in vivo *23*: 381-386 (2009)

Distribution of [^{3}H]-Corticosterone in Urine, Feces and Blood of Male Sprague-Dawley Rats after Tail Vein and Jugular Vein Injections

KLAS S.P. ABELSON[1,2], SHAHRZAD SHIRAZI FARD[1], JULIA NYMAN[1], RENÉE GOLDKUHL[1] and JANN HAU[2]

[1]*Department of Neuroscience, Division of Comparative Medicine, Biomedical Centre, Uppsala University, Uppsala, Sweden;*
[2]*Department of Experimental Medicine, Panum Institute, University of Copenhagen, Copenhagen, Denmark*

Abstract. *The present study aimed to investigate the time-course and distribution of [^{3}H]-corticosterone in urine, feces and blood of male Sprague-Dawley rats after intravenous administration of a low dose (1 μCi), and to investigate whether different intravenous routes of administration may affect the dynamics of excreted [^{3}H]-corticosterone in the feces. One μCi [^{3}H]-corticosterone was injected intravenously either through the tail vein in manually restrained rats or through a jugular vein catheter three days after surgical implantation. Urine and feces were collected at different time points over 78 h from the rats injected in the tail vein, and blood and feces were collected over 48 h from rats injected in the jugular vein. In the blood, radioactivity peaked immediately and decreased rapidly within 90 minutes. The radioactivity was excreted in urine within six h and in feces after at least 12 h. Sixty percent of the radioactivity was detected in the urine and 40% in feces during the study period of 78 h. The detected amount of radioactivity in feces was higher and displayed a more pronounced peak 12 h after injection when the substance was administered through a jugular vein catheter compared to tail vein injection. The data obtained in the present study may serve as an important benchmark when choosing time points for fecal collection for quantification of corticosterone or corticosterone metabolites as a non-invasive measure of preceding HPA-axis activation.*

Corticosterone in serum has been extensively used as a measure of adrenal activation after stressful conditions in laboratory rats. However, this method has limitations due to the procedures associated with blood sampling, which may cause a stress response in the animals either by the handling and restraint for manual sampling (1, 2) or by the surgical insertion of catheters for automated sampling (3-5). To avoid interference with endocrine functions caused by stress during studies, excreted corticosterone and corticosterone metabolites (CM) in feces may be a useful non-invasive measure of adrenal activity. This method has advantages over blood sampling. It causes no stress to the animals in connection to sampling, and involves no interaction at all during the period of interest since there is a delay between the adrenal secretion of corticosteroids into blood and the subsequent excretion in feces. It also results in a measure of corticosterone that represents a true integrated amount of the steroid over time, compared to blood samples requiring integration of areas under curves to give an estimate of changes in concentration. Several articles have been published on the fecal excretion of CM in rats, and most of these studies have demonstrated a clear circadian rhythm in both female and male rats (6-13). Lepschy *et al*. showed that adrenocorticotropic hormone (ACTH) stimulation caused a subsequent increased fecal CM excretion 4-12 h after administration in Fisher and Sprague-Dawley rats, although the actual levels in blood were unknown in this study (9). Two other studies have, independently of each other, investigated the direct relation between substantially increased corticosterone levels in blood and increased excretion of fecal CM, and found that an increased level in blood could be detected after about eight hours in feces (12, 13). These three studies all used different extraction and quantification methods. Thus, quantification of fecal CM is a promising method for non-invasive stress assessment. However, the method still requires further studies to completely understand the sensitivity and applicability of fecal CM as a measure of preceding changes in corticosteroid concentrations in the circulation. The time-course from increased levels in blood to fecal excretion and determination of the proportion of CM that is excreted in feces in relation to urine remains to be investigated. Lepschy *et al*. found that the

Correspondence to: Jann Hau, Department of Experimental Medicine, University of Copenhagen and National Hospital, Blegdamsvej 3B, 2200 Copenhagen, Denmark. Tel: +45 35327363, Fax: +45 35327399, e-mail: jhau@sund.ku.dk

