Immediate versus modified release hydrocortisone in mitotane-treated patients with adrenocortical cancer

Weigel, Marianne; Hahner, Stefanie; Sherlock, Mark ; Agha, Amar; Behan, Lucy Ann; Stewart, Paul; Arlt, Wiebke; Beier, Daniela; Frey, Kathrin; Zopf, Kathrin; Quinkler, Marcus

DOI:
10.1111/cen.13302

Document Version
Peer reviewed version

Citation for published version (Harvard):
Weigel, M, Hahner, S, Sherlock, M, Agha, A, Behan, LA, Stewart, P, Arlt, W, Beier, D, Frey, K, Zopf, K & Quinkler, M 2017, 'Immediate versus modified release hydrocortisone in mitotane-treated patients with adrenocortical cancer', *Clinical Endocrinology*, vol. 86, no. 4, pp. 499-505. https://doi.org/10.1111/cen.13302

Immediate versus modified release hydrocortisone in mitotane treated patients with adrenocortical cancer

Short title: mitotane and hydrocortisone replacement therapy

*Marianne Weigel[1], Stefanie Hahner[2], Mark Sherlock[3], Amar Agha[4], Lucy Ann Behan[3,4], Paul M Stewart[5], Wiebke Arlt[6,7], Daniela Beier[8], Kathrin Frey[2], Kathrin Zopf[1], Marcus Quinkler[1,8]

[1] Clinical Endocrinology, Charité Campus Mitte, Charité University Medicine Berlin, Germany ; [2] Endocrinology & Diabetes Unit, Department of Internal Medicine I, University Hospital of Wuerzburg, Wuerzburg, Germany; [3] Department of Endocrinology and Diabetes, Adelaide and Meath Hospitals, Incorporating the National Children's Hospital and Trinity College, Tallaght Hospital, Dublin, Ireland; [4] Department of Endocrinology, Beaumont Hospital and RCSI Medical School, Dublin Ireland; [5] Department of Endocrinology, University Of Leeds, UK; [6] Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, UK; [7] Centre for Endocrinology, Diabetes and Metabolism, Birmingham Health Partners, Birmingham, UK; [8] Endocrinology in Charlottenburg, Berlin, Germany

Correspondence:

Marcus Quinkler, MD

Endocrinology in Charlottenburg

Stuttgarter Platz 1, 10627 Berlin, Germany

Tel. ++49-30-2132004; Fax.++49-030-2132005

email: marcusquinkler@t-online.de

Keywords: cortisol, cortisol binding globulin, adrenal insufficiency, ACC

Declaration of interest: M Quinkler and S Hahner worked as a consultant for ViroPharma and had received honoraria for talks. M Quinkler received research grants from HRA pharma for studies regarding mitotane. M Sherlock has worked as a consultant and received honoraria for talks from Shire.

Funding: M Sherlock was funded by the MRC as a Clinical Research Training Fellow. Hypoadrenalism studies were supported by unrestricted research grants from Novo Nordisk; the SAI studies were supported by an unrestricted educational grant from Pfizer Endocrine Care. PM Stewart is the recipient of an ERC Advanced Grant. W Arlt received funding from the European commission (FP7 grant ENSAT-CANCER).

Word count: 2736

Abstract word count: 250

Tables: 2

Figures: 4

Abstract

Objective: Mitotane induces hepatic CYP3A4 activity, resulting in accelerated cortisol inactivation, and also increases cortisol binding globulin (CBG). Therefore, higher hydrocortisone doses are required in patients with adrenocortical cancer (ACC) on mitotane treatment. Modified release hydrocortisone has not been used in mitotane-treated ACC patients yet.

Aim: Case series to compare serum cortisol, calculated free serum cortisol and ACTH levels in ACC patients on mitotane treatment with immediate and modified release hydrocortisone.

Design: Pharmacokinetics of immediate and modified release hydrocortisone, each administered at a dose of 40-20-0mg, in 9 patients with ACC and adjuvant mitotane treatment. For comparison, ten patients with secondary adrenal insufficiency (SAI) on three different hydrocortisone regimens, and ten healthy males were included.

Methods: Serum cortisol and plasma ACTH were measured by chemiluminescent enzyme immunoassay, and CBG by RIA, followed by calculation of free cortisol.

