only limited information is available on GC metabolism, excretion and on non-invasive methods of monitoring adrenocortical activity. Recently, Bauer et al. (2008) reported the physiological validation of an enzyme immunoassay (EIA) for fecal GCM in guinea pigs, which was achieved by detecting a significant and time-delayed increase of fecal GCM concentrations after pharmacological stimulation (adrenocorticotropic hormone, ACTH) of the adrenal cortex. Their measurements did not reveal significant sex differences in GCM concentrations, but showed high inter-individual variation concerning the time delay of fecal peak concentration. This time delay is important to establish a proper sample collection protocol and to correlate changes in GC concentrations with the disturbance effects of interest (Palme 2005; Touma and Palme 2005). For example, Bauer et al.'s (2008) results have been used to help evaluate repeated stress stimuli during pregnancy in guinea pigs (Schöpper et al. 2011, 2012). GCM in feces represent a cumulative measure of GC release over a period of up to several hours (Touma and Palme 2005). However, monitoring hormone levels over longer periods of time requires a high number of fecal samples and entails continuous observation in field studies.

Hair has recently been recognized as a biomaterial that may accumulate GC hormones over weeks to months (Davenport et al. 2006; Macbeth et al. 2010). Similarly, it has long been known from forensic science literature that various steroids misused to promote growth and enhance performance of animals in meat production or in sports are detectable in hair (e.g., Anielski 2008; Boyer et al. 2007; Rambaud et al. 2005). Steroids, like many drugs, are incorporated into the hair shaft during the growing phase of the hair follicles (Cone 1996; Pragst and Balikova 2006), and therefore, GC levels in hair are thought to reflect average systemic levels over the respective growth phase by integrating baseline levels and elevated adrenocortical secretion. Consequently, the analysis of GC in hair is a potentially useful technique. It is increasingly applied to evaluate chronic exposure to various stressors or potentially stressful conditions (Accorsi et al. 2008; Comin et al. 2011; Davenport et al. 2006; Koren et al. 2002; Macbeth et al. 2010). The benefits of hair cortisol analysis are numerous, including non-invasive collection, simple handling of samples, no particular shipping and storage logistics as well as enabling retrospective analysis of endogenous cortisol exposure (Bechshøft et al. 2011; Gow et al. 2010). Transiently experienced stress and increased GC levels caused by individualization, restraint or live trapping will not be reflected in hair GC levels of the current sample but, if at all, may be relevant for samples of re-grown hair (Sheriff et al. 2011). Then again, minimally intrusive sampling techniques, as recently described by Henry and Russello (2011), may prove useful, particularly in field studies, to avoid handling, trapping, disturbing, or even observing the animals. Nevertheless, to date, there have only been a few studies addressing the issue of biological relevance of hair cortisol measurements by linking them to (systemic) GC levels measured in more established sample matrices such as saliva, feces and urine (Accorsi et al. 2008; Davenport et al. 2006; Sauvé et al. 2007). In addition, the biological validity of this technique, i.e., to evaluate if biologically meaningful changes induced by a known long-term stressor can be detected in a series of samples across time, has not yet been demonstrated. Moreover, only limited work has been done into the factors associated with variations in GC measurements (Bechshøft et al. 2011; Davenport et al. 2006;

Macbeth et al. 2010) and, consequently, if these factors are not accounted for, the results may be confounded. External contamination of hair with GC containing excreta and secretions (feces, urine, saliva, sweat, sebum) or even blood will lead to a false-positive result. Therefore, an effective and reliable decontamination protocol is essential and requires selection of an adequate wash solvent. The particular challenge is to use a solvent for GC which does not penetrate too deeply into the hair shaft, so as to prevent extraction from the interior (Davenport et al. 2006).

Another issue in hair GC analysis is the potential of epidermal cells not only to metabolize steroid hormones, but also to synthesize steroids (Bamberg et al. 2005; Taves et al. 2011; Thiboutot et al. 2003); a function typically associated with classic steroidogenic tissues (adrenal gland, ovary and testis). While sebocytes, which are sebum-producing epithelial cells most commonly found in association with hair follicles (Schneider and Paus 2010), and various other skin cell types express key steroidogenic enzymes (Slominski et al. 2004; Thiboutot et al. 2003), mammalian skin and hair follicle (keratinocytes) have been proven to synthesize glucocorticoids in situ (Ito et al. 2005; Slominski et al. 2000a). It is not known whether the cortisol produced in these cells/tissues is incorporated into the hair and, if so, to what extent it competes with GC entering the hair by diffusion from the circulation as a result of systemic HPA activity.

The aims of this experiment were, first, to substantiate information on the metabolism and excretion of GCM in guinea pigs, which enables researchers to improve future non-invasive studies utilizing the measurement of fecal GCM in this species. For this purpose, we monitored the time course of radioactivity excreted in feces and urine after multiple injections of radiolabeled ^{3}H-cortisol and used HPLC and two group-specific EIAs to characterize the metabolites excreted in the feces. Second, the radiometabolism study was designed to detect and discriminate between ^{3}H-cortisol metabolites potentially incorporated into growing hair from metabolites deposited on the hair surface by sweat and sebum or through contamination with feces or urine. As opposed to other laboratory rodents, the guinea pig is an appropriate species as it exhibits an unsynchronized hair growth cycle, which ensures some follicles to be in the anagen (growing) phase at any time (Militzer 1982). Finally, if GC were incorporated into the hair, then the intraperitoneal administration of radiolabeled cortisol would allow us to differentiate between systemic cortisol metabolites and those produced in the hair follicle.