Key Words: Stress, corticosterone, fecal corticosterone metabolites, non-invasive stress assessment, laboratory animals, rats.

proportion between urinary and fecal CM was approximately 25:75 after intravenous and per os administration of [^{3}H]-corticosterone (9). Similar proportions were found by Bamberg *et al.* after intraperitoneal administration (6). However, many technical aspects of the administration of [^{3}H]-corticosterone have not yet been studied. For instance, it is not known how rapidly the injected [^{3}H]-corticosterone is eliminated from the circulation. It is also unknown if the time-course and proportions would be affected by the dose of [^{3}H]-corticosterone injected, and what effect different intravenous injection sites and techniques may have on the measurements.

The aim of the present study was to investigate the time-course and distribution of a small dose of [^{3}H]-corticosterone in urine, feces and blood of male Sprague-Dawley rats after intravenous administration, and to investigate whether different intravenous routes of administration may affect the detection of excreted [^{3}H]-corticosterone in the feces.

Materials and Methods

Animals. All animal experiments in this study were approved by the Uppsala Animal Ethics Committee in Uppsala, Sweden. Seventeen male Sprague-Dawley rats (Scanbur B&K, Sollentuna, Sweden) with an average body weight of 310±8 g (mean±S.E.M.) were used in the study. Eleven of them were used in Experiment 1 and six in Experiment 2. Male rats were chosen to minimize confounding variables related to estrous cycle associated hormonal fluctuations in females. After arrival, the rats were kept for seven days acclimatization in animal rooms under standardized conditions: Diurnal rhythm was regulated with a 12 h light: 12 h dark cycle; temperature was kept at 20±2˚C; relative humidity at 30-60% ; air was changed approximately 15 times per hour; and clean cages were provided twice a week. Aspen chips (Finn Taipei, Kortteinen, Finland) were used as bedding material. The animals had free access to food pellets (R36 Laktamin, Stockholm, Sweden) and tap water at all times. Two days before surgery, the rats were transferred to single housing in Macrolon type III cages and moved to a designated laboratory with similar environmental conditions, where the experiment was conducted.

Experiment 1. After seven days of acclimatization, each animal was transferred to a metabolic cage and allowed to habituate to the new cage for three days. Each day during habituation, the rats were handled by the experimenter and habituated to restraint. After habituation, the rats were restrained and injected intravenously in the tail vein, with 1 μCi [^{3}H]-corticosterone ([1,2,6,7-^{3}H]-corticosterone, specific activity 2.92 TBq/mmol, 79.0 Ci/mmol, Amersham Bioscience, Uppsala, Sweden) in 0.2 mL saline, corresponding to a blood concentration of approximately 0.2 ng/mL blood in a 300 g rat. The injection was performed at 06.00 h. After injection, urine and feces were collected every 6th hour (12.00, 18.00, 00.00, 06.00 *etc.*) during four days. Body weight was recorded daily.

Experiment 2. Two days before surgery, the rats were transferred to single housing in Macrolon type III cages and moved to a designated laboratory with similar environmental conditions, where the experiment was conducted. After translocation, each rat was given Nutella® hazelnut and chocolate cream for habituation to the flavor, to facilitate future oral administration of pre-emptive analgesia (4, 12). All rats were handled regularly each day to habituate them to the experimenter.