Results: Calculated free serum cortisol levels after 40mg immediate release hydrocortisone in ACC patients (46±14nmol/l) were similar to those after 10mg immediate release hydrocortisone intake in men with SAI (64±16nmol/l) or to the physiological morning free cortisol levels in healthy subjects (31±5nmol/l), Compared to immediate release hydrocortisone, free cortisol levels after 40mg modified release hydrocortisone in ACC patients were significantly lower (12±3nmol/l; p=0.03) resulting in a generally lower AUC (98±21 vs 149±37 nmol*h/l; p=0.02).

Conclusions: 40-20-0mg immediate release, but not modified release hydrocortisone, resulted in sufficient glucocorticoid coverage in patients with ACC

under mitotane treatment. The use of equivalent doses of modified release hydrocortisone preparation should be avoided in patients on mitotane treatment.

Introduction

Adrenocortical cancer (ACC) is a rare malignancy that occurs in approximately 0.5-2 per million persons per year [1;2]. The only curative treatment is radical surgery [3]. However, even after initial curative surgery, 80% of the patients develop recurrent disease within two years [4]. Adrenolytic therapy with mitotane (o'p'-DDD) is the established adjuvant therapy for ACC patients after R0 resection [5] and also the primary treatment for patients with advanced ACC (3), either as monotherapy or in combination with cytotoxic chemotherapy. Mitotane has been shown to extend disease-free survival in retrospective analyses [6;7]. Recent data shows that mitotane confers adrenolytic activity and down-regulation of steroidogenesis by lipid-induced ER-stress through inhibition of the activity of SOAT-1 [8]. As mitotane affects all adrenocortical steroid production it is necessary to replace glucocorticoids and sometimes mineralocorticoids in treated patients. However, mitotane is a strong inducer of hepatic cytochrome CYP3A4 activity [9;10], which inactivates cortisol to 6β-hydroxycortisol. Mitotane also increases cortisol binding globulin (CBG) [11]. These effects result in both a significantly increased cortisol metabolism and reduced free, active cortisol [12-16]. Therefore, classic hydrocortisone replacement doses of 20 mg/day, as used in adrenal insufficiency, are not sufficient in ACC patients on mitotane and higher dosages (40-80mg/day) are necessary.

A modified-release hydrocortisone preparation (Plenadren©, formerly known as Duocort®), [17;18], is available across Europe for replacement therapy in adult patients with adrenal insufficiency. It is administered in the morning and contains a rapid-release part and a delayed released part of hydrocortisone suggesting that only a once daily dose of hydrocortisone might be necessary. Improvement in QoL, blood pressure and metabolic parameters was demonstrated in 64 patients with Addison's

disease [19]. In an open, prospective trial in Addison patients modified-release hydrocortisone decreased BMI and HbA1c compared with immediate release HC treatment [20].

Patients with ACC on mitotane treatment often suffer from significant impairment by the disease and associated treatment. The number of tablets that need to be ingested is mostly high, making it desirable to reduce their amount. The rapid metabolic inactivation of cortisol during mitotane treatment may not only increase the dose of hydrocortisone needed to be taken but also necessitate shortening the time intervals between the doses. Thus, a modified-release hydrocortisone might be a useful alternative in this patient group. However, although authorized for the use in patients with ACC, it is unclear how concurrent mitotane treatment influences the pharmacokinetic profile of modified-release hydrocortisone. Besides increased metabolic inactivation by CYP3A4 induction, gastrointestinal side effects that can be associated with mitotane treatment may represent a further safety issue in this patient group. Of note, even for immediate release hydrocortisone, detailed data regarding serum pharmacokinetics in mitotane treated ACC patients is not available.

In a series of ACC patients in whom modified release hydrocortisone was considered as alternative treatment to immediate release hydrocortisone, we carried out 24-h pharmacokinetic measurements comparing cortisol (serum and saliva), free serum cortisol and plasma ACTH levels during one day of immediate release hydrocortisone and modified-release hydrocortisone, respectively, both administered at a dose of 40 mg first thing in the morning and 20 mg at lunchtime.

