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Hair analyses of steroids as tool of disease control in patients with congenital adrenal hyperplasia

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Abstract

Patients with Congenital Adrenal Hyperplasia (CAH) due to 21-hydroxylase deficiency need a lifelong replacement therapy with corticosteroid hormones due to enzyme deficiency in the endogenous steroid synthesis. Despite circadian deviations and inter-individual variations, the urine and blood concentrations of steroids are utilized as clinical parameters to optimize and control medication of CAH-patients. Blood concentrations of the androgenic hormones are important to get an estimation of disease control in CAH-patients. However, by using blood analyses only short-time information are available and retrospective statements are not possible. Therefore, the usefulness of hair analyses as a method to get a long-term record was investigated. Hair concentrations of steroid hormones were measured by LC-ESI-MS/MS in six CAH-patients. The permanent medication with glucocorticoids was detectable in all patients. In case of one CAH-patient the steroid concentrations in blood and hair differed significantly compared to other patients, suggesting an insufficient dosage of glucocorticoid substitution or non-compliance in medication. In addition to the concentrations in blood, hair analysis is applicable for purposes of disease control. The detection of glucocorticoids in hair can be used for drug testing in horses where they are prohibited permanently, unlike the human sports.

Introduction

Congenital adrenal hyperplasia is a dysfunction of the adrenal cortex characterized by enzyme deficiencies, especially of the steroid 21-hydroxylase, in the steroid biosynthesis. This enzyme defect causes a decrease of endogenous synthesis of gluco-/mineralocorticoids. The negative feedback-mechanism effects an increased synthesis of steroid precursors such as progesterone and 17-OH-progesterone as well as the androgens androstenedione and testosterone (Figure 1) [1]. To correct this hormonal imbalance and prevent comorbidities, lifelong replacement of the missing steroids is essential. Cortisol can be replaced by administration of hydrocortisone, predniso(lo)ne or dexamethasone [2].
Usually, disease control is accomplished by regular analysis of steroid hormones in blood and urine. The aim of this study was to test the potential of hair analyses to complement these measurements for disease and compliance controls to get retrospective conclusions up to months.

Experimental

Sample:
Hair samples of six patients with congenital adrenal hyperplasia were used in the study. Patient characteristics as well as medication are presented in Table 1.

Hair analysis:
Hair specimens were cut into 3 cm segments, depending on hair length. Hair segments were pulverised and 50 mg were processed if available. Calibration samples were prepared using blank material spiked with a mixture of progesterone, testosterone, androstenedione, 17-OH-progesterone, cortisol, cortisone, dexamethasone and prednisone. 19-Nortestosterone-d_3 and cortisol-d_4 were added as internal standards.

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Extraction of hair samples was carried out in an ultrasonic bath at 50 °C for 4 h using 2.5 mL of methanol. After separation 20 µL of ethylene glycol were added to the supernatant and then it was evaporated nearly to dryness under nitrogen stream at 60 °C. Resuspension was carried out with 0.5 mL water, and the solution was transferred to SPE column (Agilent, Plexa, 3 mL, 60 mg). SPE column was activated with 3 mL methanol and 1 mL water before use. The loaded column was washed with 2 mL methanol/2% NH_4OH-solution (40/60; v/v) and eluted with 2 mL methanol/water (90/10; v/v). 20 µL of ethylene glycol were added to the eluate. It was evaporated nearly to dryness and resuspended in 30 µL mobile phase for HPLC-ESI-MS/MS analysis. A 4 µL volume of the resuspended extract was injected onto the column (100 x 2.1 mm, 3 µm, C18, Hypersil Gold, Thermo). The chromatographic separation was operated on the LC-system 1200 series (Agilent Technologies). A flow-rate of 300 µL/min and a linear gradient from 90 % mobile phase A to 100 % mobile phase B were used. Mobile phase A was water/acetonitrile (95/5), 2 mmol NH_4Ac and phase B water/acetonitrile (5/95) 2 mmol NH_4Ac. Total run time was 14 min. Detection was carried out by Q-Trap 5500 (AB Sciex).

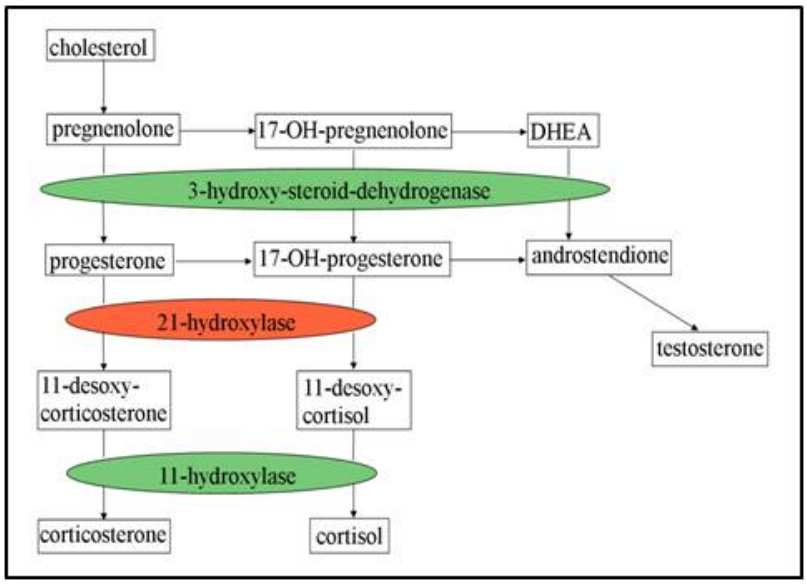

Figure 1: Localization of the 21-hydroxylase deficiency (red) in the biosynthesis of steroid hormones. For disease control the blood concentrations of 17-OH-progesterone, DHEA testosterone and androstendione were measured. For hair analyses the same steroid hormones as well as the applied corticosteroides were used as analytes.