All surgeries were commenced and completed before noon. One hour before surgery, the rats were treated for pre-emptive analgesia. Since the rats in Experiment 2 were also included in a separate study, aiming to investigate the post-operative recovery after different analgesic treatments, rats were randomly assigned to either treatment with 15 mg/kg ibuprofen (Ibumetin, Nycomed AB, Stockholm, Sweden) dissolved in Nutella (2 g/kg body weight) for oral voluntary ingestion, or no analgesic treatment. Rats were placed in an induction chamber and anesthesia was induced with 5% isoflurane (Forene®, Abbot Scandinavia, Stockholm, Sweden) delivered in 100% oxygen. Once the paw withdrawal reflex was absent, the rats were shaved at the incision sites and attached to a Simtec anesthetic mask for spontaneous respiration. Isoflurane was maintained at a level of 2.5-3% to ensure adequate anesthesia. The shaved parts were washed with iodine (Jodopax vet®, Pharmaxin AB, Helsingborg, Sweden). An incision was made in the skin of the neck and a dual cannulation was performed by catheterization of *v. jugularis externa* (for intravenous administration of ACTH) and *a. carotis communis* (for blood sampling). Catheters were filled with heparinized saline to prevent blood clotting. The catheters were secured in the vessels and led subcutaneously through a DiLab® Dacron button attached to the dorsal region of the neck. The catheters were led further through a metal spring and connected to an AccuSampler® (DiLab, Lund, Sweden) for automated blood sampling. To ensure adequate recovery, the rats' activity was observed regularly during the first hours after they regained consciousness, and daily water intake was recorded.

The experiments were conducted three days after surgery, when corticosterone is known to have returned to normal levels and cyclic rhythmicity in the circulation is restored (11, 12). At 06.00 hours, the rats were injected with 1 μCi [^{3}H]-corticosterone in 0.2 mL saline intravenously through the jugular vein catheter. After injection, blood samples (200 μL) were collected after 10, 20, 40, 60 and 90 minutes, followed by a sample every hour until 6 h after injection. After that, samples were collected every 12th hour and fecal samples were collected every 6th hour (12.00, 18.00, 00.00, 06.00 etc.) until the end of experiment 48 h after injection.

Quantification of [^{3}H]-corticosterone. As the exact proportions between intact corticosterone and corticosterone metabolites excreted in urine and feces are unknown, it is uncertain whether the tritium measured is conjugated to corticosterone or to a metabolite. Therefore, 'radioactivity' is used to describe the excreted product instead of [^{3}H]-corticosterone.

The radioactivity from urine samples was quantified without any preceding extraction, by mixing 1 mL of each sample urine with 10 mL scintillation liquid (Ultima Gold Universal LSC-cocktail, PerkinElmer, Boston, MA, USA) and radioactivity was quantified in a scintillator (1219 RackBeta Liquid Scintillation Counter, Wallac, USA).

The fecal pellets from each sampling occasion were weighed and thoroughly homogenized after adding 4 g MilliQ H_2O per gram of faces. Thereafter, [^{3}H]-corticosterone was extracted according to the following procedure: five milliliters CH_2Cl_2 were added to 1 g homogenate, and the tubes were vortexed 5×7 sec, followed by centrifugation 3000 rpm for 15 min at 4˚C. The organic phase was