Materials and methods

The experiment has been approved by the Animal Ethics Committee of the University of Veterinary Medicine Vienna and the Austrian Federal Ministry for Science and Research (BMWF-68.205/0248-II/10b/2009).

Animals and housing conditions

In this study we used eight sexually mature (4 female, 4 male) guinea pigs (*Cavia aperea f. porcellus*) from a heterogeneous multicolored stock. The animals, selected from same-sex groups of 3 or 4 individuals, had a body weight of 735 ± 39 g and were of similar age (minimum 4.2 and maximum 4.5 months). Prior to the experiment, the animals had been moved from group housing to individual cages (size 50 × 50 × 44 cm) that were equipped with absorbent paper covering the bottom and edges. During an 11-day habituation phase as well as during the experiment each animal was fed 15 g of pelletized guinea pig standard diet (Altromin 3013, Altromin GmbH&Co KG, Lage, Germany) and 40 g of fruit and vegetables daily. Hay and bottled tap water were available ad libitum. Housing was managed under a natural light–dark cycle (dawn at 6:30 h, dusk at 17:45 h), at an average room temperature and relative humidity of 21.9 ± 0.3 °C and 35.6 ± 2.1 %, respectively.

Administration of radiolabeled cortisol and sampling of hair, urine and feces

On day 1 of the experiment (10:00 h) 243.6 kBq (6.58 µCi) ^{3}H-cortisol (1,2,6,7-[^{3}H]-hydrocortisone, specific activity 2.6788 TBq/mmol, PerkinElmer, Boston, MA, USA), the major GC in the guinea pig (Malinowska and Nathanielsz 1974), diluted in 500 µl sterile isotonic saline solution was administered intraperitoneally to each animal. The injection was repeated at the same time of day on each of the two following days (day 2 and 3; Fig. 1). After the second injection, hair was shaved off very close to the skin over an area measuring approximately 4.5 × 6 cm on the animals' back, using an electric clipper (Andis®, Ultraedge blade size 50 SS, 0.2 mm cutting depth). On day 7 newly grown hair from the same patches was obtained by clipping again to the level of the skin. During the first 12 h after each injection, all urine voided and absorbed by the paper in the cages was removed and absorbent paper was immediately renewed during each sampling (every 30 min). The wet parts of the paper were cut out, air-dried and stored at −18 °C in plastic pouches. On day 1, all feces voided was collected at 4, 6, 8, 10 and 12 h post-injection followed by 1-h intervals during the next 12 h right until injection on day 2. Subsequently, sampling was performed in 2-h intervals during the first 12 h on day 2 and 3 continued by a pooled sample for feces voided during the second half of day 2 and 3. Fecal pellets voided during the nights of day 2 and 3 were pooled and

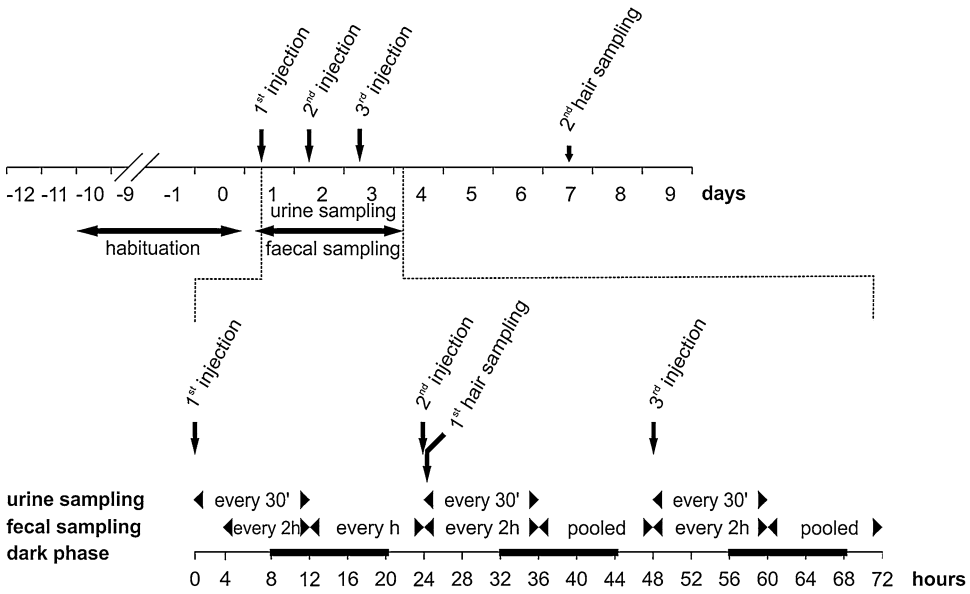


Fig. 1 Time schedule of the experiment

assigned to 3 different time slots according to their moisture level, which was roughly determined by assessing the surface (texture) and handling. All fecal pellets were frozen at −18 °C within 15 min and after collection of pooled samples, respectively, for storage. For determination of possible background radioactivity hair, urine and fecal samples of each animal were taken prior to the first injection of ^{3}H-cortisol.