Methods

Study Population

Nine adult patients with histologically confirmed ACC on stable mitotane treatment for at least 4 months in whom alternative replacement therapy with modified release hydrocortisone was considered, were investigated. ENS@T stage at diagnosis was 2 (n=8) and 3 (n=1). The reason for mitotane treatment was adjuvant therapy (n=5) or metastatic disease (n=4; abdominal lymph nodes and lung) treatment. All patients had an Eastern Cooperative Oncology Group (ECOG) performance status 0 to 2 and no signs of severe renal or hepatic insufficiency. None of the patients had other concomitant anticancer treatment except from mitotane. However, two patients had received streptozotocin chemotherapy at an earlier stage. Immediate release hydrocortisone replacement therapy was taken for at least 4 months prior to the pharmacokinetic assessment. No patient received supplementary mineralocorticoid treatment. No patient experienced signs of hypocortisolism at time of investigation. Six patients had mitotane plasma levels within the proposed therapeutic range (14-20mg/l), three patients had levels below this range (6.6-9.6mg/l). Immediate release and modified release hydrocortisone are freely available for treatment of patients with adrenal insufficiency in Germany. All patients provided written informed consent for additional evaluation of blood, saliva and urine samples.

Design and assessments

On study day 1, ACC patients on mitotane treatment were taking 60mg conventional hydrocortisone (40-20-0mg), during study day 2 they took the same dose of modified-release hydrocortisone (40-20-0mg). On both days the timing of hydrocortisone tablet intake was 8:00h and 12:00h. Mitotane tablets were taken during meal times at 9:00

and 13:00. Blood and saliva samples were taken at 7:30, 8:00, 9:00, 10:00, 11:00, 12:00, 13:00, 14:00, 15:00, 16:00, 17:00. Saliva samples were collected with the Salivette system (Sarstedt, Etten-Leur, The Netherlands) in accordance with the manufacturer´s instructions.

24-hour urines were collected for analysis of urinary cortisol metabolites two weeks prior to the pharmacokinetic measurements while the patients were on 60mg conventional hydrocortisone (40-20-0mg).

Laboratory Measurements

Serum cortisol was measured by a solid-phase competitive chemiluminescent enzyme immunoassay (Siemens Healthcare, Erlangen, Germany), with an analytical sensitivity of 5.5 nmol/l (0.2 μg/dl). The intra- and interassay coefficients of variability (CVs) of the cortisol assay were 6.9% and 7.3%, respectively. Plasma ACTH was measured by a solid-phase, two-site sequential chemiluminescent assay (Siemens Healthcare). The intra- and interassay CVs were 9.6% and 8.8%, respectively. Salivary cortisol was analysed by electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics, Germany).

Serum CBG was measured by radioimmunoassay (MyBioSource, San Diego, California, USA). The intra- and interassay coefficients of variability (CVs) of the CBG RIA assay 3.9% and 5.5%, respectively. Calculated free cortisol was derived using the Coolens' equation: $U2 \times K (1 + N) + U \times [1 + N + K(T - C)] - C = 0$, where C is total cortisol (μM), T is CBG (μM), U is unbound cortisol, and K is the affinity of CBG for cortisol at 37^{o}C [21]. N is the ratio of albumin bound to free cortisol, and 1.74 is the value used. For K a value of $3x10^{-7}$ M^{-1} was assumed. This results in the formula

$U=(Z^2 + 0.0122*C)^{½} – Z(\mu M)$ with $Z=0.0167+0.182(T-C)$. We did not use the free cortisol index (calculated free unbound cortisol) as published by others [22].

Urine steroid metabolite excretion analysis was carried out by gas chromatography/mass spectrometry as previously described [23].

Mitotane levels at baseline were measured as previous described, by using a gas chromatography-electron capture detection assay [24].

Control cohort

For comparison with hydrocortisone pharmacokinetics in non mitotane-treated patients we included data from a recently published prospective study [25] investigating ten adult male patients with secondary adrenal deficiency on three different hydrocortisone regimens (20mg 8:00 + 10mg 16:00; 10mg 8:00 + 10mg 16:00; 10mg 8:00 + 5mg 16:00) and ten healthy male controls. Measurements and analytical methods were described previously [25]. In addition, 24-h urines were collected in all control subjects.

