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Mass spectrometric quantification of salivary metanephrines – a pilot study in healthy controls

Thamara E Osinga[1], **Anouk NA van der Horst-Schrivers**[1], **Martijn van Faassen**[2], **Michiel N Kerstens**[1], **Robin PF Dullaart**[1], **Karel Pacak**[3], **Thera P Links**[1], and **Ido P Kema**[2]

[1]Department of Endocrinology and Metabolic Diseases,University of Groningen, University Medical Center Groningen, P.O. Box 30.001, 9700 RB Groningen, The Netherlands [2]Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, P.O. Box 30.001, 9700 RB Groningen, The Netherlands [3]Program in Reproductive and Adult Endocrinology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

Determination of metanephrine (MN), normetanephrine (NMN) and 3-methoxytyramine (3-MT) in saliva could be of diagnostic value in patients with pheochromocytoma. This preliminary study was set out to determine metanephrine concentrations in saliva from healthy subjects compared to their simultaneously measured plasma levels. In addition, we studied the possible influence of pre-analytical conditions such as a collection device, awakening, position, and eating on the salivary metanephrine levels. We included 11 healthy volunteers. Fasting blood and saliva samples were collected in seated position and after 30 minutes of horizontal rest. Saliva samples 30 minutes after eating were also collected. Saliva was collected with and without the use of a polyethylene salivette. 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 the supine position. We found no significant correlation between the MN, NMN and 3-MT concentrations in saliva and plasma in the seated or supine position. In addition, there was no difference between MN, NMN and 3-MT concentrations collected with or without a collection device. Plasma MN, NMN, 3-MT and salivary NMN concentrations collected in seated position were significantly higher compared concentrations of samples collected in supine position (all $P<.05$). In conclusion, salivary metanephrines can be detected with LC-MS/MS with sufficient sensitivity and precision. Our findings warrant evaluation of salivary metanephrine measurement in the work-up of patients who are suspected to harbor pheochromocytoma.

Corresponding author and requests for reprints: I.P. Kema, PhD, Department of Laboratory Medicine (EA61), University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9700 RB, Groningen, the Netherlands, Telephone: +31503613779, Fax: +31503612290, i.p.kema@umcg.nl.

This study or parts of it have not been presented elsewhere.

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Keywords

saliva; metanephrines; pheochromocytoma; paraganglioma

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), 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 (Lenders *et al.* 1995, Lenders *et al.* 2014). However, plasma metanephrines can be affected by several pre-analytical factors such as position (Lenders *et al.* 2007), age (Eisenhofer *et al.* 2013), coffee (Robertson *et al.* 1978, Deutschbein *et al.* 2010), diet (de Jong *et al.* 2009) and salt intake (Kerstens *et al.* 2012), certain drugs (depending on the method used) (e.g. mesalamine, sulfasalazine, and tricyclic antidepressants) (Bouhanick *et al.* 2010, Neary *et al.* 2011, Lenders *et al.* 2014), previous adrenalectomy (Osinga *et al.* 2013) and even the season of the year (Pamporaki *et al.* 2014). Plasma NMN and MN samples collected in seated position are 30% and 27% higher, respectively, compared to samples drawn after 30 minutes of supine rest (Lenders *et al.* 2007). Therefore, the United States Endocrine Society recommends that patients rest for 20–30 minutes in a supine position before blood sampling (Lenders *et al.* 2014). This requires reference values in supine position and moreover, increases costs for venipuncture and is more cumbersome (Chortis *et al.* 2014).

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 part of routine diagnostics in diagnosing Cushing's syndrome mostly for logistic reasons (Nieman *et al.* 2008, Guignat and Bertherat 2010). Salivary 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 (Chiappin *et al.* 2007). Determination of metanephrines in saliva could for logistic reasons be useful in patients suspected of harboring a pheochromocytoma/paraganglioma. These patients could then collect saliva at home, which would enhance patient comfort and obviates the need of extra hospital facilities and costs.

