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Full Length Research Paper

Salivary nicotine and cotinine concentrations in unstimulated and stimulated saliva

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Salivary nicotine and salivary cotinine is widely used in clinical and epidemiological studies to validate smoking cessation. However, the reported collection for salivary nicotine and salivary cotinine vary by technique and duration. This study investigated the influence of salivary collection by unstimulation and stimulation technique of the concentration of salivary nicotine and salivary cotinine. It was found that unstimulated technique produced the highest salivary nicotine concentration, whereas stimulated technique produced the highest salivary cotinine concentration. The results of this study suggest that it is important to standardise salivary nicotine and cotinine collection technique.

Key words: Saliva, smoker, nicotine, cotinine, technique, biological marker, oral, verify, passive, tobacco, smoke.

INTRODUCTION

Determining the concentration of nicotine and cotinine in biological fluids is widely practiced in both epidemiological and clinical smoking studies (Hatsukami et al., 2003). Both nicotine and cotinine concentrations are used to estimate tobacco consumption, to determine exposure to environmental smoke and to validate abstinence in smoking cessation programmes (Hatsukami et al., 2003; Schneider et al., 1997). Nicotine, when smoked in cigarettes is absorbed across buccal and nasal membranes. The drug has a fast onset of action with a half-life of 2 h and can be detected in blood, saliva and urine (Hatsukami et al., 2003). As nicotine is a weak base (pKa of 8.0), it is present mainly in the non-ionised form in alkaline pH, and hence more easily absorbed with increased pH levels (Ciolino et al., 2001a). Thus, changes in salivary pH will affect the amount of nicotine that is absorbed across the buccal mucosa (Zevin et al., 1998).Cotinine, the major metabolite of nicotine, is widely used for estimating exposure to nicotine. This pharmacologically inactive compound has a half-life of 20 h (15 - 40 h), is slowly cleared from the body and is specific to tobacco (Hatsukami et al., 2003; Patterson et al., 2003). Cotinine has been reported to have a pKa<5.0, and can also be detected in urine, blood and saliva (Beckett et al., 1972; Benowitz, 1996). Urinary levels of cotinine have been shown to be quite variable, due to the difference in nicotine metabolism among individuals (Yang et al., 2001). Blood provides quantitative results that can be more accurately related to dosing. However, collection of blood samples is more invasive. In many nicotine treatment trials, saliva collection is favoured over blood and urinary measures as it is easy to obtain and non-invasive (Hatsukami et al., 2003). Saliva samples are useful for determining compliance with medication (especially in paediatric patients), for analysing the concentration of free drugs and in situations where repeated sampling is necessary.

Salivary nicotine and cotinine concentration is reported to be dependent upon a number of factors. One of the factors where variability reportedly arises in salivary nicotine and cotinine concentrations is the difference in sample collection methods (Curvall et al., 1990; Schneider et al., 1997). There have been a number of techniques used to collect saliva. Saliva can be collected under unstimulated (resting) or stimulated conditions. Among the reported disadvantages of collecting unstimulated saliva was insufficient volume. Most studies have employed

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sampling devices that aim to stimulate the production of saliva. Among the stimulated techniques, the method of stimulation has varied between using wax, sugar, lemon juice or other acidic drinks (Curvall et al., 1990; Jarvis et al., 2003; Torano and van Kan, 2003; Zevin et al., 2000). The use of stimulated saliva has an advantage over unstimulated saliva as a larger volume sample could be obtained in a short period of time. The importance of standardising saliva collection has been highlighted for research and clinical practice (Curvall et al., 1990; Di Giusto and Eckhard, 1986; Schneider et al., 1997). Schneider et al., (1997) reported lowered salivary cotinine concentration when the saliva collection was stimulated with wax or sugar compared to when saliva was collected without stimulation. No difference in salivary cotinine concentration was observed with consecutive unstimulated saliva sampling within the same subject. However, other earlier studies were less clear cut and found no difference in salivary cotinine levels whether the sample was collected stimulated or without stimulation (Curvall et al., 1990). This study aimed to determine the influence of stimulated saliva collection (compared to unstimulated collection) on salivary nicotine and cotinine concentrations.

METHOD

Subjects

Twelve subjects were recruited by on-site poster advertisement. The participants were current cigarette smokers (defined as someone who had smoked >100 cigarettes in their lifetime and who at the time of the study reported that they were smoking) (U.S. Department of Health and Human Services 1996). Ethical approval was obtained from the South London and Maudsley NHS Trust and Institute of Psychiatry Joint Research Committee (Ref 110/03). Subjects were asked to complete a self-administered questionnaire concerning the number and type of cigarettes smoked per day (cig/day) and their smoking pattern in a day. Their height and weight measurements were also taken to calculate their body mass index (BMI).