Statistical analyses

Area under the curve (AUC) was calculated by using the linear trapezoid method for the study time 8:00 until 17:00. Data are shown in mean ± SEM if not stated differently. P-values <0.05 were considered statistically significant. Independent samples t-test was used for two groups comparison and the Mann-Whitney U test for variables which were not normally distributed. Statistical analyses were performed using SPSS 15.0 (IBM, Armonk, NY, USA).

Results

Nine patients with ACC on mitotane treatment and on a stable daily immediate release hydrocortisone dose of 60mg were investigated. The clinical characteristics of the patients are shown in **Table 1**.

Serum cortisol levels showed a wide variation in the supraphysiological range (1300-3900nmol/l) after immediate release hydrocortisone intake, and a more homogenous pattern, but unanimously much lower profile, after modified release hydrocortisone intake (570-1630nmol/l) in ACC patients (**Figure 1**). After 40mg of immediate release hydrocortisone intake the serum cortisol level peaked significantly higher than with 40mg modified release hydrocortisone (2105±298 vs 1015±120nmol/l; p=0.003). The morning peak of immediate release hydrocortisone appeared one hour after the tablet intake, whereas the peak after the modified release hydrocortisone appeared two hours after tablet intake. The AUC of the serum cortisol levels was significantly higher with immediate release hydrocortisone than with modified release hydrocortisone (10577±1393 vs 8047±1021nmol*h/l,; p=0.03).

Most of the ACC patients showed suppressed ACTH levels with no differences between the two hydrocortisone preparations (data not shown).

Urine analysis confirmed the previously described [10] enhanced cortisol metabolism with an increased CYP3A4 activity and reduced 5α-reductase activity, and an overall increased total glucocorticoid excretion in our ACC patients on mitotane (**Table 2**).

Most of the cortisol (80-85%) is bound to the specific binding protein corticosteroid binding globulin (CBG), approx. 10% to albumin and the remaining is present in the free form. It is assumed that the bioavailability of endogenous glucocorticoids is predominantly influenced by CBG, and in situations where CBG levels are altered,

e.g. mitotane treatment, total cortisol levels may not adequately represent the free cortisol fraction. It is assumed that the biologically active level of cortisol to which tissues are exposed is free cortisol. Therefore we measured CBG, and showed that serum CBG levels in ACC patients were high (median 2.50 μmol/l). By correcting for the sex-specific upper-limit of the normal range, the CBG levels were 1.36- (0.87-3.0) fold (mean (range)) above sex-specific upper-limit of normal.

In ACC patients, calculated free cortisol levels showed more pronounced peaks (12-144nmol/l) after tablet intake of immediate release hydrocortisone (**Figure 2a**), whereas the peaks were blunted after intake of modified release hydrocortisone (5-33nmol/l) (**Figure 2b**). This resulted in significant, fourfold higher mean free cortisol levels after immediate release than after modified release hydrocortisone intake (46±14 vs 12±3nmol/l; p=0.03) (**Figure 3a**).

For comparison with immediate release hydrocortisone pharmacokinetics in non mitotane-treated patients, we included data from ten adult male with secondary adrenal insufficiency and ten healthy controls. In men with secondary adrenal insufficiency, a morning dose of immediate release hydrocortisone resulted in a mean free cortisol level of 64±16nmol/l (10mg dose) and 128±22nmol/l (20mg dose), respectively (**Figure 3b**). Physiological morning free cortisol levels peaked around 31±5nmol/l in ten healthy men (**Figure 3b**).

The AUC of the calculated free cortisol levels was significantly higher with immediate release hydrocortisone than with modified release hydrocortisone (149±37 vs 98±21nmol/l/h, respectively; p=0.02). Compared to the available data from men with secondary adrenal insufficiency, the AUC of the calculated free cortisol levels during treatment of immediate release hydrocortisone was slightly higher in ACC patients under mitotane than the AUC achieved by a 10mg-10mg-0mg treatment in secondary

male AI patients but lower than values achieved by a 20mg-10mg-0mg regime. The AUC of calculated free cortisol levels with 40-20-0mg modified release hydrocortisone (70±11nmol/l) resembled the levels achieved by a 10mg-5mg immediate release hydrocortisone regimen in male secondary AI patients (114±14nmol).