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

Participants and Methods

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 (Lenders *et al.* 2014).

The first saliva sample was collected at home directly after awakening (between 6.00 and 7.00 AM) in supine position (T0). Participants visited the outpatient clinic at 8.00 AM in a fasting state. Blood pressure was measured in seated and after 5 minutes in supine position using an automatic blood pressure measurement device. Blood and saliva samples were collected while participant were 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 minutes of recumbency (T3). 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 waved because the purpose of this study was to calibrate metanephrine values in saliva to the values in plasma and therefore according to the Dutch Medical Research Involving Human Subjects Act no further Institutional Review Board approval was required. All participants gave oral informed consent.

Saliva collection

Saliva was collected in two ways, either by direct spitting saliva into a collection tube (without a collection device) or by using a polyethylene swab (Salivettes®; 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 minutes. 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.

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 minutes at 2500

g and saliva samples for 2 minutes at 1000 g. Samples were subsequently stored at −80 °C until processing.

Plasma free and saliva MN, NMN, and 3-MT were analyzed by HPLC-MS/MS with automated solid phase extraction sample preparation, exactly as described by de Jong *et al.* (de Jong *et al.* 2007). Established reference intervals 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 (de Jong *et al.* 2007).

The intraassay and interassay analytical variation coefficients were 2.5% to 4.8% and 3.6% to 5.6% for free plasma MN, 5.1 to 6.2% and 4.2% to 7.1% for free plasma NMN, and 4.5% to 11.1% for free plasma 3-MT, respectively.

Statistical Analysis

Data are presented as mean ±standard deviation (SD) or as median with inter quartile 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. MN/NMN and NMN/3-MT ratios in plasma and saliva were calculated by dividing NMN by MN and 3-MT by NMN. 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).

Results

Participant characteristics

Four men and 7 women with a mean age (±SD) of 39±16 years participated. Mean blood pressure and pulse in seated and supine position were 126±17/80±8 mmHg, 70±9/min and 123±19/71±9 mmHg, 62±10/min, respectively.

Metanephrine concentrations simultaneously measured in saliva and plasma samples

Median concentrations of metanephrines in saliva and plasma at the different time points are shown in Table 1. Seated NMN/MN and 3-MT/NMN ratios were not significantly different between plasma and saliva, 1.94 [1.27–2.46] vs. 7.21 [5.33–8.16] ($P=0.08$) and 0.05 [0.04–0.06] vs. 0.12 [0.08–0.14] ($P=0.08$). Supine NMN/MN and 3-MT/NMN ratios were not significantly different between plasma and saliva, 1.49 [1.15–2.08] vs. 4.92 [3.62–5.25] ($P=0.08$) and 0.05 [0.04–0.10] vs. 0.11 [0.08–0.16] ($P=0.17$).

Influence of collection device on the concentration of metanephrines

There were no differences in concentrations of metanephrines between samples collected with or without a saliva collection device in either seated position or recumbent position (data not shown). Therefore, all samples described in this study are calculated based on samples taken with a polyethylene swab as device (Salivettes®).

Correlations between salivary and plasma metanephrines

There was no significant correlation between the MN, NMN, and 3-MT concentrations in saliva and plasma in the seated position (T1), ρ_s= .08 (P=0.81), ρ_s=0.33 (P= 0.32) and ρ_s= −0.32 (P=0.37) respectively (Figure 1).

There was also no significant relationship between MN, NMN, and 3-MT concentration in saliva and plasma in supine position (T3), ρ_s=0.31 (P=0.36), ρ_s=0.57 (P=0.07) and ρ_s=−0.33 (P=0.36) (Figure 1).

Influence of posture during sampling on salivary and plasma metanephrines

Plasma MN, NMN, and 3-MT concentrations collected in supine position were significantly lower compared to samples collected in seated position (MN −16.4%, NMN −27.5% and 3-MT −8.3% respectively, P<0.05; Table 1, Figure 2). Salivary NMN collected in supine position was also significantly lower compared to samples collected in seated position (NMN 38.4%, P<0.05). In contrast, there were no significant effects of posture during sampling on the salivary MN and 3-MT concentrations (P=0.53 and P=0.10; Table 1, Figure 2).