Procedure

The subjects were asked to come for the study after an overnight smoking abstinence (9 h). An overnight smoking abstinence of 9 h was required as a wash out period from the previous day smoking (half-life nicotine = 2 h). Thus the nicotine measured in the study was an estimation of the cigarette smoked at the time of the study. The time for saliva collection was also standardised at 9 am in the morning. This was because the properties of saliva were not constant and varied within an individual (as high as 50%) in a day (Dawes, 2005a; Dawes and Kubieniec, 2004). For instance, the viscosity of unstimulated saliva was highest in the morning and lowest at 5 pm. (Rantonen and Meurman, 1998).

This variation in the properties of saliva throughout the day may have potential impact on salivary drug concentrations. This study thus standardised the time of saliva collection to eliminate the potential impact of time of day on salivary drug concentration. Prior to sample collection, they were asked to rinse out their mouths. An unstimulated saliva sample was collected by asking the subjects to place a neutral Salivette, (Sarstedt; Numbrecht, Germany) cotton wool roll in the mouth (between the gum and cheek) for 2 min, within 5 min after smoking a cigarette. This was followed immediately by a stimulated saliva sample, where the subjects were asked to chew a second neutral Salivette cotton roll for 2 min.

The order of testing was not expected to affect nicotine and cotinine levels because the combined collection time was less than 10 min (2 min for unstimulated and 2 min for stimulated). This is within the half-life of nicotine, which is approximately 2 h, and the half-life of cotinine, 20 h (Hatsukami et al., 2003). Therefore, there should not be a significant change in both nicotine and cotinine levels within this time frame by either collection method. The saliva samples were collected in Salivettes, labelled, centrifuged, immediately frozen and couriered in dry ice to the Department of Molecular Pharmacology and Pharmacogenetics, Division of Clinical Sciences, University of Sheffield for analysis by LCMS (Liquid Chromatography Mass Spectrometry). Statistical analysis was carried out by SPSS using Paired t-test for dependent samples.

RESULTS

Subject characteristics

Twelve healthy volunteers (9 males) were recruited for this study. Subject characteristics can be found in Table 1. The volunteers were current smokers, mean age 34.7 ± 10.2 years (range 22 - 54). Six of the subjects smoked filtered cigarettes and 6 subjects smoked hand - rolled cigarettes. The mean number of cigarettes smoked per day (cig/day) was 13.3 ± 5.3 (range 2 - 20) and the mean BMI was 25.7 ± 5.1 (range 19.9 - 39.7).One subject smoked mainly in the evenings, all the other subjects smoked consistently from morning to bedtime. The subjects were from different ethnic groups: 9 Caucasians, 2 Mixed Ethnicity and 1 Oriental.

Unstimulated vs. stimulated saliva samples

Mean weight for saliva collected by unstimulated technique was 1.4 ± 0.7g (range 0.3 -3.3) and mean weight for saliva collected by stimulated technique was 1.6 ± 0.7g (range 0.3 - 3.3). Subjects showed a significant reduction in salivary nicotine levels when collected by stimulation technique (mean 1070.6 ± 728.5 ng/ml: range 367.3 - 2496.4) as compared to unstimulated technique (mean 2348.7 ± 1261.3 ng/ml: range 899.9 - 4611.5) ($t_{11} = 4.28$, $p=0.001$). Salivary cotinine concentrations, however, showed an opposite change. Salivary cotinine collected by stimulated technique (387.9 ± 382.3 ng/ml, range 28.4 -1449.7) was significantly higher than unstimulated technique (257.5 ± 275.8 ng/ml, range 9.6 - 1019.9) ($t_{11} = -2.95$, $p=0.013$).

Figure 1 illustrates the changes in salivary nicotine and salivary cotinine concentrations when collection by unstimulated and stimulated technique. The unstimulated sample collection method resulted in higher nicotine concentration but lower cotinine concentration than stimulated method.

Table 1. Nicotine and cotinine concentrations in unstimulated and stimulated saliva samples.