Salivary cortisol levels showed large variations after tablet intake and reached very high levels in some patients (**Figure 4**), exceeding normal salivary cortisol morning levels (1.3-25.7nmol/l) 20-50-fold. No significant difference in salivary cortisol levels was observed between immediate or modified release hydrocortisone intake.

Discussion

The main finding in our ACC patient group with mitotane treatment is that modified release hydrocortisone resulted in much lower total and free cortisol levels than immediate release hydrocortisone despite the administration of identical doses. In addition, modified release hydrocortisone in ACC patients lacked the physiological morning free cortisol peak seen in healthy subjects. In contrast, immediate release hydrocortisone in ACC patients provided a similar morning free cortisol peak as seen in healthy males and in males with secondary adrenal insufficiency with a 10mg immediate release hydrocortisone morning dose [25].

In a previously published pharmacokinetic study with modified release hydrocortisone the time to reach a clinically significant plasma concentration of cortisol (>200 nmol/l) was within 20 min and a mean peak of 431 nmol/l was obtained within 50 min after administration of the 20 mg tablet [18]. A 20mg tablet at a fasted state resulted in an AUC of 3634 nmol*h/l in healthy subjects [18]. In comparison to that study, here we administered the three-fold dose of modified release hydrocortisone in our patients and received a mean AUC of 8047 nmol*h/l. The slightly lower than expected rate might be explained by the fact that the absorption and bioavailability is not fully dose proportional. This is most likely due to reduced dissolution rate kinetics of hydrocortisone in the intestinal lumen at the higher dose that will in turn reduce the absorption rate [18]. In addition, we studied ACC patients on mitotane treatment and not healthy volunteers. Mitotane is known to have a wide range of gastrointestinal side effects [26] possibly influencing intestinal hydrocortisone absorption. Furthermore CYP3A4 induction by mitotane results in rapid inactivation of more than 50% of administered hydrocortisone, explaining the need for at least doubling of the hydrocortisone replacement dose in mitotane-treated patients to achieve a

comparable glucocorticoid exposure as is reached in subjects not treated with CYP3A4 inducing compounds [10]. Comparing mean serum total cortisol AUC of immediate and modified release hydrocortisone in ACC patients on mitotane resulted in a trend to lower AUC for modified release hydrocortisone (10577±4179 nmol/l*h vs. 8047±3066 nmol/l*h, p=0.163), suggesting that glucocorticoid exposure achieved by modified release hydrocortisone might be lower compared to immediate release hydrocortisone. This difference is similar to what has been observed in patients with Addison's disease receiving either glucocorticoid replacement.

Interestingly, the total levels of cortisol achieved by a treatment with 40-20-0 immediate release regimen are high (**Figure 1**), suggesting that daily doses of 40-50mg of immediate release hydrocortisone might be sufficient. However, this dose reduction would not be justified by our observation that free cortisol levels were comparable to the levels obtained with a 10mg immediate release morning dose in patients with adrenal insufficiency (**Figure 2-3**) [25]. In general, deduced from the cortisol levels measured in these patients, glucocorticoid coverage was achieved by the 40-20-0mg immediate release hydrocortisone regimen which may be regarded as sufficient and without cortisol trough levels below a value that would pose a safety issue.

In case of administration of modified release hydrocortisone, a once daily administration is generally recommended. On the background of the rapid metabolic inactivation of cortisol due to increased CYP3A4 activity by mitotane, we assumed that a once daily regime might not be sufficient to secure sufficient glucocorticoid coverage over the whole day and decided to test a twice daily regimen. However, considering our measurements we believe that even a 40mg-20mg-0mg modified

release hydrocortisone regimen did not provide sufficient free cortisol levels in mitotane treated ACC patients.

Salivary cortisol is regarded as CBG-independent measurement reflecting the free serum cortisol. Therefore. it is thought to be a good tool to monitor glucocorticoid replacement therapy in ACC patients on mitotane. Our results show massively increased salivary cortisol levels with a 40mg-20mg-0 hydrocortisone replacement therapy in ACC patients on mitotane. A recent study showed that the ECLIA immunoassay possesses a quite large cross-reactivity with endogenous cortisol precursors and metabolites, especially with 6β-hydroxycortisol (148% cross reactivity) [27], the main cortisol metabolite in mitotane treated patients. Therefore, we believe that our salivary results are influenced and overestimated by this cross-reactivity. This is endorsed by another study showing a significant difference in salivary cortisol levels measured with the ECLIA method and LC-MS/MS [28]. Therefore we suggest that salivary cortisol levels should be measured only with LC-MS/MS in mitotane treated ACC patients.

