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Mass spectrometric quantification of salivary metanephrines—A study in healthy subjects☆

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ABSTRACT

Background: Determination of metanephrine (MN), normetanephrine (NMN), and 3-methoxytyramine (3-MT) in saliva may offer potential diagnostic advantages in diagnosing pheochromocytoma.

Methods: In this preliminary study, we determined metanephrine concentrations in saliva of healthy subjects and the relationship with simultaneously measured plasma metanephrines. We also studied the possible influence of pre-analytical conditions such as a collection device, awakening, posture, and eating on the salivary metanephrine levels.

Results: Eleven healthy subjects were included. Fasting blood and saliva samples were collected in seated position and after 30 min of horizontal rest. Plasma and salivary MN, NMN, and 3-MT concentrations were determined using a high-performance liquid chromatography tandem mass spectrometric technique (LC–MS/MS) with automated solid phase extraction sample preparation. Metanephrines were detectable in saliva from all participants both in seated and supine position. No significant correlations were observed between the MN, NMN, and 3-MT concentrations in saliva and plasma in seated or supine position. Furthermore, there was no difference between MN, NMN, and 3-MT samples collected with or without a collection device.

Conclusion: Metanephrines can be detected in saliva with LC–MS/MS with sufficient sensitivity and precision. Our findings warrant evaluation of salivary metanephrine measurement as a novel laboratory tool in the work-up of patients suspected of having a pheochromocytoma.

1. Introduction

Sympathetic paragangliomas (either adrenal, i.e. pheochromocytoma, or extra-adrenal (sympathetic paragangliomas)) are neuroendocrine tumors that are known for their overproduction of catecholamines, i.e., epinephrine, norepinephrine, and dopamine. The biochemical diagnosis of sympathetic paragangliomas is based on the demonstration of elevated metanephrine (MN),[1] normetanephrine (NMN), or 3-methoxytyramine (3-MT) in plasma and/or urine. Measurement of plasma-free metanephrines is currently considered to be the most accurate method for diagnosing these tumors [1,2]. However, plasma metanephrines can be affected by several pre-analytical factors such as position [3], age [4], coffee [5,6], diet [7] and salt intake [8], certain drugs (depending on the method used) (e.g. mesalamine, sulfasalazine, and tricyclic antidepressants) [2,9,10], previous adrenalectomy [11], and even the season of the year [12]. Plasma NMN and MN samples collected in seated position are 30% and 27% higher, respectively, compared to samples drawn after 30 min of supine rest [3]. Therefore, the United States Endocrine Society recommends that patients rest for 20–30 min in a supine position before blood sampling [2]. This not only requires reference values in supine position but is also more cumbersome and increases costs for venipuncture [13].

Catecholamines and metanephrines are biogenic amines that are easily transported over the salivary gland membrane and should therefore be detectable in saliva. Assessment of late-night cortisol in saliva is now recommended as part of routine diagnostics in diagnosing Cushing's syndrome mostly for logistic reasons [14,15]. Salivary

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[1] MN, metanephrine; NMN, normetanephrine; 3-MT, 3-methoxytyramine; LC–MS/MS, Liquid Chromatography tandem mass spectrometric technique; SD, standard deviation; IQR, inter quartile range; ELISA, enzyme-linked immune sorbent assay; HVA, homovanillic acid; MHGP, 3-methoxy-4-hydroxyphenylglycol.

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catecholamines and metanephrines are expected to reflect their respective concentrations in plasma. High-performance liquid chromatography tandem mass spectrometric technique (LC–MS/MS) with automated solid phase extraction sample preparation is a highly sensitive technique that enables the measurement of very low concentrations of catecholamines and metanephrines in saliva. Free unconjugated metanephrines in plasma filtrate into saliva through gap junctions between cells of secretory units [16]. Determination of metanephrines in saliva could be useful in patients suspected of harboring a sympathetic paraganglioma for logistic reasons. If patients could collect saliva at home, this would improve patient convenience and obviate the need of extra hospital facilities and costs.

This pilot study was initiated to determine metanephrines in saliva of healthy subjects. In addition, we tested the influence of several pre-analytical factors such as collection device, position, awakening, and food consumption on the salivary concentrations of metanephrines. Also, the relationship between salivary and plasma metanephrines concentrations was established.

2. Participants and methods

2.1. Study population and design

In this single center study, we examined 11 healthy nonsmoking volunteers older than 18 years. All healthy volunteers were seen at the Department of Endocrinology of the University Medical Center Groningen. Participants were not allowed to use medication known to interfere with metanephrines, such as mesalamine, sulfasalazine, and tricyclic antidepressants [2]. Participants did not have periodontal disease.