Extraction and determination of ^{3}H-GCM

Urine samples

The dried urine-soaked paper was cut into small pieces (approximately 2 × 2 cm) and mixed with 20 ml of 60 % methanol. The vials were vortexed for approximately 12 h, and 1 ml aliquots (in duplicates) of the solution were filled into scintillation vials, each mixed with 12 ml scintillation fluid (Quicksafe A, No. 100800, Zinsser Analytic, Maidenhead, UK). Radioactivity was measured in a liquid scintillation counter (Tri-Carb 2100TR, Packard Instruments, Meriden, CT, US) for 5 min while simultaneously running a quench compensation program. Given the difficult implementation of urinary GCM measurement in non-invasive studies, we refrained from characterization of GCM in urine samples.

Fecal samples

For determination of the amount of radioactivity excreted into the feces, the samples were dried in a cabinet dryer at 80 °C for approximately 3 h. Thereafter, each sample was homogenized, weighed and an aliquot of 0.1 g was extracted with 2 ml of 80 % methanol on a vortex (Vortex Evaporator, Labconco, Kansas City, USA). After centrifugation (10 min at 2,500*g*), 0.25 ml aliquots (duplicates) of the supernatant (mixed with 6 ml scintillation fluid) were measured in the liquid scintillation counter as described above.

Background radioactivity was accounted for, and the amount of radioactivity measured in fecal samples was multiplied by the total weight of each sample. The total recovery of radioactivity per animal was calculated as the ratio of the sum of recovered radioactivity in all urine and feces collected to the total amount of radioactivity administered during the whole experiment.

Wash procedure and extraction of GCM in hair samples

All hair samples (19–95 mg/animal) were stored in plastic pouches at −20 °C until analysis. The samples were washed two times (10 min per wash) with 5 ml 100 % *n*-hexane and afterwards two times with 5 ml 100 % methanol using a vortex (Thermomixer, stage 5 of 10, room temperature; Eppendorf, Germany). The *n*-hexane supernatants and the methanol supernatants, respectively, of each wash were combined, evaporated to dryness and reconstituted with 1 ml of 100 % methanol for further measurements. A 500 µl aliquot was then mixed with 6 ml scintillation fluid and measured (30 min) in a liquid scintillation counter as described before. For extraction, washed hair samples were immersed with 5 ml 100 % methanol and incubated at room temperature for 24 h with gentle rotation. After centrifugation (15 min at 2,500*g*) the methanol supernatant was removed, evaporated to dryness and reconstituted with 1 ml of 100 % methanol. A 20 µl aliquot of each sample was measured in the liquid scintillation counter.

Following extraction and removal of the methanol supernatant, the hair samples were moistened with 100 µl water for 20 min and solubilized for 24 h with 1 ml of Biolute S (No. 1310200, Zinsser Analytic, Maidenhead, UK). The samples were then neutralized with 1 ml 1 M HCl and 18 ml scintillation fluid for measurement of radioactivity (30 min).

Characterisation of radioactive metabolites in fecal and hair samples

For the determination of the amount of conjugated and non-conjugated metabolites in feces, a solvent extraction using diethyl ether was performed. For this purpose, 0.5 ml of the supernatant remaining from the preceding extraction of each of 40 fecal samples (5 of each animal) was concentrated by evaporation. The residue was diluted with its own volume of water and the solution was extracted with 3 ml of diethyl ether as described by Teskey-Gerstl et al. (2000) and Schatz and Palme (2001). Radioactivity of both the organic phase (non-conjugated steroids) dried down and reconstituted in methanol, and the aqueous phase (conjugated steroids) was measured as described earlier.

On the basis of the results of the solvent extraction, reversed-phase HPLC was performed to further characterize ^{3}H-GCM excreted in the feces of guinea pigs. Extracts of samples of 4 randomly chosen animals (2 of each sex) were subjected to a Novapak C18 column (3.9 × 150 mm, Millipore Corporation, Milford, MA, USA) with a Mini-Guard column (C18 Guard-Pak TM, WAT085825, Waters Corp., UK) used at room temperature with the following gradient: 50 % methanol over 5 min, and 50–75 % methanol over 30 min. The flow rate was 1 ml per min and three fractions per minute were collected (cf. Teskey-Gerstl et al. 2000). Additionally, elution positions of cortisol, cortisone and corticosterone were determined in the HPLC system to allow comparison with the elution profile of the ^{3}H-GCM (all steroids were obtained from Steraloids, Wilton, NH, USA). The presence of radioactivity in the HPLC fractions was measured by counting an aliquot of each fraction (50 µl of an 1:5 dilution with assay buffer) in a liquid scintillation counter (TopCount, Packard Instruments, Meriden, CT, USA) after adding 200 µl of scintillation fluid (Microscint PS, Art. No. 6013631, Packard Instruments, Meriden, CT, USA) to each well. Immunoreactivity of the fractions was determined in a cortisol EIA (Palme and Möstl 1997) and an 11-oxoetiocholanolone EIA measuring GCM with a 5β-3α-ol-11-one structure (Möstl et al. 2002). Characteristics of these EIAs including the respective antibody cross-reactivities with relevant steroids are provided in Möstl et al. (2002) and Palme and Möstl (1997).

