Development of a new non-invasive method to detect the cortisol level in human skin

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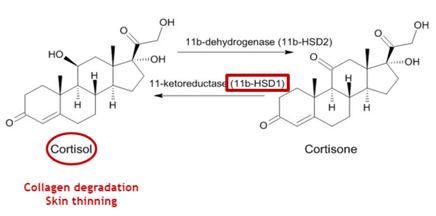
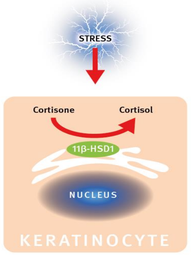
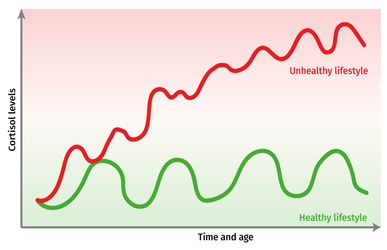
Introduction:

In skin care recently mental stress has come in the scope of risk factors for skin damages both in short term (e.g. barrier disruption) (1) but also in long term as a pro-aging factor based on the finding that 11betaHSD1 is upregulated in aged skin and by UV irradiation (2, 3). Cortisol is recognized as key stress related hormonal marker in saliva, blood, hair and other tissues. So far little is known about cortisol level in skin however it has been shown that cortisol can be synthesized locally via an HPA-axis equivalent (4) as well as by activation of 11betaHSD1 that converts to cortisol from inactive cortisone. Since it has been shown that 11betaHSD1 is upregulated in aged skin and photoaged skin it is assumed that local cortisol levels are also upregulated in these skin conditions. Previous attempts to detect cortisol from skin were of limited success mostly due to lack of sensitivity for cortisol detection (5, 6).

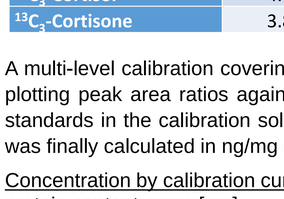




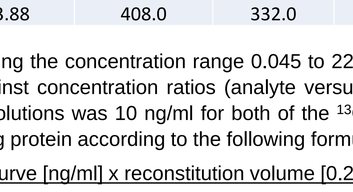


In order to investigate and control the cortisol level in skin the aim was to develop a robust analytical method for cortisol detection that is more sensitive than existing methods. The aim was also to make the analytical method applicable for sample material that was collected in vivo directly from human subjects with the simple but non-invasive tape stripping method. An additional goal was to use this method to measure samples form a human in vivo study with a formulation containing biphenyl azepanyl methanone (BAM), an inhibitor of 11betaHSD1.

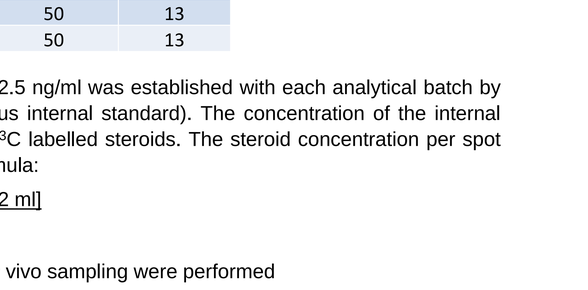
Materials & Methods:



6 times tape strip sampling as described elsewhere (7-9)



Protein content by method of Voegeli (10) using a Squame Scan™ 850A IR densitometer



Sampling via tape strips (D-squames) and processing is shown in the diagram. The methanolic extract (5µl) was injected for LC-MS/MS analysis onto an Agilent 6495 triple quadrupole MS system equipped with electrospray ionization source operating in negative mode. Separation was achieved on a column switching system comprising a two-position six-port valve, two UHPLC pumps, a Poroshell HPH C18 (2.0 x 50 mm, 2.7 um) analytical column and a trapping column (2.1 x 5 mm) of the same material. The mobile phase consisted of water (A) and methanol (B), both containing 5 mM ammonium acetate, and 100% acetonitrile (C). A gradient was developed on the analytical column from 28% B to 95% B in 4.3 min before the it was flushed with 100% acetonitrile for 2 minutes and equilibrated again to 28% B for another 3 minutes. Signals were recorded in multiple reaction monitoring mode using the transitions listed in **table 1**.