The first saliva sample was collected at home directly after awakening (between 6.00 and 7.00 AM) in supine position (T0). Participants were allowed to brush their teeth after the saliva collection at T0. There were at least 30 min between tooth brushing and the saliva collection at T1. Participants visited the outpatient clinic at 8.00 AM in a fasting state. Blood pressure was measured in seated and after 5 min in supine position using an automatic blood pressure measurement device. Blood and saliva samples were collected in seated position (T1). Thereafter, saliva samples were collected directly after changing into a supine position (T2). The second blood sample and third saliva sample were collected after 30 min of recumbency (T3). Blood samples at T1 and T3 were drawn immediately after collection of the saliva sample. Ten minutes after blood sample collection at T3, saliva was again collected while remaining in supine position (T4). Thereafter, participants ate a standard breakfast but were not allowed to smoke, drink caffeine-containing products such as coffee or tea, or consume food products with a high (catechol)amine content such as walnuts, pineapple, or bananas. Thirty minutes after finishing breakfast, participants were asked to collect saliva for the fifth time (T5). Salivary and plasma samples were stored on ice until transportation to the department of laboratory medicine.

Approval of the study by the Medical Ethics Committee of the University Medical Center of Groningen in the Netherlands was requested but waived because the purpose of this study was to compare metanephrine values in saliva to the values in plasma and the burden for the participants was considered to be very low and therefore, no further Institutional Review Board approval was required according to the Dutch Medical Research Involving Human Subject Act. All participants gave oral informed consent.

2.2. Saliva collection

Saliva was collected in two ways, either by directly spitting saliva into a collection tube (without a collection device) or by using a polyethylene swab (Salivette®; Sarstedt, Nümbrecht, Germany), while participants were either in seated or supine position. Participants needed to chew or suck gently on the polyethylene swab for 2–3 min. Collected samples were immediately put on ice for transportation to the department of laboratory medicine and were subsequently stored at −80 °C until further processing.

2.3. Analytical methods

Blood samples were taken via venipuncture, with the participant either in the seated or supine position, using 4 ml Vacutainer Tubes (Becton Dickinson®) containing K_2-EDTA as anticoagulant. Collected samples were immediately put on ice for transportation to the department of laboratory medicine. Blood samples were centrifuged for 12 min at 2500 g and saliva samples for 2 min at 1000 g. Samples were subsequently stored at −80 °C until processing.

Plasma-free and saliva MN, NMN, and 3-MT were analyzed by high-performance LC–MS/MS with automated solid phase extraction sample preparation, essentially as described by de Jong et al. [17]. The only difference was that in this study, a new-generation and thus more sensitive mass spectrometer was used (Waters XEVO TQ instead of Waters Quattro Premier). Established reference intervals (in seated position) for plasma-free metanephrines were MN 0.07–0.33 nmol/L, NMN 0.23–1.07 nmol/L, 3-MT < 0.17 nmol/L [17].

The intra-assay and inter-assay analytical variation coefficients (CVs) were 2.5%–4.8% and 3.4%–5.6% for free plasma MN, 5.1%–6.2% and 4.2%–7.1% for free plasma NMN, and 4.4%–8.0% and 4.5%–11.1% for free plasma 3-MT, respectively. The analytical method, which has been extensively validated for plasma-free metanephrines, was validated for saliva, using a limited validation protocol in which relevant parameters were tested. We used three different saliva pools with low, medium, and high concentrations of MN, NMN, and 3-MT. The low saliva pool was unspiked saliva (MN 0.070 nmol/L, NMN 0.40 nmol/L, 3-MT 0.020 nmol/L), medium (MN 0.93 nmol/L, NMN 2.6 nmol/L, 3-MT 0.57 nmol/L), and high (MN 10 nmol/L, NMN 27 nmol/L, 3-MT 6.8 nmol/L) saliva pools were spiked with increasing concentrations of MN, NMN, and 3-MT. The following parameters were tested: intra-assay (n = 10), inter-assay (n = 6 different days), lower limit of quantification (LLOQ) by serially dilution of the low saliva sample and analyzing the dilutions on four different days. LLOQ was set at where CV ≥ 20%. Recovery was determined by spiking three different levels of MN, NMN, and 3-MT on the low and medium saliva pools on four different days.

Intra- and inter-assay CVs were 1.4%–7.0% and 3.2%–8.3% for free salivary MN, 1.7%–1.8% and 1.7%–3.2% for free salivary NMN, and 1.4%–6.1% and 1.4%–7.0% for free salivary 3-MT, respectively. LLOQs in saliva were 0.03, 0.035, and 0.010 nmol/L for MN, NMN, and 3-MT, respectively. Recovery for MN was 106 ± 10%, for NMN 101 ± 5%, and 3-MT 102 ± 6%. Linearity was excellent over the different calibration ranges (MN 0.03–10 nmol/L, NMN 0.035–26 nmol/L, 3-MT 0.010–6.6 nmol/L) with $R^2 > 0.99$.

2.4. Statistical analysis

Data are presented as mean ± standard deviation (SD) or as median with interquartile range [IQR] where appropriate. Differences between salivary metanephrine samples collected with and without a collection device, in seated and supine position, before and after breakfast and after awakening were calculated with the Friedman's two-way ANOVA analysis.

Non-parametric correlation analysis (Spearmans ρ) was used to examine the relationship between blood and saliva samples. NMN/MN and 3-MT/NMN ratios in plasma and saliva were calculated. A two-sided $P < 0.05$ was considered statistically significant. Analyses were performed with SPSS statistics (version 22.0; IBM/SPSS, Armonk, New York) and Analyse-it Software Ltd. (Ver.2.30, Leeds, United Kingdom).