Based on the low radioactivity measured in the individual hair samples (see the following section), the methanol extracts of all individuals were pooled. The extract was evaporated to dryness and reconstituted with 100 µl of 40 % methanol. Subsequently, the extract was subjected to reversed-phase HPLC (cf. Teskey-Gerstl et al. 2000 and above). However, in variance to that, a linear gradient of 40–60 % methanol was used for separating the steroids. The presence of radioactivity in the HPLC fractions was measured by counting a 300 µl aliquot of each fraction mixed with 6 ml scintillation fluid for 30 min in a liquid scintillation counter. A 2 µl aliquot of each fraction was used to determine immunoreactivity with a cortisone EIA and a cortisol EIA as described by Rettenbacher et al. (2004) and Palme and Möstl (1997), respectively.

Results

Recovery and time course of fecal and urinary excretion of GCM

The overall amount of radioactivity recovered from both urine and feces after multiple ^{3}H-cortisol injections was 88.1 ± 9.15 %. Only a small proportion thereof (13.3 % ± 3.7) could be recovered in the feces, whereas most of the radioactivity (86.7 % ± 3.7) was found in the urine.

Peak radioactivity in urine was repeatedly detected in the first samples (median = 2, 2.5 and 2 on day 1, 2 and 3, respectively; overall range = 1–5) corresponding to 2.5 h (median; ranging from 1.5 to 8 h) after the injection. In feces maximum radioactivity after each injection was reached later. Here a delay of 8 h (median; ranging from 6 to 12 h) for peak concentrations was observed. Excretion into the feces did not show a marked accumulation of radioactive metabolites in the course of the multiple-dose administration of ^{3}H-cortisol (for 3 consecutive days; Fig. 2). Radioactive metabolites almost reached background levels within 24 h of any of the injections.

No significant differences between sexes could be found concerning the recovery (overall recovery, t test, $n_{\text{males}} = n_{\text{females}} = 4$; $^{**}P = 0.843$), the route (urine vs. feces, t test, $n_{\text{males}} = n_{\text{females}} = 4$; $P = 0.522$) and peak excretion after each injection, neither in urine nor in feces (t test, $n_{\text{males}} = n_{\text{females}} = 4$; $0.097 < P < 1$).

Characterization of metabolites in fecal samples

Ethyl ether extraction

The proportion of conjugated metabolites found in fecal samples near peak radioactivity was only small (23.0 ± 6.0 %) compared to unconjugated metabolites extractable with diethyl ether (77.0 ± 6.0 %).

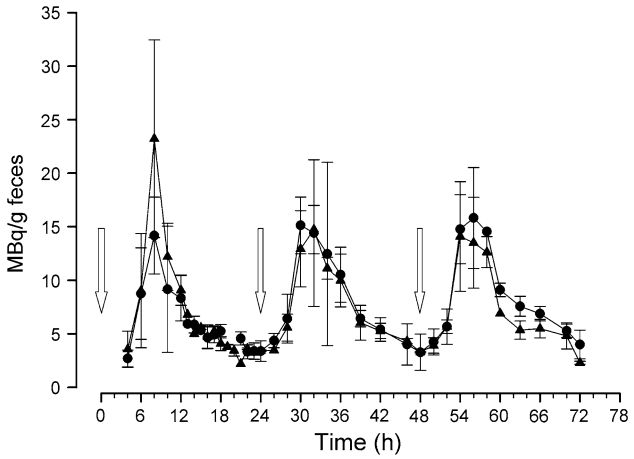


Fig. 2 Time course of excreted ^{3}H-cortisol metabolites (mean ± SD) in feces of male (*circles*) and female (*triangles*) guinea pigs after repeated i.p. administration of ^{3}H-cortisol. The *arrows* mark the injection times (10:00 h)

HPLC

HPLC-immunograms of fecal samples showed only minor variation between individuals and sexes (Fig. 3). One major radioactive peak co-eluting with the corticosterone standard (fraction 29) was present. Both EIAs showed no immunoreactivity at this position. Furthermore, a minor radioactive and a dominant immunoreactive peak (cortisol EIA) could be detected eluting around fraction 19 at the elution position of the cortisol standard. The 11-oxoetiocholanolone assay detected one major peak around fraction 53 co-eluting with a radioactive peak. Two additional but rather small peaks (eluting around fractions 24 and 46) were detected by the same assay.