Subject	Ethnic group	No of cig/day	BMI	Type of cigarette smoked	Unstimulated		Stimulated	
					Nic (ng/ml)	Cot (ng/ml)	Nic (ng/ml)	Cot (ng/ml)
1	Caucasian	15	19.9	Filtered	4611.5	332.2	1722.8	338.4
2	Caucasian	20	26.9	Filtered	3406.0	367.2	1431.4	691.3
3	Mixed Ethnicity	10	39.7	Filtered	3414.8	1019.9	1431.4	1449.7
4	Caucasian	13	26.5	Filtered	3865.6	399.1	2496.4	335.3
5	Oriental	2	20.5	Filtered	1179.4	9.6	510.2	28.4
6	Mixed Ethnicity	10	28.5	Filtered	1654.9	289.8	645.4	286.7
7	Caucasian	15	22.3	Hand-rolled	1590.8	37.8	1280.0	175.8
8	Caucasian	20	25.6	Hand-rolled	899.9	211.4	604.3	483.5
9	Caucasian	13	26.3	Hand-rolled	3095.8	118.3	1001.9	193.8
10	Caucasian	20	24.8	Hand-rolled	1001.9	189.5	1070.0	425.5
11	Caucasian	16	25.5	Hand-rolled	1440.1	80.1	887.0	191.8
12	Caucasian	20	22.3	Hand-rolled	2024.0	35.5	367.3	54.0

Nic-nicotine, Cot-cotinine.

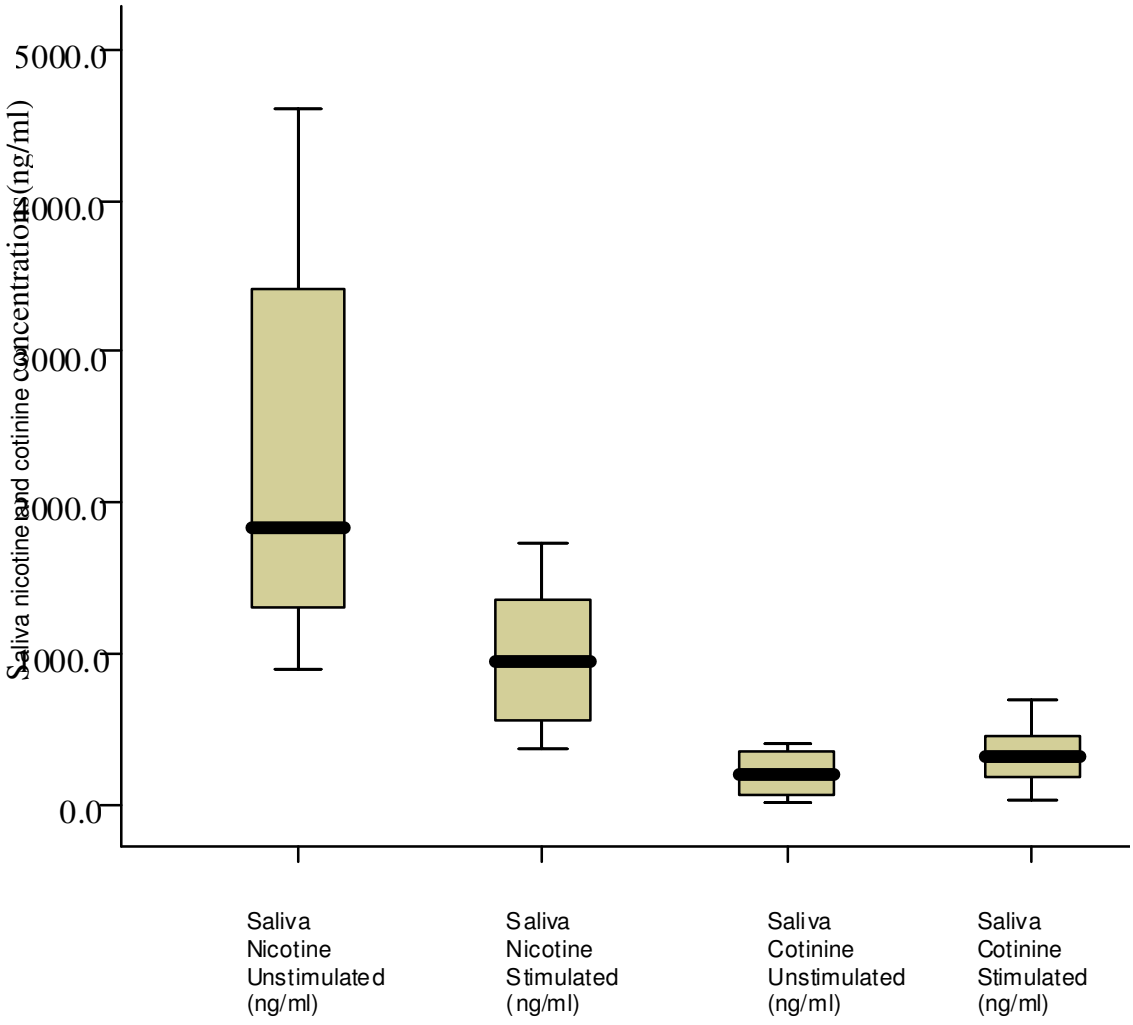

Figure 1. Salivary nicotine and cotinine concentration by unstimulated and stimulated technique.

