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The effect of sampling procedures on the urate and lactate concentration in oral fluid

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ABSTRACT

This study was aimed at evaluating the influence of sampling procedure on the determination of uric acid and lactate in oral fluid. Samples of non-stimulated and stimulated oral fluid were collected from 22 healthy volunteers. Different frequencies of stimulation were obtained by moving a polyester swab within the mouth at 50, 100 and 150 min^{-1}. Three oral fluid samples were consecutively collected from a subgroup of 5 volunteers at a constant stimulation (70 min^{-1}) and at a self-selected pace to evaluate reproducibility.
The urate concentration in oral fluid decreased with the increase of the stimulation and oral fluid flow rate ($r = -0.98$, $p = 0.01$). Also, the lactate concentration was much ($p = 0.03$, two tailed) lower in samples collected under a mild stimulation (50 min^{-1}) than in samples collected without stimulation. Nevertheless, it progressively increased at higher stimulations (100 and 150 min^{-1}). A transfer process mediated by membrane carriers (i.e. urate transporter and organic anion transporters) was hypothesized to explain these results. Finally, a reduced variability (relative standard deviation below 10%) of the urate concentration was obtained when oral fluid was sampled at constant stimulation (70 min^{-1}), but it increased remarkably (20–50%) in case of sampling at self-selected pace. Nevertheless, expressing the salivary excretion of urate as a function of time (μg min^{-1}), the variability of sampling procedure at self-selected pace was lower than 15%.

1. Introduction

The correlation between drug concentration in oral fluid and the corresponding concentration of the unbound and pharmacologically active fraction in blood [1] has recently made oral fluid analysis attractive for many researchers working in the field of pharmacokinetic [2] and therapeutic drug monitoring (TDM) [3,4]. In addition, the minimally invasive sampling and the simpler matrix compared to blood has made oral fluid analysis popular for human biomonitoring [5].

In humans, oral fluid mainly originates from three pairs of major salivary glands (parotid, sublingual and submandibular) and from a large number of minor salivary glands [6]. Healthy adults normally produce 500–1500 mL of oral fluid per day at an approximate rate of 0.3–1.0 mL min^{-1}, but several physiological and pathological conditions can modify this secretion rate [7]. The non-invasive sampling that does not require trained personnel is the main advantage of oral fluid analysis compared to the analysis of blood and its derivatives (e.g. plasma).

Several procedures for collecting non-stimulated and stimulated oral fluid samples are currently available [8]. Non-stimulated oral fluid samples can be collected by draining, spitting, suction and/or adsorption into swab, whereas secretion can be chemically stimulated with few drops of citric acid (0.1–0.2 M) on the tongue or mechanically stimulated by asking the patient to chew paraffin wax, parafilm, rubber bands or chewing gum. After stimulation, the oral fluid can be spat out, suctioned or absorbed [8]. Assuming a unit density for oral fluid [9], flow rate (mL min^{-1}) is estimated from the ratio of sample weight (grams) to sampling time (min). Swab saturation and swallowing of saliva from the patient should be avoided in order to have a reliable estimate of the flow rate. Stimulation allows to collect large sample volumes (>1 mL) in a short time (30–60 s) and limits the variability of salivary pH, as most samples' pH values lie in a narrow range centred at pH 7.5 [10].

Generally, the transfer of a compound from blood to oral fluid occurs by passive transport through the salivary membrane or active processes mediated by a protein carrier. The actual mechanism (or combination of mechanisms) depends on the physical and chemical properties of the compound (e.g. molecular size, pKa and lipid solubility), as well as on the oral fluid pH and flow rate [11]. When passive transport is involved,