Influence of eating on salivary metanephrines

Salivary MN and NMN concentrations were significantly lower after breakfast (T5) compared to samples collected fasting in seated position (T1) (both P<0.05) (Table 1). Only salivary MN was significantly lower after breakfast (T5) compared to samples collected fasting after 30 minutes of supine rest (T3) (P<0.05).

Awakening response

Salivary MN, NMN and 3-MT concentrations on awakening (T0) (around 6:00–7:00 AM) were significantly higher than after 30 minutes of recumbency in the hospital (around 9:00 AM) (T3) (all P<0.05) (Table 1).

Discussion

In the present study, we show that metanephrines can readily be measured in saliva of healthy volunteers using mass spectrometry based techniques. We investigated several pre-analytical conditions and found that both position during sample collection and eating are of importance, moreover, we found that there was only a weak correlation between saliva and plasma concentrations.

Whole saliva is a clear, slightly acidic (pH 6.0–7.0 or 5.5−6.0 when not stimulated) and complex biological fluid composed of secretions from salivary glands and a variety of enzymes, hormones, antibodies, antimicrobial constituents, and growth factors entering the saliva from the blood by either passive or active intracellular diffusion or extracellular ultrafiltration (Vining and McGinley 1986, Kaufman and Lamster 2002). Catecholamines and unconjugated metanephrines in plasma filtrate into saliva through gap junctions between cells of secretory units (Chiappin *et al.* 2007, Groschl 2008). The hormone concentration in

saliva is 300–3000x lower than in plasma (Chiappin *et al.* 2007). We found average concentrations of free metanephrines to be comparable to concentrations measured in blood.

Stefanescu *et al.* measured salivary free MN and NMN levels in 30 patients with a pheochromocytoma and compared these to 70 normotensive healthy controls using an enzyme-linked immunosorbent assay (ELISA) (Stefanescu *et al.* 2011). The reported concentrations in healthy controls were similar to ours. Both salivary and plasma MN and NMN levels were increased in patients with pheochromocytoma when compared to healthy controls. In addition, they found a linear relationship between salivary and plasma MN and NMN levels (Stefanescu *et al.* 2011). In the present study, we used LC-MS/MS with automated solid phase extraction sample preparation to analyze both salivary and plasma samples. We could not reproduce this observation, as we only found a tendency towards a statistical significant correlation between plasma and saliva NMN in supine position, but there was no statistical significant correlation between MN, NMN in seated position, and 3-MT concentrations in saliva and plasma. Beside differences in group size, differences in techniques used for quantification of metanephrines could be an explanation. Differences in sample clean up could result in different recoveries from saliva, as it has been shown that binding proteins such as albumin and proline rich protein are present in saliva as well (Fiers *et al.* 2014).

The tendency towards difference in NMN/MN ratio between plasma and saliva can be an indication that there is a decreased filtration of the free MN fraction from plasma into saliva, or secretion of catecholamines by the salivary glands and subsequent metabolization of these catecholamines by COMT in saliva ((Uhlen *et al.* 2015), http://www.proteinatlas.org/ENSG00000093010-COMT/tissue, date: 05/26/2015). As we saw no difference between passive drewling and salivette collected saliva, it is unlikely that the use of a device (polyethylene Salivettes®) influenced the recovery of metanephrines. In addition, polyethylene Salivettes® have been found to be suitable for determining several (polar) hormones and therapeutic drugs in saliva (Groschl *et al.* 2008).

We found a decrease of salivary metanephrines 30 minutes after eating breakfast compared to the values during fasting. This could be explained by the stimulation of saliva production by chewing. Higashi et al. found a lower concentration of catecholamine end products (homovanillic acid (HVA) and 3-methoxy-4-hydroxyphenylglycol (MHGP)) after use of chewing gum (Higashi *et al.* 2012). Therefore, we believe it is appropriate to recommend that salivary samples should be obtained while fasting or at least 30 minutes after eating.