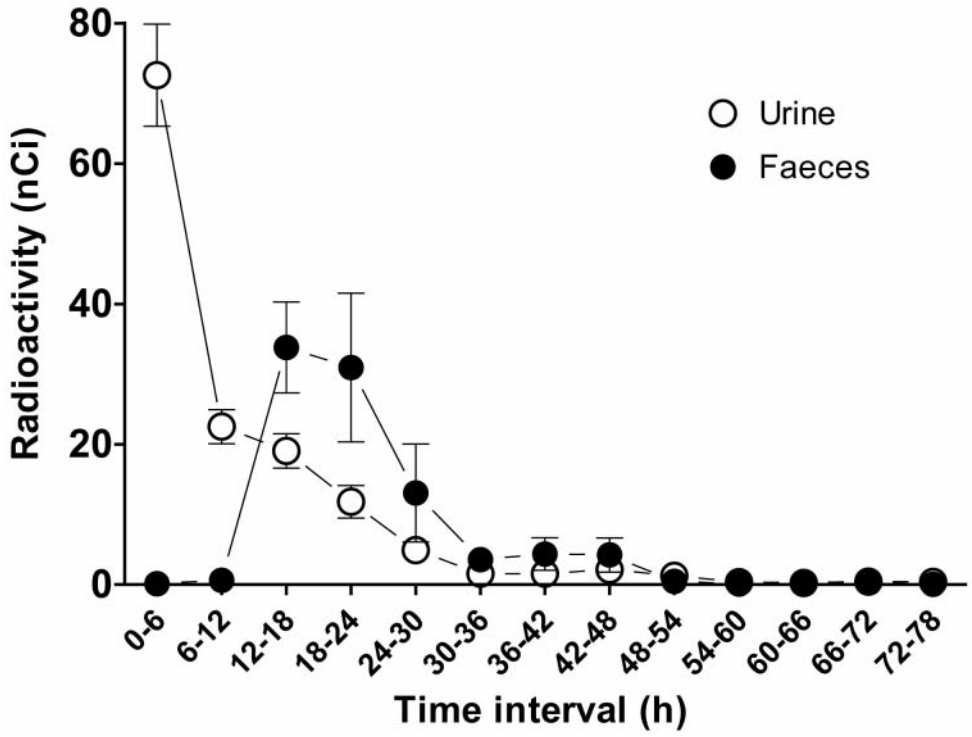

Figure 1. *Detected radioactivity in urine and feces during 72 h, after tail vein injection of [^{3}H]-corticosterone. The excretion is expressed as the total amount nCi (mean±S.E.M.) detected during each sampling interval.*

transferred to a clean tube, 1 mL 0.1 M NaOH was added and the tubes were vortexed and centrifuged as above. The samples were washed with 1 mL MilliQ H_2O, vortexed and kept on ice for 10 min. The hydrophilic layer was removed and the washing repeated once. Two milliliters of the organic phase were mixed with 10 mL scintillation liquid and the radioactivity was quantified as above.

Blood samples were centrifuged and the obtained serum was mixed with 10 mL scintillation liquid followed by quantification of radioactivity.

Standard calibration curves for [^{3}H]-corticosterone in urine, feces and blood were established by adding [^{3}H]-corticosterone in amounts of 10 pCi – 100 nCi followed by quantification as described above. This calibration accounted both for the efficiency of the extraction method and for quenching of radioactivity during the scintillation. The recovery of added [^{3}H]-corticosterone was 89±8% (mean±standard error of the mean [SEM]) for urine, 74±18% for feces and 82±9% for blood.

Statistical methods. To investigate whether the fecal corticosterone and CM excretion differed after tail vein and jugular vein injection respectively, an independent *t*-test was performed using SPSS version 14.0.

Results

Experiment 1. The excretion of [^{3}H]-corticosterone in urine and feces after tail vein injection is shown in Figure 1. The total recovery of radioactivity was 23%. The excretion detected during 78 h was 139±14 nCi (13.9% of injected [^{3}H]-corticosterone) for urine and 92±13 nCi (9.2% of injected [^{3}H]-corticosterone) for feces. The proportion of [^{3}H]-corticosterone between urinary and fecal excretion was 60:40. Average peak concentrations were observed in the time interval 0-6 h in urine and in the time interval 12-24 h in feces.

Experiment 2. The detected excretion of [^{3}H]-corticosterone in feces after jugular vein injection is shown in Figure 2. The excretion detected during 48 h was 157±20 nCi (15.7% of injected [^{3}H]-corticosterone). The choice of experimental endpoint at 48 h in Experiment 2 was based on the observation in Experiment 1 that [^{3}H]-corticosterone levels were undetectable in both urine and feces after this time point. Average peak concentration was observed in the time interval 12-18 hours. When compared to tail vein injection, the [^{3}H]-corticosterone was found to peak earlier, and the accumulated excretion detected was 70% higher after jugular vein injection. This difference was statistically significant, determined by an independent t-test ($t_{[7.57]}$=−2.61; p=0.033).