Characterization of metabolites in hair samples

Wash procedure and extraction efficiency

All samples in this study were free of visible contamination. The *n*-hexane and methanol supernatants of both washes contained no radioactivity at all. In the pooled methanol extracts 11.68 Bq were measured. In the pooled solubilized hair samples, in total, another 13.0 Bq were detected. Thus, taking the combined sum of the methanol extracts and the solubilized samples, the methanol extraction procedure provides a recovery of 47.4 %.

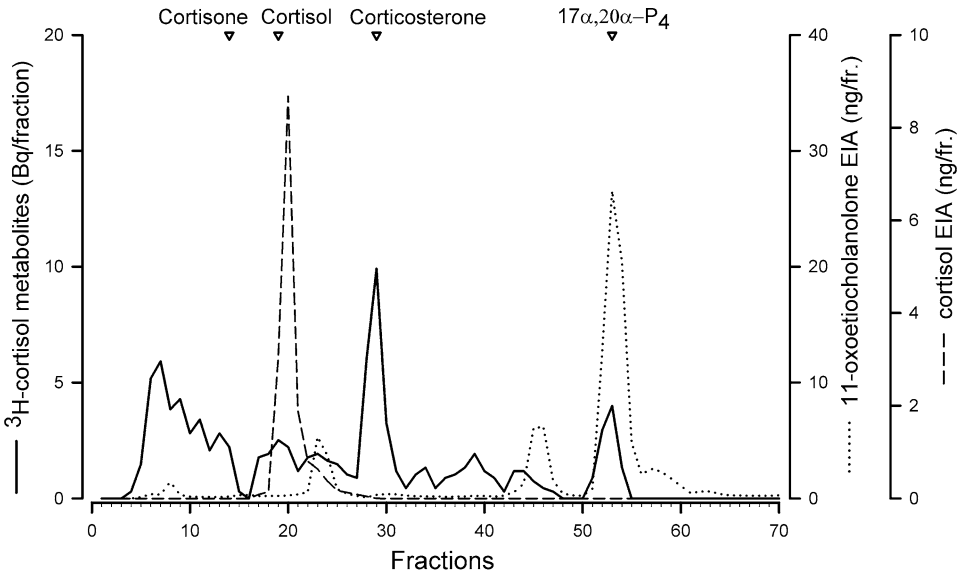
HPLC immunogram

To characterize ^{3}H-cortisol metabolites in the hair, all of the individual samples from day 7 were pooled due to very low radioactivity (11.68 Bq in total) and subjected to HPLC (see "Materials and methods"). The HPLC separation revealed four dominant peaks of radioactivity (Fig. 4). Two rather polar metabolites eluted in fractions 6 and 12. The third radioactive peak eluted around the position of the cortisone standard (fraction 49). The fourth was detected between cortisone and cortisol standards (fraction 58).

Both the cortisol EIA and the cortisone EIA revealed immunoreactive metabolites in several HPLC fractions (Fig. 3). Both assays showed major immunoreactivity around fraction 6 and 49 each coinciding with a radioactive peak. Immunoreactivity was also present in fraction 45. Furthermore, the cortisol EIA showed expressed immunoreactivity around fraction 61, which corresponds with the position of the cortisol standard. Additionally, the cortisone assay showed two dominant peaks: one of them being rather polar (fraction 8) and the other being rather apolar, co-eluting with the corticosterone standard (peak in fraction 89).

Discussion

The present radiometabolism study is characterized by a high recovery rate of radioactivity (88 %), which is comparable to experiments in other species (Lepschy et al. 2007; Teskey-Gerstl et al. 2000; Touma et al. 2003). This may be attributed to a minimum loss of feces and urine due to a frequent and efficient sampling schedule. Moreover, the recovery rate demonstrates that the methanolic extraction procedure modified from Bauer et al. (2008) is adequate for cortisol metabolites from feces of guinea pigs. Several studies have shown that the route and time lag of excretion of GCM in the feces varies greatly between species (reviewed in Palme et al. 2005). We found that GCM were mainly excreted via the urine (>85 %), which is similarly reported in the squirrel, in the hare and in larger mammals such as dogs, pigs and sheep (Bosson et al. 2009; Dantzer et al. 2011; Palme et al. 1996; Schatz and Palme 2001; Teskey-Gerstl et al. 2000), whereas in other laboratory rodents (like rats and mice) or carnivores (like cats and mink) most of the metabolites were excreted in the feces (Lepschy et al. 2007; Malmkvist et al. 2011; Schatz and Palme 2001; Touma et al. 2003). Our findings on fecal elimination of cortisol metabolites indicate peak concentrations after a time lag of about 8 h post-injection and a near-complete decrease to baseline between the injections and, hence, are at variance with the time course shown by Bauer et al. (2008). In an experiment to physiologically validate a group-specific EIA for monitoring adrenocortical activity in feces of guinea pigs, the authors observed peak concentrations of cortisol metabolites approximately 18 h after the challenge. They used a subcutaneous injection of a sustained release ACTH analogue to stimulate endogenous