It must be taken into account that both replacement regimes were tested only for one day, and we do not have data on longer treatment periods with modified release hydrocortisone in mitotane-treated ACC patients. However, it would be interesting to further investigate patient acceptance and quality of life in mitotane treated ACC patients depending on the form of hydrocortisone replacement therapy. In addition, a longer duration of observation would have provided information on the impact of potential gastrointestinal effects of mitotane on hydrocortisone kinetics. Longer study durations must also include safety aspects such as the frequency of adrenal crisis in those patients. The relevance of the lacking morning cortisol peak and the low free

cortisol levels during the day in ACC patients treated with modified release hydrocortisone further remains to be clarified.

In conclusion, here we show that immediate release but not modified release hydrocortisone at a dose of 60mg per day result in sufficient glucocorticoid coverage in patients with ACC under mitotane treatment. Glucocorticoid exposure in mitotane-treated patients receiving modified release hydrocortisone appears to be considerably lower compared to the same dose of immediate release hydrocortisone, suggesting that the use of equivalent doses of modified release hydrocortisone preparation should be avoided in patients on mitotane treatment.

Reference List

(1) Soreide, J.A., Brabrand, K., Thoresen, S.O. (1992) Adrenal cortical carcinoma in Norway, 1970-1984. *World J Surg* **16,**pp. 663-667.

(2) Brennan, M.F. (1987) Adrenocortical carcinoma. *CA Cancer J Clin* **37(6),**pp. 348-365.

(3) Lafemina, J., Brennan, M.F. (2012) Adrenocortical carcinoma: Past, present, and future. *J Surg Oncol*,p. 10.

(4) Sullivan, M., Boileau, M., Hodges, C.V. (1978) Adrenal cortical carcinoma. *J Urol* **120,** (6),pp. 660-665.

(5) Berruti, A., Fassnacht, M., Baudin, E., Hammer, G., Haak, H., Leboulleux, S., et al. (2010) Adjuvant therapy in patients with adrenocortical carcinoma: a position of an international panel. *J Clin Oncol* **28(23),**p. e401-e402.

(6) Hahner, S., Fassnacht, M. (2005) Mitotane for adrenocortical carcinoma treatment. *Curr Opin Investig Drugs* **6,** (4),pp. 386-394.

(7) Terzolo, M., Angeli, A., Fassnacht, M., Daffara, F., Tauchmanova, L., Conton, P.A., et al. (2007) Adjuvant mitotane treatment for adrenocortical carcinoma. *N Engl J Med* **356(23),**pp. 2372-2380.

(8) Sbiera, S., Leich, E., Liebisch, G., Sbiera, I., Schirbel, A., Wiemer, L., et al. (2015) Mitotane Inhibits Sterol-O-Acyl Transferase 1 Triggering Lipid-Mediated Endoplasmic Reticulum Stress and Apoptosis in Adrenocortical Carcinoma Cells. *Endocrinology* **156(11),**pp. 3895-3908.

(9) van Erp, N.P., Guchelaar, H.J., Ploeger, B.A., Romijn, J.A., Hartigh, J., Gelderblom, H. (2011) Mitotane has a strong and a durable inducing effect on CYP3A4 activity. *Eur J Endocrinol* **164(4),**pp. 621-626.

(10) Chortis, V., Taylor, A.E., Schneider, P., Tomlinson, J.W., Hughes, B.A., O'Neil, D.M., et al. (2013) Mitotane therapy in adrenocortical cancer induces CYP3A4 and inhibits 5alpha-reductase, explaining the need for personalized glucocorticoid and androgen replacement. *J Clin Endocrinol Metab* **98(1),**pp. 161-171.

(11) Alexandraki, K.I., Kaltsas, G.A., le Roux, C.W., Fassnacht, M., Ajodha, S., Christ-Crain, M., et al. (2010) Assessment of serum-free cortisol levels in

patients with adrenocortical carcinoma treated with mitotane: a pilot study. *Clin Endocrinol (Oxf)* **72(3),**pp. 305-311.

