be possible to detect other polymorphic markers more amenable to quantification by real-time PCR.

References

Measurement of Cortisol in Small Quantities of Saliva, Carolina de Weerth,1* Gerard Graat,2 Jan K. Buitelaar,3 and Jos H.H. Thijsse4 1Child and Adolescent Psychiatry, University Medical Center Utrecht, HP A01.468, Postbox 85500, 3508 GA Utrecht, The Netherlands;2Endocrinology Laboratory, University Medical Center Utrecht, HP KC.03.063.0, Postbox 85090, 3508 AB Utrecht, The Netherlands;3Department of Psychiatry, University Medical Center Nijmegen, HP 333, Postbox 9101, 6500 HB Nijmegen, The Netherlands;4author for correspondence: fax 31-30-2505487, e-mail C.deWeerth@psych.azu.nl)

The determination of cortisol in saliva has become popular for human research on stress reactions (1–5). Depending on the sensitivity and reliability of the assays used, the required sample volume varies between 0.025 and 2 mL of saliva (6–8). Infants and toddlers, however, often produce only small amounts of saliva and are usually sampled by swabbing the mouth with cotton dental rolls (5) or commercial cotton swabs (Salivette; Sarstedt Inc.) (9), or by pipettes or alternative devices that aspirate saliva from the floor of the mouth (10–13). Cotton rolls must either be centrifuged to obtain saliva (9) or be placed in the barrel of a syringe (needleless), from which the saliva is expressed into a vial by compression of the plunger (5). With these procedures, saliva remaining in the swabs is thus lost for analysis. When we tested seven different types of cotton rolls, we found that, depending on the individual type, 135–450 µL of saliva could not be centrifuged from the rolls.

Oral stimulants (such as presweetened Kool-Aid crystals) can increase saliva production, but they affect the concentration of cortisol (14). Finally, in the case of Salivettes, the material covering the cotton swab is hard and makes sampling unpleasant.

In this report, we present a new method that uses soft cotton swabs without hard covering material and solvent extraction of cortisol from saliva in the cotton.

Saliva was collected from volunteers in the laboratory and from infants and toddlers participating in studies on cortisol and behavior. Volunteers and the parents of the infants gave informed consent. These studies had been approved by the Medical Ethical Committee of the University Medical Center Utrecht. After collection, either direct or with use of cotton rolls, the samples were stored in closed containers at −20 °C for periods of up to several weeks. We placed 4-cm cotton rolls with a diameter of 8 mm (article no. 900-2005; Henri Schein) individually in disposable 5-mL syringes (PE + PP; Becton Dickinson), closed the syringes with a small plastic cap, and weighed them. For the saliva collection, the cotton roll was taken out of the syringe and the child’s mouth was swabbed by introducing one end of the cotton roll into the buccal cavity. The experimenter moved the roll in the child’s mouth, trying to induce sucking. To obtain as much saliva as possible, after 1–2 min, the experimenter took the roll out of the child’s mouth, turned it around, and introduced the dry end into the child’s mouth. After an additional 1–2 min, the cotton roll was put back in the syringe. The syringe was stored in the dark at −18 to −20 °C and later transported to the laboratory where it was once again weighed. The increase in weight was caused by the amount of saliva on the cotton, 1 mg being equivalent to 1 µL of saliva.

When the volume of saliva was 50–250 µL, cortisol was extracted from the cotton by opening the syringe at both sides and rinsing the cotton roll in the syringe with 1 mL of 960 mL/L ethanol, followed by centrifugation of the syringe at 1500 g for 5 min. The resulting liquid was evaporated, and when the volume of saliva was <0.1 mL or the volume was equivalent to the volume of saliva collected, the residue was dissolved in 100 µL of 0.01 mol/L phosphate-buffered saline (pH 7.0) containing 2 g/L bovine serum albumin. After the solution had stood for at least 15 min with repeated mixing with a vortex-mixer, 25 µL was used for the measurement of cortisol by RIA (15).

Direct measurements of cortisol in saliva required 25 µL of saliva. The detection limit of the direct assay was 0.5 nmol/L; the within-assay imprecision (CV) was 4% at 10 nmol/L (n = 10), and the between-assay CV was 9% at 4 nmol/L (n = 69) and 5% at 10 nmol/L (n = 69).

Over an experimental range of 2–20 nmol/L cortisol, concentrations measured after extraction were highly comparable to those measured directly. At the lowest volumes, the imprecision of the measurements increased, but the concentrations with and without extraction did not differ significantly: with solvent extraction they were 117% (22%), 98% (12%), and 107% (11%) of the values measured directly for volumes of 50, 100, and 200 µL, respectively (n = 24 at each volume).
Methanol, isopropanol, and acetone were also tested as possible alternative solvents, but the highest recoveries (>95% of added cortisol) were consistently obtained with ethanol.

The smallest amount of saliva that could be used for the extraction technique with reliable results was evaluated by calculating the mean cortisol concentrations obtained for a series of 459 samples collected in 1- to 3-year-old children screened for possible developmental problems. The absolute amounts of saliva per sample ranged from 10 μL to 1057 μL. The cortisol concentrations were then sorted by the volumes of collected saliva, from smallest to largest, and the running means for 25 consecutive cortisol values were calculated (Fig. 1). The means (SD) were significantly higher for volumes <50 μL [13.7 (7.0) nmol/L; n = 95] than for samples >50 μL [8.6 (3.6) nmol/L; n = 364; P < 0.0001, unpaired t-test with Welch correction, not assuming equal variances].