DISCUSSION

This study highlights the importance of standardising the salivary collection technique. Salivary nicotine concentration showed a significant reduction when collected by stimulation as compared to unstimulated technique. In contrast, salivary cotinine concentration showed increased concentration when collected by stimulation technique.

The findings in this study could be explained by how drugs such as nicotine and cotinine are absorbed across the lipophilic oral mucosa (Kidwell et al., 1998). This absorption process is by passive diffusion, which is dependent on the proportion of the drug present in the unionised or unchanged form in the oral cavity. In the unionised form, passive diffusion of a drug is faster than in the ionised form. This is because unionised or uncharged molecules have greater solubility in the lipophilic cellular membranes found in the oral mucosa. The proportion of the unionised form of a drug also depends on the dissociation constant of the compound and the pH of the biological medium. Therefore, the concentration of nicotine and cotinine in saliva will be dependent upon their existence in an unionised form which can be altered by manipulating the pH of the oral cavity (Kidwell et al., 1998).

In order to explain the effects that different stimulation techniques have on salivary concentration of drugs, an understanding of how stimulation affects salivary flow rates is needed. Salivary flow rates vary significantly both between individuals and under different conditions (Liu and Delgado, 1999). Salivary flow rates affect pH of saliva (Kidwell et al 1998; Schneider et al 1997) and saliva collected by stimulation (e.g. chewing) produces higher salivary flow rates, generates carbon monoxide, and a higher bicarbonate concentration which causes a higher pH value. It was reported that pH of saliva increases with stimulation. For example, the pH of unstimulated saliva range between 5.6 - 7.0, but with stimulation it could increase up to 8.0 (Ciolino et al., 2001b). It was also reported that an increase of salivary flow rates from 0.55 ml/min to 0.88 ml/min changed salivary pH from 7.12 to 7.17 (Polland et al., 2003). Dawes (1969, 2005) showed that the bicarbonate concentration of saliva increased with flow rates. At salivary flow rates of 0.5 ml/min, salivary pH was 7.3 and at salivary flow rates of 1.0 ml/min, salivary pH increased to 7.5 (Dawes, 1969; Dawes, 2005b). This alkaline environment facilitates reabsorption of drugs with high pKa, producing lower salivary drug concentrations, but reduces the reabsorption of drugs with low pKa, producing higher salivary concentrations. Therefore, nicotine with pKa of 8.0 will be in unionised form at alkalinised salivary pH, hence facilitating reabsorption into buccal mucosa, and causing reduced nicotine concentration in saliva (Kidwell et al., 1998; Zevin et al., 1998; Zevin et al., 2000). However, cotinine with pKa < 5.0 (Beckett et al., 1972), will be present in a more ionised form at alkalinised salivary pH, hence resulting in less reabsorption across buccal mucosa, and an increase in salivary cotinine concentration.

In this study, salivary nicotine collected by unstimulated technique showed a wide range of concentrations. This could be due to inter-individual variability of nicotine intake when smoking a cigarette. It could also reflect the wide range of tobacco consumption as the number of cigarettes smoked ranged from 2 to 20 cig/day or could be possibly due to contamination from recent smoking. However, this could not be attributed to the effect of passive smoking as the reported mean salivary nicotine concentration from passive smoking was 4.8 ng/ml and mean salivary nicotine concentration from cigarette smoking was 672.5 ng/ml (Jarvis, 1984). Thus passive smoking could not be responsible for this wide interindividual variability of salivary nicotine collected by unstimulated technique. The pH of saliva was also not measured in this study. However, other factors which could affect the pH of saliva and the concentration of nicotine and cotinine in saliva such as time of day and duration of collection of saliva were standardised. The subjects were also asked to rinse their mouth prior to the procedure to eliminate trace of nicotine in the oral cavity from a prior cigarette.

The findings of this study suggest that collection using unstimulated methods produced the highest salivary nicotine concentration, whereas collection by stimulation technique produced the highest salivary cotinine concentrations. This is most likely due to the different influence the different techniques has on salivary pH. Therefore, in research and clinical practice, it is important to standardise salivary collection techniques, taking into account that different collection techniques may produce contrasting results. When contemplating which technique to be used, investigators need to consider a technique which would serve their purpose best. Thus unstimulated technique is recommended for salivary nicotine estimation and stimulated technique is recommended for salivary cotinine estimation.

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