The detected levels of [^{3}H]-corticosterone in blood during 48 h after jugular vein injection are shown in Figure 3. It was found that the levels rapidly decreased and stabilized at low levels after 90 min.

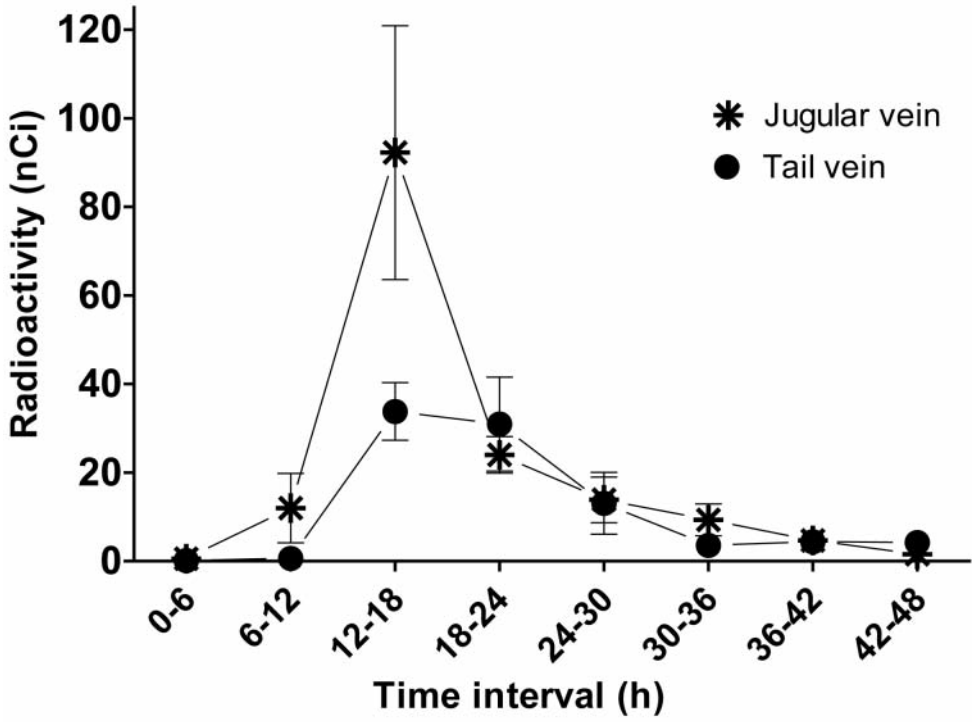

Figure 2. *Detected radioactivity in feces during 48 h, after tail vein and jugular vein injection of [^{3}H]-corticosterone. The excretion is expressed as the total amount nCi (mean±S.E.M.) detected during each sampling interval.*

Discussion

The present study investigated methodological issues of the time course and distribution in urine, feces and blood of a low dose of [^{3}H]-corticosterone administered intravenously by the tail vein and jugular vein in male Sprague-Dawley rats.

The recovery of the administered radioactivity was as low as 23%. After 48 h, the levels were close to zero. This is explained by the low levels of radioactively labeled corticosterone administered. With the exception of the peak levels, most of the radioactivity in urinary and fecal excretions was simply present at far too low levels to be detected by the scintillation counter. The recovered radioactivity from the samples, however, was high (74-89%). In the study by Lepschy *et al*., a much larger amount [^{3}H]-corticosterone was administered (62 μCi with a specific activity of 2.61 TBq/mmol), corresponding to a concentration of approximately 14 ng/mL of blood in a 300 g rat. This is a concentration approximately 70 times higher than in the present study, and by using that magnitude of concentration, the levels in urine and feces are probably detectable with a higher precision. However, in the present study, the detection of peak values was the most relevant, and a low dose was chosen in order not to reach potential maximal capacities for renal or bile excretion of CM at any time point. When adding all detectable radioactivity levels excreted during the study period, it was found that 60% of all recovered radioactivity was found in urine. This is in agreement with the findings of Eriksson *et al*., who observed that the proportions of the CMs excreted in urine and feces were rather similar (50:50) during daytime followed by a predominant urinary excretion during night time (8). It is, however, in contrast to other studies discussed above, where it was found that only approximately 20-25% of the detected radioactivity was found in urine compared to feces (6, 9). At present, there is no explanation for these differences, for which this issue should be further investigated.