	gender	age [years]	length of hair [cm]	medication	daily dose [mg]
P-1	f	19	25	prednisone	7.5
P-2	m	39	5	dexamethasone	0.375
P-3	m	32	2	prednisone	7.5
P-4	f	26	22	hydrocortisone	12.5
P-5	m	33	2.5	hydrocortisone	30
P-6	m	22	2	hydrocortisone	40

Table 1: Patients characteristics

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Results and Discussion

The limits of detection of this method are 1 pg/mg for androstenedione, 2 pg/mg for cortisone, cortisol and dexamethasone, 5 pg/mg for testosterone and 6 pg/mg for progesterone, 17-OH-progesterone and prednisone.
The applied glucocorticoids were detectable in all hair segments of the patients, but concentrations decline along the hair shaft from roots to tips. Glucocorticoid decrease is probably the result of the wash-out effect. Li et al. showed that hair concentrations of glucocorticoids, e.g. cortisol, decline significantly after washing with water (and shampoo) and by UV-irradiation [3]. Therefore, retrospective analyses of glucocorticoid steroids in hair are limited to the previous 3 months, and do not allow conclusions on the applied dosage.
If the androstenedione and/or testosterone blood concentrations are above the normal range it will commonly show a false adjustment of the daily dose or an irregular intake of medicine. To get more information, the steroid precursor concentrations in blood were also ascertained. In case of the CAH-patient P-6 the blood concentration of androstenedione was significantly above the normal range. The 17-OH-progesterone concentration was also increased compared to the other CAH-patients (Table 2).
The hair analysis showed, that the concentrations are increased, too. Because of these findings it can be supposed that disease control in patient P-6 was insufficient either due to a too low glucocorticoid replacement dose or due to non-compliance during the last 2 months.
In three of the other patients (P-2, P-3 and P-5) the 17-OH-progesterone concentration in blood and hair was also above the normal range. In clinical diagnostics, only an increased concentration of this steroid precursor in addition to an abnormal androgen concentration is interpreted as an advice for insufficient disease control.

	17-OH-progesterone		**androstenedione**	
	blood [ng/ml]	hair [pg/mg]	blood [ng/ml]	hair [pg/mg]
P-1	3.2	15.7	1.2	< 1
P-2	24.5	20.2	2.8	< 1
P-3	8.1	70.7	3.7	< 1
P-4	0.3	< 6	< 0.3	< 1
P-5	10.7	30.7	2.3	< 1
P-6	35.5	110.9	6	13
normal range	0.1 - 3.0 [4]	1.3 – 6.9 [5]	0.3 – 3.3 [4]	4.9 – 14.6 [5]

Table 2: Blood and hair concentrations (proximal segment) of 17-OH-progesterone and androstenedione in CAH-patients compared to the normal ranges in healthy people

Conclusions

The results of the present study show, that hair analyses can be used as supplementary method to blood analyses. The possibility to detect corticosteroids over a longer period [6,7] can be used for doping controls; especially in horses (e.g. stallion licensing and acquisition). Due to the low detection limits, it is possible to identify o corticosteroid use or misuse up to 3 months retrospectively.
In case of the CAH-patients the advantage over blood is the possibility to evaluate disease control over a longer time in the proximal hair segment. Androstenedione and 17-OH-progesterone seem to be appropriate markers to monitor efficient medication in CAH using hair samples

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References

[1] Migeon CJ, Wisniewski AB. (2001) Congenital adrenal hyperplasia owing to 21-hydroxylase deficiency. Growth, development, and therapeutic considerations. *Endocrinol Matab Clin North Am* **30**, 193-206.
[2] Merke DP, Cutler GB Jr. (2001) New ideas for medical treatment of congenital adrenal hyperplasia. *Endocrinol Metab Clin North Am* **30**, 121-135.
[3] Li J, et al. (2012) Time course of cortisol loss in hair segments under immersion in hot water. *Clin Chim Acta*, **413**, 434-440.
[4] Reisch N, et al. (2009) High prevalence of reduced fecundity in men with congenital adrenal hyperplasia. *J Clin Endocrinol Metab* **94** *(5)*, 1665-1670.
[5] Scherer CR, Reinhardt G.(1995) Kongressbericht der 74.Jahrestagung der Deutschen Gesellschaft für Rechtsmedizin, (Ed.H.Althoff), Aachen.
[6] Bévalot F, et al. (2000) Analysis of corticosteroids in hair by liquid chromatography –electrospray ionization mass spectrometry. *J Chromatogr B Biomed Sci Appl* **740**, 227-236.
[7] Cirimele V, et al. (2000) Identification of ten corticosteroids in human hair by liquid chromatography-ionspray mass spectrometry *Forensic Sci Int*. **107**, 381-388.

 ISBN 978-3-86884-038-4