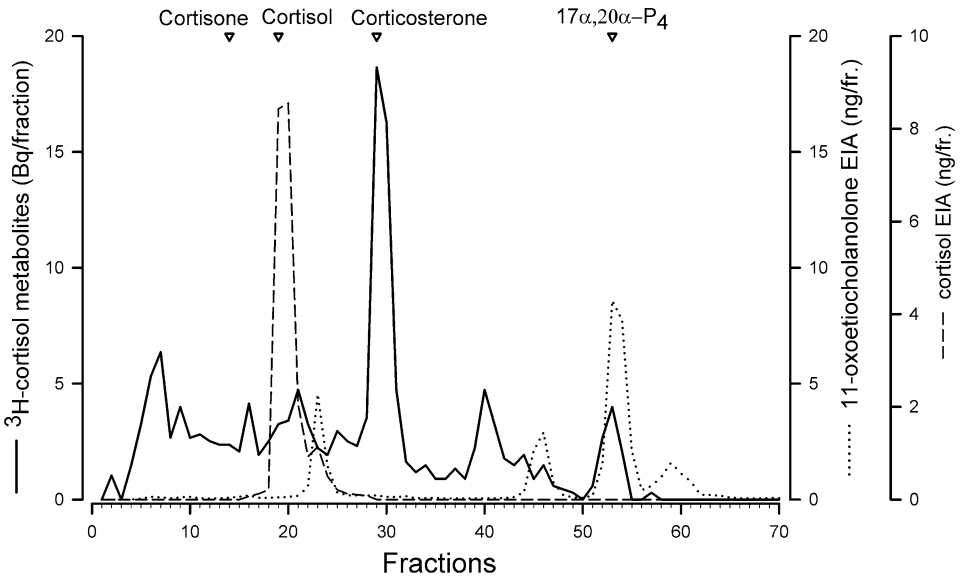


Fig. 3 Reversed-phase high-performance liquid chromatographic (RP-HPLC) separation of fecal ^{3}H-cortisol metabolites (peak concentration sample) in the feces of a male (*upper panel*) and a female (*lower panel*). Radioactivity of each fraction was determined by liquid scintillation counting and immunoreactivity was measured in a cortisol EIA and an 11-oxoetiocholanolone EIA. *Open triangles* indicate the approximate elution positions of respective standards (*17α,20α-P$_4$* 17α,20α-dihydroxyprogesterone)

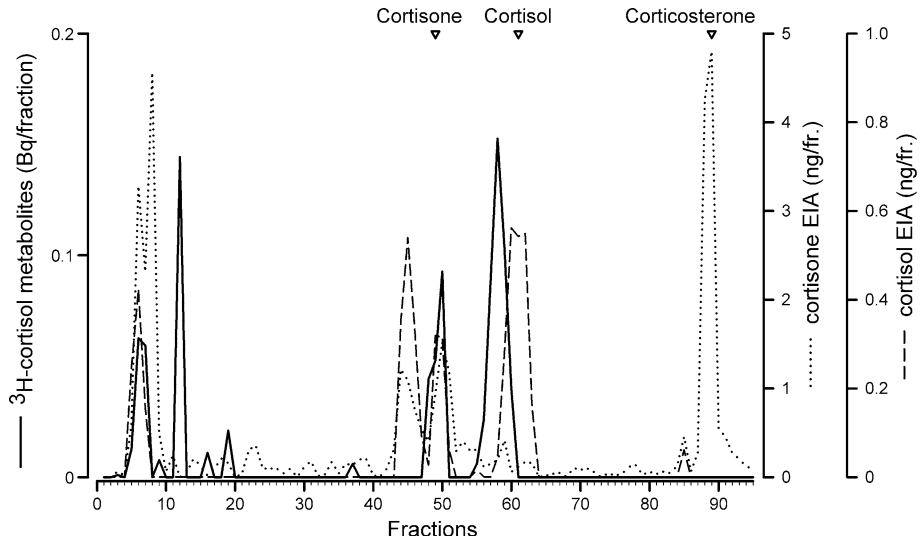


Fig. 4 Reversed-phase high-performance liquid chromatographic (RP-HPLC) separation of ^{3}H-cortisol metabolites (pooled sample) in the hair. Radioactivity of each fraction was determined by liquid scintillation counting and immunoreactivity was measured in a cortisol EIA and a cortisone EIA. *Open triangles* indicate the approximate elution positions of respective standards

adrenal cortisol secretion as opposed to intraperitoneally injected aqueous ^{3}H-cortisol. This may explain the difference in the time delay in fecal excretion. Moreover, the secretion of GCM may also be significantly influenced by the animals' daily activity rhythm. Based on Touma et al.'s (2003) findings in mice, we assume that more frequent defecation due to higher activity might have contributed to an earlier appearance of metabolites in the feces, but confirmation is lacking as activity has not been assessed and data on the defecation rate is not given by Bauer et al. (2008).