Despite the fact that the recovery is lower and that the proportions are different than those shown in studies using higher [^{3}H]-corticosterone doses, the time course is similar. Peak radioactivity levels were found in urine within six hours followed by peaks in feces after 12-24 hours. Thus, using a concentration as low as 1 μCi [^{3}H]-corticosterone should be valid for studying time-course of CM excreted in urine and feces of rats.

When comparing the detected radioactivity excreted in feces after the two injection techniques, *i.e.* tail vein injection during restraint and jugular vein injection after catheterization, it was found that the amount detected was significantly higher after the jugular injection than after the tail vein injection. Since no data of urinary excretion were available from this experiment, it is not known whether the proportions between

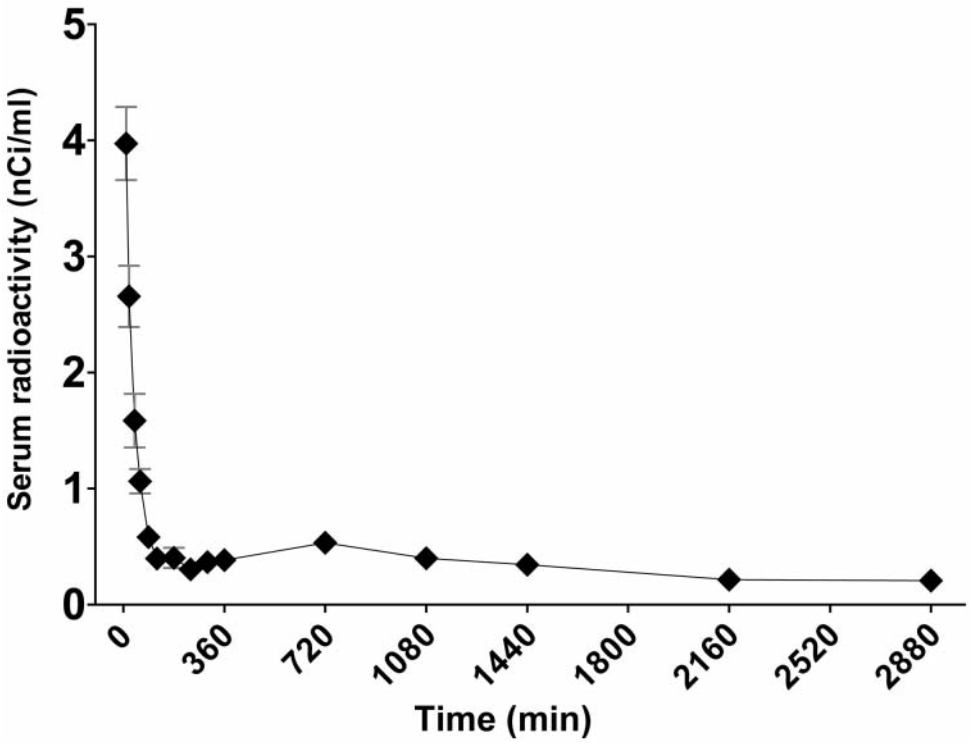

Figure 3. *Detected radioactivity in serum after jugular vein injection of [^{3}H]-corticosterone. The concentration is expressed nCi/mL serum (mean±S.E.M.) detected at each sampling point.*