In addition, we calculated the imprecision for 100- and 50-μL samples. For three 100-μL samples from healthy adults, the interassay CV for the individual samples was 3.4–8.5% (n = 5–16). For 50-μL samples, the CV was 19% (n = 24), close to the limits of precision for the RIA. Therefore, only samples >50 μL were used.

To test the stability of cortisol in the cotton at room temperature and during storage in a refrigerator or freezer for periods of up to 3 days, we absorbed known temperature and during storage in a refrigerator or freezer for a series of 459 samples collected in 1- to 3-year-old children screened for possible developmental problems. The safety of cortisol in saliva at −20 °C was excellent: aliquots pooled samples of saliva used for internal quality assessment for >1-year-old yielded CVs between 5.1% at 3.8 nmol/L and 3.4% at 19.1 nmol/L (n = 24).

For some assays (16), contamination of saliva with milk affects the measured cortisol. We tested samples of human milk (n = 8) and eight different formula milk preparations at a 1:10 (100 μL of milk or formula and 900 μL of saliva) dilution. At these concentrations, formula milk had no effect on the concentration of cortisol measured, the mean values being 99% (2.4%) of the original saliva concentrations, whereas high amounts of human milk caused a slight decrease in cortisol values, to 91% (6%) of the original concentrations.

As an illustration, the technique as described has been used to evaluate the effects of a known stressor for infants of 11 days, i.e., a standardized physical examination (17) that takes ~20 min. Saliva samples from 114 healthy, normally developing infants were collected before (representing basal cortisol) and 40 and 60 min after the start of the examination (representing stress and recovery cortisol, respectively). Salivary sample sizes were 23–1025 μL of saliva; 2% of the samples were <50 μL (and were therefore not included), and 45% were 50–200 μL. During stress, cortisol increased significantly, from 10.5 (5.0) to 17.4 (9.2) nmol/L (Wilcoxon signed-ranks test, z = −6.2; P < 0.001), decreasing significantly after the examination, to 14.6 (7.4) nmol/L (z = −5.2; P < 0.001).

Although we elected to use our in-house RIA, it is conceivable that the quantitative extraction technique may be readily adapted for cortisol determinations with other, more sophisticated, commercially available methods.

In practice, volumes collected on the cotton rolls varied between 10 and 1057 μL of saliva with the percentage of samples with a volume <200 μL varying between 20% and 80%, depending on the experience of the sampler, the sampling population, and other factors. For example, in the data set used to estimate the smallest amount of saliva that can be used, 45% of the 459 samples contained 50–100 μL of saliva and 22% of the samples contained <50 μL. The extraction method enables analysis of low-volume samples that cannot be assayed by established methods, substantially increasing the number of samples for which results can be obtained; for example, the 58% of samples in our example data set that had volumes of 50–250 μL.

In conclusion, the new method enables reliable measurements of cortisol in small amounts of saliva (as are often obtained from young infants and toddlers) and thus avoids the use of saliva production stimulants and loss of data because of insufficient sampling volume. The method can be used for any clinical or research purpose in which cortisol assessments in saliva are required.
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References

Malonic aciduria (OMIM 248360) is a rarely diagnosed autosomal-recessive inborn error of metabolism caused by congenital deficiency of malonyl-CoA decarboxylase (MCD; EC 4.1.1.9). Hypoglycemia, seizures, developmental delay, and cardiomyopathy are among the most common symptoms, but both clinical signs and the time of presentation of patients with MCD deficiency can be variable. To date, only eight cases have been reported in the literature (1–8), and recently molecular defects within the MCD gene (MLYCD) have been elucidated for the first time in some of these cases (8–11). At least in part, clinical heterogeneity might be caused by the fact that MCD is expressed in different compartments of the cell and that MLYCD mutations with different effects on the subcellular localization of the MCD protein may thus affect different metabolic pathways (Wightman et al., submitted for publication).

To date, only symptomatic patients with MCD deficiency have been detected, and many of them were already severely handicapped at the time of diagnosis because of residues of acute metabolic crises or from episodes of cardiac decomposition, which may develop as a consequence of secondary carnitine deficiency. Typically, the key to diagnosis is the excessive amount of malonic acid in the patient’s urine, which can be detected by gas chromatography–mass spectrometry. This then leads to a confirmatory test, such as the measurement of MCD activity in cell extracts, or to molecular genetic testing.

Detection of the carnitine ester of malonic acid has been mentioned previously in single cases of malonic aciduria (4, 6, 7). In the study reported here, we systematically investigated the concentration of malonylcarnitine in the blood of MCD-deficient patients by electrospray ionization tandem mass spectrometry (ESI-MS/MS). Because MCD deficiency may be amenable to dietary and medical treatment, we also evaluated whether an increase in blood malonylcarnitine is detectable in the neonatal period, which would be mandatory for early presumptory diagnosis.

Details of the method used by our group have been reported previously (12). In summary, acylcarnitines were analyzed by ESI-MS/MS with an API 365 ESI-MS/MS system equipped with a Turbolon spray device, Series 200 µHPLC pump, and a Series 200 autosampler (PE-SCIEX). For that purpose, 3.2-mm (1/8-inch) spots were punched from Guthrie test cards by an automated punching device and transferred to a 96-well microtiter plate. After the addition of internal standard solution (containing 7.5 pmol of L-[2H3]octanoylcarnitine), methanol was added, and after elution, the methanolic extract was evaporated to dryness. Acylcarnitines were derivatized to the corresponding butyl esters with butanolic hydrochloric acid. Samples were redissolved in acetonitrile–water–formic acid (50:50:0.025 by volume) and introduced into the ionization chamber. Acylcarnitines were measured in multiple-reaction monitoring (MRM) mode with the ion pairs 347.4/85.0 Da and 360.4/85.0 Da for

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