Comparable to salivary cortisol, we found elevated levels of metanephrines immediately after awaking. The cortisol awakening response was found to be decreased in patients with chronic pain or psychiatric conditions (posttraumatic stress disorders, chronic fatigue syndrome or sleep disorder), while healthy subjects can exhibit elevated cortisol response under some stress conditions (such as job stress) (Clow *et al.* 2004, Fries *et al.* 2009). Similar to cortisol, an increase of catecholamine levels directly after awakening has been described (Schofl *et al.* 1997). In addition, there is an indication that catecholamine show diurnal variation(Prinz *et al.* 1979). Concentrations of metanephrines were elevated immediately after awakening compared to values determined in supine position between

8.00 and 9.00 am. This suggests that salivary metanephrines could also represent an easy to collect biomarker to detect a diurnal rhythm or stress-related disorders to which an individual is exposed.

Assessment of salivary metanephrines might become a novel and clinically useful biochemical tool for the diagnosis of pheochromocytoma. Obviously, this requires determination of salivary metanephrines in a sufficient number of healthy controls and patients with a pheochromoyctoma in order to establish reference ranges for these metabolites.

Measurement of metanephrines in saliva instead of plasma could offer several advantages for clinical practice. It is noninvasive and free of harmful effects such as pain or hematoma formation and it gives the possibility to determine other hormones such as cortisol at the same time. This would make determination of salivary metanephrines very suitable for children as well as for periodic screening in patients with a hereditary pheochromocytoma/paraganglioma. In addition, it can readily be repeated at various intervals and patients can also collect the sample at home, which is likely to be more cost-effective compared to an in-hospital venipuncture (Chortis *et al.* 2014). We found no differences in concentration of metanephrines between saliva samples collected with or without a collection device. Taking into account that the collection of saliva with a device is more convenient for the patient, we would recommend the use of a polyethylene or polyester salivette® (Groschl *et al.* 2008).

In conclusion, LC-MS/MS enables to detect salivary MN, NMN and 3-MT. In order to become a new tool for the biochemical diagnosis of pheochromocytoma/paraganglioma, more samples need to be collected including those of pheochromocytoma/paraganglioma patients, taking into account the above described pre-analytical conditions.

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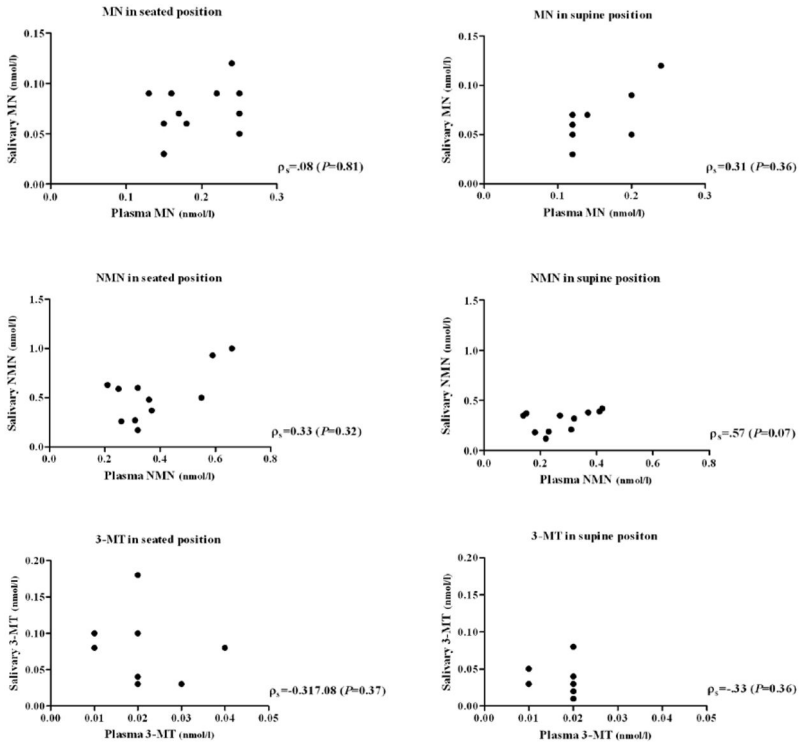

Figure 1.