HPLC analysis of fecal samples revealed very similar patterns in all tested animals. Immunoreactivity and radioactivity eluting around the position of the cortisol standard might be interpreted as native, unmetabolized cortisol. However, some cross-reacting 5α/5β-reduced cortisol metabolites (5α/5β-di- or tetrahydrocortisol) elute close to cortisol in our HPLC system and previous studies in other species did not show substantial radioactivity at the position of cortisol either. Hence, the existence of native cortisol in feces of guinea pigs is very unlikely, but cannot be ruled out. A dominant peak of radioactivity could be found at the position of the corticosterone standard, which should not be interpreted as native corticosterone. In fact, the cortisol antibody (6.9 % crossreactivity with corticosterone) was not able to measure any immunoreactivity at the position of corticosterone. As, to date, no metabolic pathway is known to allow the conversion from cortisol to corticosterone, it appears unlikely that native corticosterone is responsible for the peak. Further investigations are needed to identify this metabolite.

Though we did not find any distinct accumulation of radioactivity in the feces as a result of multiple dosing, we were expecting to detect radioactivity in or on hair providing a matrix for the (long-term) deposition of exogenous and endogenous steroids. Based on the time course studies of anabolic steroids in hair by Shen et al. (2009), we investigated the (secretion and) possible incorporation of ^{3}H-cortisol metabolites into/onto the hair after multiple-dose administration. Hair samples collected for cortisol analysis in animals may be contaminated by various endogenous sources of cortisol and its metabolites such as urine, feces and saliva. Additionally, it is assumed that cortisol may be bound on the surface of the hair or possibly enter the hair by diffusion through sweat and sebum secretions (Cone 1996). However, sweat can be ruled out as possible source in guinea pigs, since they have sweat glands only in isolated areas (e.g., footpads; Adelmann et al. 1975; Hesselmann 2010). A wash procedure using two (subsequent) short *n*-hexane/methanol washes was applied to hair samples and did not reveal any measurable radioactivity in any of the washes. These findings indicate that samples had not been contaminated by urine or feces during the sampling period, which may be due to hair sampled only from the animals' back. Moreover, the probability of hair contamination was reduced to a minimum due to a frequent sampling protocol for excreta.

While a wash protocol is considered to be effective when removing all visible contamination and measurable cortisol or its metabolites from the surface of the hair, the wash solvent is also required to be safe as characterized by a low risk of extracting GC from the interior of the hair shaft. Since radioactivity in the *n*-hexane/methanol washes was as low as in background samples, the use of these successive washes did not entail removal of ^{3}H-cortisol or its metabolites from the interior of the hair shaft nor from sebum (secretions) on the external hair shaft and, therefore, can be interpreted as suitable. First, this may be attributed to *n*-hexane being highly lipophilic and unable to penetrate as deeply into the hair shaft as reported for lower alcohols or aqueous washing media (Eser et al. 1997). Second, the level of radiolabeled metabolites measured in the hair extract was very low such that only little or even undetectable amounts of the unbound steroid fraction present in the circulation may have been secreted in sebum.

The majority of the work measuring cortisol in hair has used methanol for the extraction procedure (Accorsi et al. 2008; Ashley et al. 2011; Bechshøft et al. 2011; Comin et al. 2011; Davenport et al. 2006; Kalra et al. 2007; Kirschbaum et al. 2009; Koren et al. 2002; Macbeth et al. 2010; Sauvé et al. 2007). Whereas a few studies have determined the extraction recovery by spiking and retrieving methods (Accorsi et al. 2008; Macbeth et al. 2010; Sauvé et al. 2007), almost no information is available on the true efficiency of the applied extraction protocol. Macbeth et al. (2010), in turn, found the extraction in methanol for 24 h to be effective, since no further cortisol could be measured when the procedure was reapplied to the same samples. The solubilization of the hair matrix provides an excellent method to measure the amount of GC remaining in the hair after the extraction. Along with radiolabeling, the efficiency of the extraction protocol can be evaluated notwithstanding any instability of the steroids due to hydrolysis during solubilization. The amount of extractable radioactivity in the pooled hair samples obtained on day 6 was 47.5 %, which is a relatively low efficiency. Importantly, the hair samples in the present study were not pulverized prior to extraction. This might be a possible explanation for the relatively low efficiency, and is supported by Davenport et al. (2006) who found enhanced cortisol recovery from powdered hair versus minced hair. Nevertheless, measurement of high amounts of unlabeled GCM in multiple fractions of the performed HPLC separation indicates that methanolic extraction provides sufficient efficiency for analysis.

As the mean radioactivity detected in the individual hair samples was very low, samples were pooled prior to HPLC analysis. Interestingly, a prominent radioactive peak coincided with the cortisone standard and with expressed immunoreactivity in the cortisone EIA. These findings strongly suggest that the radioactive peak represents cortisone. While there was major immunoreactivity of the respective EIA at the position of the cortisol and corticosterone standard, radioactivity was absent in these fractions. This pattern strongly implies that the radiolabeled cortisol administered intraperitoneally had been metabolized prior to incorporation into the hair shaft, and cortisol measured in the EIAs did not originate from systemic production. Considering the capability of cultured, isolated melanocytes or dermal fibroblasts to produce cortisol (Bamberg et al. 2005) and/or corticosterone in response to corticotropin releasing hormone (CRH) and ACTH stimulation (Slominski et al. 2005a, b), the cortisol and corticosterone detected in the EIAs are likely to be a result of local synthesis in the hair follicle. GC synthesis in cell populations of mammalian skin is evident from several studies (Slominski et al. 1999, 2000a, 2005a, 2006). While in isolated, cultured immortalized keratinocytes the sequence of CRH stimulating proopiomelanocortin (POMC) gene expression and production of ACTH could not be detected (Slominski et al. 2002), cultured primary human keratinocytes (Hannen et al. 2011) and keratinocytes of microdissected organ-cultured human scalp hair follicles have been found to engage in cortisol synthesis and secretion enhanced by either ACTH or CRH (Ito et al. 2005).