Table 1: MRM transitions, collision energies and dwell time for analytes and ^{13}C labelled internal standards

Analyte	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Dwell time (msec)	CE (V)
Cortisol (quantifier)	4.05	407.2	331.1	50	13
Cortisol (qualifier)	4.05	407.2	297.0	50	35
Cortisone (quantifier)	3.88	405.2	329.2	50	13
Cortisone (qualifier)	3.88	405.2	301.2	50	21
$^{13}C_3$-Cortisol	4.05	410.0	334.0	50	13
$^{13}C_3$-Cortisone	3.88	408.0	332.0	50	13

A multi-level calibration covering the concentration range 0.045 to 22.5 ng/ml was established with each analytical batch by plotting peak area ratios against concentration ratios (analyte versus internal standard). The concentration of the internal standards in the calibration solutions was 10 ng/ml for both of the ^{13}C labelled steroids. The steroid concentration per spot was finally calculated in ng/mg protein according to the following formula:

$$\frac{\text{Concentration by calibration curve [ng/ml] x reconstitution volume [0.2 ml]}}{\text{protein content curve [mg]}}$$

To validate the assay method a few pilot tests with time dependent in vivo sampling were performed

BAM Study: Human in vivo study to measure cortisol levels after application of formula with 11beta-HSD1 inhibitor (BAM):

Informed consent by subjects and ethical principles according to Helsinki protocol were followed.

30 subjects used the placebo formulation, and another 30 subjects used a formulation containing 20ppm biphenyl azepanyl methanone (BAM), a 11beta-HSD1 inhibitor (commercial product from DSM Nutritional Products). Most of volunteers had self-perceived dry skin with mean age 55.5 ± 4.8y. 6 tape strips were taken in a row on the cheek, 3cm beneath the outer edge of the eye at t = 0 and after 12 weeks treatment on other side of the face. Samples stored frozen at -80°C. Trans epidermal water loss (TEWL) was determined with a Tewameter® TM300 at t=0, then after 4 and 12 weeks of product application. Measures were taken on same skin position used for tape stripping but before tape-stripped and skin density was measured too by DermaScan ® C (Cortex Technology).

Results & Discussion:

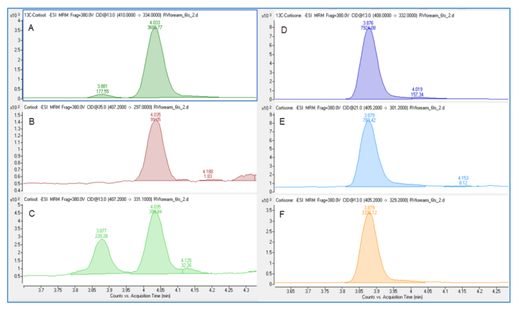


Figure 1: Typical MRM chromatograms from a biological sample: 6 skin strips sampled from the volar forearm of a 53 year old were processed according to the method. A= ^{13}C-Cortisol peak at 4.033 min; B= Cortisol qualifier at 4.035 min; C= Cortisol quantifier at 4.035 min; D= ^{13}C-Cortisone peak at 3.876; E= Cortisone qualifier at 3.879 min; F= Cortisone quantifier at 3.879 min

Technical limits of quantification (LOQ) of 14.5 pg/mL for cortisone and 25.1 pg/mL for cortisol were obtained. Linearity of the calibration curve was given over a concentration range of 0.045 – 22.5 ng/mL for cortisone and cortisol. The repeatability of the method was below 6% relative standard deviation (RSD). With this LOQ cortisol levels as low as 12pg/mg protein could be detected from stratum corneum samples. **Figure 1** depicts typical LC-MS chromatograms from a human stratum corneum sample

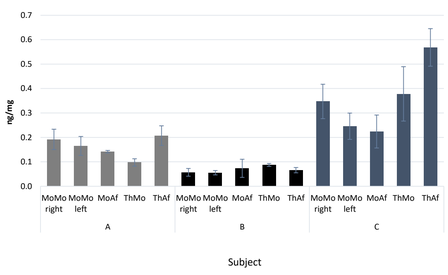


Figure2: Cortisol detection (ng/mg skin protein) after in vivo tape stripping in triplicates, error bars correspond to standard deviation, MoMo, MoAf = Monday morning/afternoon, ThMo, ThAf = Thursday morning/afternoon, three subjects A, B, C, sampling area left and right volar forearm

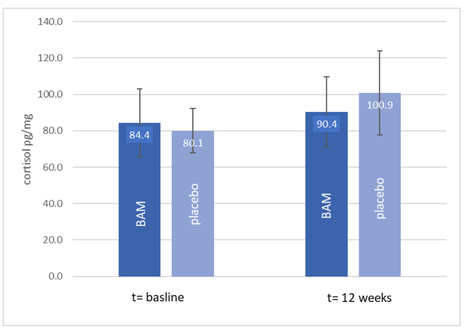


Figure 3, from BAM Study: Average cortisol levels measured in the samples from placebo group and BAM treated group Error bars = SEM