(12) Allolio, B., Fassnacht, M. (2006) Adrenocortical Carcinoma: Clinical Update. *J Clin Endocrinol Metab*.

(13) Haak, H.R., Hermans, J., van, d., V, Lentjes, E.G., Goslings, B.M., Fleuren, G.J., et al. (1994) Optimal treatment of adrenocortical carcinoma with mitotane: results in a consecutive series of 96 patients. *Br J Cancer* **69,** (5),pp. 947-951.

(14) Hague, R.V., May, W., Cullen, D.R. (1989) Hepatic microsomal enzyme induction and adrenal crisis due to o,p'DDD therapy for metastatic adrenocortical carcinoma. *Clin Endocrinol (Oxf)* **31(1),**pp. 51-57.

(15) Kroiss, M., Quinkler, M., Lutz, W.K., Allolio, B., Fassnacht, M. (2011) Drug interactions with mitotane by induction of CYP3A4 metabolism in the clinical management of adrenocortical carcinoma. *Clin Endocrinol (Oxf)* **75,** (5),pp. 585-591.

(16) Van Seters, A.P., Moolenaar, A.J. (1991) Mitotane increases the blood levels of hormone-binding proteins. *Acta Endocrinol (Copenh)* **124(5),**pp. 526-533.

(17) Johannsson, G., Filipsson, H., Bergthorsdottir, R., Lennernas, H., Skrtic, S. (2007) Long-acting hydrocortisone for glucocorticoid replacement therapy. *Horm Res* **68 Suppl 5,**pp. 182-188.

(18) Johannsson, G., Bergthorsdottir, R., Nilsson, A.G., Lennernas, H., Hedner, T., Skrtic, S. (2009) Improving glucocorticoid replacement therapy using a novel modified-release hydrocortisone tablet: a pharmacokinetic study. *Eur J Endocrinol* **161,** (1),pp. 119-130.

(19) Johannsson, G., Nilsson, A.G., Bergthorsdottir, R., Burman, P., Dahlqvist, P., Ekman, B., et al. (2012) Improved cortisol exposure-time profile and outcome in patients with adrenal insufficiency: a prospective randomized trial of a novel hydrocortisone dual-release formulation. *J Clin Endocrinol Metab* **97(2),**pp. 473-481.

(20) Quinkler, M., Miodini, N.R., Zopf, K., Ventz, M., Oksnes, M. (2015) Modified-release hydrocortisone decreases BMI and HbA1c in patients with primary and secondary adrenal insufficiency. *Eur J Endocrinol* **172(5),**pp. 619-626.

(21) Coolens, J.L., Van, B.H., Heyns, W. (1987) Clinical use of unbound plasma cortisol as calculated from total cortisol and corticosteroid-binding globulin. *J Steroid Biochem* **26(2),**pp. 197-202.

(22) Bonte, H.A., van den Hoven, R.J., van, d.S., V, Vermes, I. (1999) The use of free cortisol index for laboratory assessment of pituitary-adrenal function. *Clin Chem Lab Med* **37(2),**pp. 127-132.

(23) Arlt, W., Biehl, M., Taylor, A.E., Hahner, S., Libe, R., Hughes, B.A., et al. (2011) Urine steroid metabolomics as a biomarker tool for detecting malignancy in adrenal tumors. *J Clin Endocrinol Metab* **96(12),**pp. 3775-3784.

(24) Moolenaar, A.J., Niewint, J.W., Oei, I.T. (1977) Estimation of o,p'-DDD in plasma by gas-liquid chromatography. *Clin Chim Acta* **76(2),**pp. 213-218.

(25) Behan, L.A., Rogers, B., Hannon, M.J., O'Kelly, P., Tormey, W., Smith, D., et al. (2011) Optimizing glucocorticoid replacement therapy in severely adrenocorticotropin-deficient hypopituitary male patients. *Clin Endocrinol (Oxf)* **75,** (4),pp. 505-513.

(26) Fassnacht, M., Kroiss, M., Allolio, B. (2013) Update in adrenocortical carcinoma. *J Clin Endocrinol Metab* **98(12),**pp. 4551-4564.