Keratinocytes and melanocytes constitute major components of the hair follicle (Eurell et al. 2006; Pragst and Balikova 2006) referred to as matrix cells. As they proliferate, they give rise to cells that keratinize, incorporate melanin pigments and migrate towards the surface of the epidermis to form a new hair (Eurell et al. 2006; Pragst and Balikova 2006). Thus, sequestration of GC or GCM in the hair shaft derived from local production in hair follicle cells seems to be comprehensible. Most of the research published on cortisol concentrations in hair used samples excluding hair follicles, using the hair shafts only (Accorsi et al. 2008; Ashley et al. 2011; Comin et al. 2011; Davenport et al. 2006; Kalra et al. 2007; Kirschbaum et al. 2009; Macbeth et al. 2010; Raul et al. 2004; Sauvé et al. 2007; Steudte et al. 2011), which may not exclude measured cortisol levels to originate from local production in the hair follicle. Based on the formation of hair in a follicle, this technique (i.e., using hair shafts) will not exclude cortisol levels measured to originate from local production in the hair follicle.

To date, passive (or active) diffusion from blood is generally accepted as the primary mechanism for the incorporation of exogenously delivered drugs into the hair (Cone 1996), which is also assumed for (systemic) cortisol measured in hair. The very low levels of radiolabeled metabolites we detected in newly grown hair of guinea pigs, even after multiple injections of ^{3}H-cortisol, suggest that the predominant way of incorporation of GC into the hair shaft is not via the blood capillaries. Though, a multilayer basal lamina of the capillaries in the dermal root sheath, which is reported as a morphological feature specific to the hair bulb of guinea pigs (Parakkel 1966), might have promoted the low concentrations in the hair shaft. Conversely, the high levels of unlabeled metabolites including intact cortisol suggest local production in the hair follicles and subsequent sequestration in the hair shaft.

There is evidence from forensic drug literature that hair color affects the incorporation and retention of drugs in hair (Cone 1996; Rothe et al. 1997; Uematsu et al. 1992). Melanin in hair is attributed the role of a principal binding site for drug incorporation in hair (Cone 1996) and may also be responsible for the retention of endogenous GC in hair. Given the small amount of radioactivity found in hair, the color effect could not be addressed in our study, but hair color as a potential factor confounding the measurement and interpretation of cortisol in hair needs to be further investigated.

In addition to cortisol synthesis and secretion, a CRH signaling system by cell type-specific POMC-derived mediators like ACTH, α-melanocyte-stimulating hormone or β-endorphins has been demonstrated in defined skin cell populations (e.g., Boehm et al. 2005; Paus et al. 1999; Slominski et al. 2000b), including a negative feedback regulation by cortisol on CRH expression in organ-cultured human scalp hair follicles (Ito et al. 2005). Thus, a hierarchically structured peripheral HPA axis equivalent in the skin has become apparent, and likewise, the mediators of this axis are proposed to regulate the cutaneous response to local stressors (Slominski et al. 2007). Our findings in hair of guinea pigs are consistent with the proposed functionality of the cutaneous HPA axis homolog, but it is still not clear, whether hair cortisol production is also affected by the mediators of the central HPA axis. Current evidence is persuasive for the hypothesis that local GC synthesis in the skin (as well as in other tissues) is independent of patterns of adrenal GC synthesis and allows for functional consequences at the level of the target organ or even on a smaller scale (Sharpley et al. 2011; Taves et al. 2011). Against this background, we want to point out that local stressor demands (as induced by physical or mechanical skin irritation) may enhance local GC production in hair follicle cells regulated by locally expressed HPA mediators, and may well explain localized effects or, if present concurrently with systemic stressors, result in increased hair cortisol levels when exposed to chronic stress. On the other

hand, enhanced hair cortisol levels may arise in response to circulating ACTH operating peripherally in the hair follicle, which may be the reason for the increase in hair cortisol following ACTH challenges (González-de-la-Vara et al. 2011). If hair follicle cells were responsive to pituitary ACTH, the measurement of GC in hair might provide a novel tool for the diagnosis of Cushing's syndrome, caused by excess levels of cortisol in the blood predominantly due to ACTH producing tumors.

In summary, our results demonstrate that only negligible amounts of systemic cortisol, preferentially as more lipophilic metabolites, are incorporated into hair. Moreover, they indicate that measurement of cortisol levels in hair may not necessarily reflect adrenal cortisol synthesis and secretion, but local synthesis in the hair follicle. Our study represents an important contribution to unravel the origin of GC levels found in hair. It reveals the complexity of evaluating long-term stress responses by hair cortisol analysis and encourages further investigations into this topic.

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