Individual relationship between salivary and plasma metanephrines in seated and supine position

MN, metanephrine; NMN, normetanephrine; 3-MT, 3-methoxytyramine

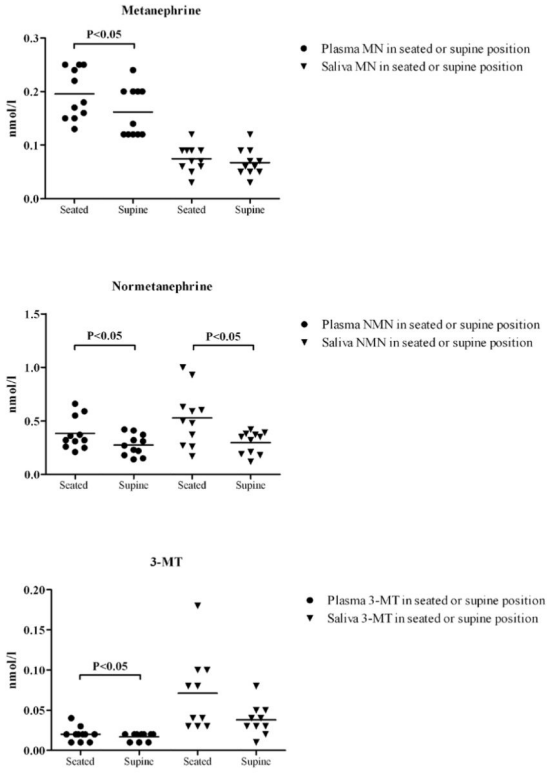

Figure 2.

Comparison between salivary and plasma metanephrines in seated and supine position

MN, metanephrine; NMN, normetanephrine; 3-MT, 3-methoxytyramine

Table 1

Median plasma and salivary concentrations of metanephrines in 11 healthy volunteers

	Plasma			Saliva		
	Metanephrine	**Normetanephrine**	**3-Methoxytyramine**	**Metanephrine**	**Normetanephrine**	**3-Methoxytyramine**
T0 (on awakening)	NA	NA	NA	0.12 [0.07–0.14]	0.47 [0.33–0.59]	0.23 [0.10–0.35]
T1 (seated)	0.18 [0.15–0.25]	0.32 [0.26–0.55]	0.019 [0.014–0.024]	0.07 [0.06–0.09]	0.50 [0.27–0.63]	0.06 [0.03–0.10]
T2 (immediately after supine position)	NA	NA	NA	0.08 [0.05–0.09]	0.37 [0.25–0.54]	0.04 [0.03–0.08]
T3 (30 minutes supine position)	0.14 [0.12–0.20] *	0.27 [0.18–0.37] *	0.015 [0.013–0.021] *	0.06 [0.05–0.09] ¶	0.35 [0.19–0.38] *,¶	0.04 [0.03–0.05] ¶
T4 (10 minutes after T3)	NA	NA	NA	0.07 [0.06–0.09]	0.29 [0.22–0.38]	0.04 [0.02–0.06]
T5 (30 minutes after breakfast)	NA	NA	NA	0.05 [0.04–0.05] †	0.26 [0.21–0.37] †	0.05 [0.02–0.36] †

Data in median with interquartile ranges between brackets. Concentrations of plasma and salivary metanephrine, normetanephrine and 3-methoxytyramine in nmol/L.

* $P < 0.05$ seated vs. supine position;

† $P < 0.05$ before vs. after breakfast;

¶ $P < 0.05$ on awakening vs. after 30 minutes of supine rest in the hospital NA, not available.