(27) Krasowski, M.D., Drees, D., Morris, C.S., Maakestad, J., Blau, J.L., Ekins, S. (2014) Cross-reactivity of steroid hormone immunoassays: clinical significance and two-dimensional molecular similarity prediction. *BMC Clin Pathol* **14,**pp. 33-14.

(28) Carrozza, C., Lapolla, R., Gervasoni, J., Rota, C.A., Locantore, P., Pontecorvi, A., et al. (2012) Assessment of salivary free cortisol levels by liquid chromatography with tandem mass spectrometry (LC-MS/MS) in patients treated with mitotane. *Hormones (Athens)* **11(3),**pp. 344-349.

Table 1: Patients´ characteristics Median (Range). BMI, body mass index; ACC, adrenocortical cancer; SAI, secondary adrenal insufficiency. na = not available

Characteristics	**ACC patients**	**SAI patients**	**Healthy controls**
Male/female	6/3	10/0	10/0
Age (years)	54 (27-75)	48 (26-71)	45 (27-67)
Time since diagnosis (months)	44 (6-192)	na (36-216)	-
BMI (kg/m^2)	24.0 (17.2-33.3)	28.4 (25.4-40.1)	28.3 (25.3-38.5)
Duration of mitotane treatment (months)	34 (6-87)	-	-
Daily mitotane dose (mg)	1500 (0-2500)	-	-

Table 2: 24-hour urine analysis by gas chromatography/mass spectrometry in 9 adrenocortical cancer (ACC) patients on mitotane, 10 male patients with secondary adrenal insufficiency (SAI) on different hydrocortisone (HC) dose regimen, and 10 healthy male subjects. F = cortisol; THF = tetrahydro-cortisol. Means ± SD.

	6β-OH-F/F ratio (CYP3A4 activity)	**5αTHF/THF ratio** (5α reductase activity)	**Total glucocorticoid excretion** (μg/24h)
ACC patients on 40-20mg HC and mitotane	22.6 ± 4.1	0.05 ± 0.02	27634 ± 6940
SAI patients on 20-10mg HC	2.0 ± 0.2	1.9 ± 0.4	21474 ± 4843
SAI patients on 10-10mg HC	1.9 ± 0.2	1.9 ± 1.1	13502 ± 2979
SAI patients on 10-5mg HC	1.8 ± 0.8	1.9 ± 0.7	11891 ± 3053
Healthy controls	2.3 ± 0.5	1.1 ± 0.4	14651 ± 7648

Figure 1: Serum-Cortisol in nine ACC patients on mitotane receiving 40mg (8.00) and 20mg (12.00) of a) immediate release hydrocortisone or b) modified release hydrocortisone. Black line = median. Black arrow = time of tablet intake. To convert to conventional units, divide cortisol by 27.59 (μg/dl).

Figure 2: Calculated free serum cortisol (nmol/l) in nine ACC patients on mitotane receiving 40mg (8.00) and 20mg (12.00) of a) immediate release hydrocortisone or b) modified release hydrocortisone. Black line = median. Black arrow = time of tablet intake. To convert to conventional units, divide cortisol by 27.59 (μg/dl).

Figure 3: Mean calculated free serum cortisol (nmol/l) in a) nine ACC patients on mitotane receiving 40mg (8.00) and 20mg (12.00) of immediate release hydrocortisone (blue line) or modified release hydrocortisone (red line), and b) in ten male patients with secondary adrenal insufficiency on 20mg-10mg-0 (blue line), 10mg-10mg-0 (red line) or 10mg-5mg-0 (green line) immediate release hydrocortisone dose regimen and in ten healthy male subjects (pink line). Black arrow = time of tablet intake (not valid for healthy subjects). Mean ± SEM. To convert to conventional units, divide cortisol by 27.59 (μg/dl).

Figure 4: Mean salivary cortisol (nmol/l) in nine ACC patients on mitotane receiving 40mg (8.00) and 20mg (12.00) of immediate release hydrocortisone (blue line) or modified release hydrocortisone (red line). Black arrow = time of tablet intake. Mean ± SEM. To convert to conventional units, divide cortisol by 27.59 (μg/dl). The range of normal salivary cortisol morning levels (1.3-25.7nmol/l) is shown as grey area.