urine and feces were the same as after tail vein injection. Hence, it remains unknown whether the total recovery would be higher than, or equal to that of Experiment 1. Interestingly, however, there was a more pronounced peak in detected radioactivity in the time-interval 12-18 hours after jugular vein injection than after tail vein injection. Although all tail vein injections were performed by an experienced experimenter in the present study, there is always a risk of loss or misplacement of the injected substance, due to leakage and partial subcutaneous administration. The possible variation caused by this was the reason for including more animals in Experiment 1 than in Experiment 2. The excretion might have been affected by a small proportion of the injected substance being placed subcutaneously in the tail instead of entering the circulation, and thereby being excreted later and to a lesser extent. In addition, when injected through the tail vein, the substance travels through smaller and longer vessels before reaching the liver than after jugular vein injection, and is more likely to bind to different binding sites to a larger extent. This may affect the excretion rate resulting in a delayed excretion at lower concentrations. Thus, both the higher recovery in feces and the more distinct peak after jugular catheter injection may indicate that this method is more accurate and precise for intravenous injection of low concentrations of [^{3}H]-corticosterone. However, it should be pointed out that variation in stress levels between animals could partly explain dynamic differences in radioactive clearance which were observed in the present study, since the adrenal production of endogenous hormone may influence the steroid hormone clearance rates. The animals subjected to tail vein injection were restrained prior to the injection. Restraint is known to cause an acute stress response, with a rapid increase of corticosterone in the circulation (1, 2). On the other hand, injection through the jugular vein in freely moving rats in the AccuSampler-system, has been shown to cause only minor increase of serum corticosterone (12).

It is noteworthy that in many studies including this, the highest levels of fecal radioactivity after administration of [^{3}H]-corticosterone were detected after at least 12 h, while stress-induced or ACTH-induced high levels of corticosterone result in peak levels in fecal CM after approximately 7-9 h (9, 12, 13). When [^{3}H]-corticosterone has been administered, this has often been done using amounts representing normal basal levels of less than 1 ng/mL as in the present study, or 10-15 ng/mL as in the study by Lepschy *et al*.. When corticosterone secretion is stimulated the concentrations reach maximal levels (12, 13). Thus, it seems as if the excretion rate is dependent on the magnitude of circulating corticosterone concentrations, which should be considered when choosing fecal sampling

intervals for stress assessments. However, more studies are necessary to scrutinize this issue.

The radioactivity was detected in serum samples over 90 minutes, followed by hardly detectable levels throughout the remainder of the study. Using a higher concentration would probably have extended the duration of detectable levels, but the rapid decline in [^{3}H]-corticosterone levels would most likely have been the same. This suggests that a certain amount of corticosterone in blood is removed from the circulation within 90 minutes, being excreted primarily *via* the kidney in urine within 6 hours, followed by excretion *via* the bile and liver in feces after at least 12 hours. This may serve as a good benchmark when choosing time points for fecal corticosterone or CM measurements. However, considering the differences in fecal peak values depending on concentrations as discussed above, further studies should be undertaken to evaluate this in detail. In addition, since different strains of rats show significantly different reactivity and response to stress and corticosteroids (14-17), strain specific excretion patterns should also be examined.

In conclusion, the present study demonstrates that intravenously injected [^{3}H]-corticosterone in a low dose (<1 ng/mL) results in detectable radioactivity in blood during 90 minutes, in urine within 6 h and in feces after at least 12 h. Sixty percent of the radioactivity was detected in the urine and 40% in feces during the study period of 72 h. The detected amount of radioactivity in feces was higher and displayed a more pronounced peak 12 h after injection when the substance was administered through a jugular vein catheter compared to tail vein injection during restraint, suggesting that the jugular injection method is the more accurate of the two.

Acknowledgements

The present investigation was supported by generous grants from the Swedish Research Council. DiLab kindly donated two AccuSampler® systems.

References

1 Gärtner K, Büttner D, Döhler K, Friedel R, Lindena J and Trautschold I: Stress responses of rats to handling and experimental procedures. Lab Anim *14*: 267-274, 1980.

2 Vachon P and Moreau JP: Serum corticosterone and blood glucose in rats after two jugular vein blood sampling methods: comparison of the stress response. Contemp Top Lab Anim Sci *40*: 22-24, 2001.