Assay validation by in vivo sampling:

As the technical repeatability of the cortisol analysis was very good, we also evaluated the intrinsic assay variance when doing the complete process from in vivo sampling via tape strips to the LC-MS/MS analysis. For this we did a small pilot study on three different subjects. We did the tape stripping on the forearms in triplicates as most nearby as possibly to minimize skin site specific effects. We repeated the sampling in the morning and afternoon of the same day and on two different weekdays. The results are shown in **figure 2**. The standard deviations obtained from the triplicates were low demonstrating a good reproducibility for the complete assay process. The variance with time from morning to afternoon or between weekdays and in particular the inter individual variance was higher than the standard deviations of the triplicates.

BAM- study results in vivo after treatment with BAM, the 11betaHSD1 inhibitor:

Cortisol levels could be detected in 82 samples resulting in high variability between individuals from 11pg/mg to 530pg/mg and two outliers one at 3.76ng/mg and one with 186ng/mg. These high levels were most probably due to illicit use of cortisol containing medication during the study. For the results see also **figure 3**.

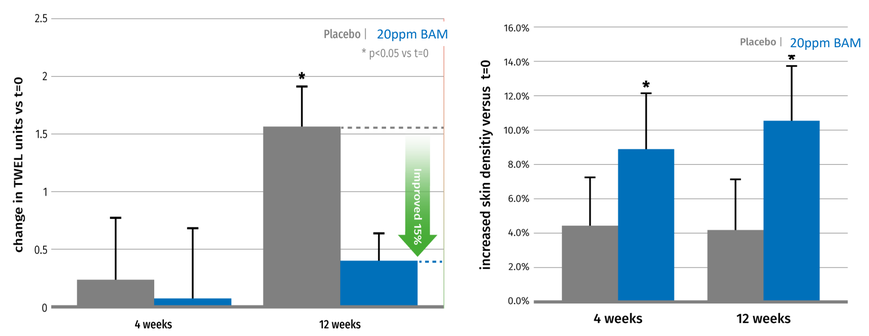


Figure 4 (BAM study) left diagram: TEWL values measured on cheeks for the placebo group and the group treated with the BAM formulation. Changes in TEWL units versus baseline are shown, * p<0.05 significant change vesus baseline, Error bars = SEM, right diagram % increase in skin density versus baseline, * p<0.05 significant change vesus baseline, Error bars = SEM

After 12 weeks treatment with cosmetic formulation including 20ppm of BAM the mean cortisol levels remained almost stable and for the placebo group it increased by 26% from 80pg/mg to more than 100pg/mg cortisol.
In the same study the TEWL and the skin density was measured on the cheek area with significant changes and better skin parameters for the BAM treated group (**figure 4**).

Conclusions:

A novel LC-MS/MS method alongside an appropriate sample preparation was developed offering a reliable measurement of cortisone and cortisol in stratum corneum. Pooled sample material from 6 tape strips were necessary to get a robust result for both cortisol and cortisone concentration. This method is more sensitive than previous studies using LC-MS or ELISA immunodetection (5, 6). Our validation study with *in vivo* sampling demonstrated that the assay reproducibility was in a good range and the standard deviations from triplicates lower than the variance with time and with individual changes.
After topical treatment of the skin for 12 weeks with cosmetic formulation containing 20ppm of BAM (11β HSD1-inhibitor) the mean cortisol level remained almost stable whereas after 12 weeks of placebo use the cortisol levels increased over time by about 26%. However other individual factors seemed to considerably modulate cortisol levels since the inter-individual variability of analytes was very high and therefore statistical significance on change of cortisol levels was not achieved in this study. However, in tendency the results correlated well to TEWL values and skin density. TEWL values increased for the placebo group (+20%, $p<0.05$) but remained stable during the study for the BAM treated group. We speculate the facial TEWL increased for the placebo group due to seasonal change of weather conditions (September to December in Switzerland) whereas this negative trend was mostly prevented in the groups using the BAM formulation and thus the BAM formulation very well protected the skin barrier potentially via inhibition of excess of cortisol formation. Also, we observed a significant increase in skin density that could be assigned to inhibition of catabolic effects by cortisol.
Further studies need to be done to better understand cortisol modulation in skin by e.g. optimizing sampling method, defining key inclusion/exclusion criteria for volunteers, eventually including individual lifestyle information and/or using larger cohorts.

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