3 Abelson KSP, Adem B, Royo F, Carlsson HE and Hau J: High plasma corticosterone levels persist during frequent automatic blood sampling in rats. In Vivo *19*: 815-819, 2005.

4 Goldkuhl R, Carlsson HE, Hau J and Abelson KSP: Effect of subcutaneous injection and oral voluntary ingestion of buprenorphine on post-operative serum corticosterone levels in male rats. Eur Surg Res *41*: 272-278, 2008.

5 Shavit Y, Weidenfeld J, Dekeyser FG, Fish G, Wolf G, Mayburd E, Meerson Y and Beilin B: Effects of surgical stress on brain prostaglandin E(2) production and on the pituitary-adrenal axis: Attenuation by preemptive analgesia and by central amygdala lesion. Brain Res *1047*: 10-17, 2005.

6 Bamberg E, Palme R and Meingassner JG: Excretion of corticosteroid metabolites in urine and faeces of rats. Lab Anim *35*: 307-315, 2001.

7 Cavigelli SA, Monfort SL, Whitney TK, Mechref YS, Novotny M and McClintock MK: Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. *184*: 153-163, 2005.

8 Eriksson E, Royo F, Lyberg K, Carlsson H-E and Hau J: Effect of metabolic cage housing on immunoglobulin A and corticosterone excretion in faeces and urine of young male rats. Exp Physiol *89*: 427-433, 2004.

9 Lepschy M, Touma C, Hruby R and Palme R: Non-invasive measurement of adrenocortical activity in male and female rats. Laboratory Animals *41*: 372-387, 2007.

10 Pihl L and Hau J: Faecal corticosterone and immunoglobulin A in young adult rats. Lab Anim *37*: 166-171, 2003.

11 Royo F, Björk N, Carlsson HE, Mayo S and Hau J: Impact of chronic catheterization and automated blood sampling (AccuSampler) on serum corticosterone and fecal immunoreactive corticosterone metabolites and immunoglobulin A in male rats. J Endocrinol *180*: 145-153, 2004.

12 Siswanto H, Hau J, Carlsson HE, Goldkuhl R and Abelson KSP: Corticosterone concentrations in blood and excretion in faeces after ACTH administration in male Sprague-Dawley rats. In Vivo *22*: 435-440, 2008.

13 Thanos PK, Cavigelli SA, Michaelides M, Olvet DM, Patel U, Diep MN and Volkow ND: A non-invasive method for detecting the metabolic stress response in rodents: Characterization and disruption of the circadian corticosterone rhythm. Physiol Res, in press, 2008.

14 Baumann MH, Elmer GI, Goldberg SR and Ambrosio E: Differential neuroendocrine responsiveness to morphine in Lewis, Fischer 344, and ACI inbred rats. Brain Res *858*: 320-326, 2000.

15 Dhabhar FS, McEwen BS and Spencer RL: Stress-Response, Adrenal-Steroid Receptor Levels and Corticosteroid-Binding Globulin Levels – a Comparison between Sprague-Dawley, Fischer-344 and Lewis Rats. Brain Res *616*: 89-98, 1993.

16 Marissal-Arvy N, Gaumont A, Langlois A, Dabertrand F, Bouchecareilh M, Tridon C and Mormede P: Strain differences in hypothalamic pituitary adrenocortical axis function and adipogenic effects of corticosterone in rats. J Endocrinol *195*: 473-484, 2007.

17 Oitzl MS, van Haarst AD, Sutanto W and Ron de Kloet E: Corticosterone, brain mineralocorticoid receptors (MRS) and the activity of the hypothalamic-pituitary-adrenal (hpa) axis: The Lewis rat as an example of increased central MR capacity and a hyporesponsive HPA axis. Psychoneuroendocrinology *20*: 655-675, 1995.

Received January 27, 2009
Revised March 17, 2009
Accepted March 30, 2009