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the concentrations of lipid-soluble acidic or basic compounds in oral fluid depend on the degree of ionization in plasma and oral fluid. Since salivary membranes are not permeable to charged "ionized" molecules, permeability is governed from the pH-partition hypothesis [11]. The Rasmussen model allows to calculate the theoretical oral fluid to plasma concentration ratio for a compound crossing the salivary membrane by a rapid diffusion through the acinar cells. Fast diffusion makes the concentration of such molecules in oral fluid independent of flow rate [12]. On the contrary, a hydrophilic compound is a poor membrane-permeant due to its limited solubility in lipids. For this reason, it slowly enters oral fluid only via the tight junctions of the acinar cells (the paracellular route) making its concentration in such fluid significantly influenced by flow rate (an increased salivation dilutes the compound and decreases concentration) [11]. In this case, the "tube" model of capillary diffusion is needed to estimate the oral fluid to plasma concentration ratio [10]. In the case of an active process, a specialized membrane protein guarantees the movement of a compound across the salivary membrane against the concentration gradient. In primary active transport, these proteins require energy in the form of adenosine triphosphate, whereas in the secondary transport the electrochemical gradient is responsible for the transfer of the compound [13].

The inter- and intra-subject variability of oral fluid pH and flow rate affects the oral fluid to plasma concentration ratio and limits the use of oral fluid analysis for therapeutic monitoring to a restricted set of drugs (e.g. unconjugated steroids) characterized by a high permeability through the salivary membranes [14]. Other drugs require ad hoc collection protocols, as recently discussed for warfarin monitoring [15], to make sure that data are representative of the actual conditions of patients as well as to compare data collected at different times.

Uric acid and lactic acid are two important metabolites produced from the xanthine oxidase enzyme via the purine metabolism pathways [16] and from pyruvate via anaerobic glycolysis respectively [17]. Several papers proposed to monitor the concentrations of these two compounds because of correlations with several pathological conditions (e.g. chronic heart failure) [18–22] or therapies (e.g. haemodialysis) [23]. Uric acid is a weak acid (pKa equal to 5.4 at 20 °C) [24] distributed throughout the extracellular fluid compartment by protein carriers, namely urate transporter (URAT1) and organic anion transporters (OATs). Lactic acid (pKa equal to 3.9 at 20 °C, [17]) is produced by lactate dehydrogenase in skeletal muscles, liver and red blood cells under anaerobic conditions [16]. At physiological pH (7.40 ± 0.02) [25], uric acid and lactic acid are mainly dissociated to urate and lactate respectively.

In the kidney, URAT1 and OATs mediates the uptake of urate from the renal tube into the renal tubular cells in exchange of organic anions, such as lactate and nicotinate [26,27]. Recently, Ikarashi et al. found that URAT1 and OATs are expressed both in the ductal cells (i.e. OAT1, 2 and 4) and in the acinar cells (i.e. OAT2 and 3) of the salivary glands [28]. Sato et al. reported that the reabsorption mechanism of urate by URAT1 is influenced by different anions, among which hydroxyl and chloride [29]. Since stimulation increases the oral fluid concentrations of both these anions up to ten times [30,31], it is reasonable to speculate that the transfer mechanism of urate from blood to oral fluid may be affected.

Based on this information, the objective of this study was to i) compare the composition of non-stimulated and stimulated oral fluid samples collected at different frequencies of stimulation (50, 100 and 150 min^{-1}) and then ii) set up a reliable sampling protocol for the non-invasive monitoring of urate and lactate.

2. Materials and methods

2.1. Statement of ethics and study subjects

The study was approved by the Ethics Committee of the Azienda Ospedaliero-Universitaria Pisana ("A co-operative mHEALTH environment targeting adherence and management of patients suffering from Heart Failure", protocol number: 643694). Twenty-two nominally healthy subjects (12 males and 10 females) volunteered to participate and signed a written informed consent.

2.2. Chemicals and materials

Uric acid, i.e. 7,9-Dihydro-1H-purine-2,6,8(3H)-trione (purity ≥ 99%), EHNA hydrochloride, i.e. erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride (purity ≥ 98%), dipyridamole (purity ≥ 98%), sodium hydroxide (purity ≥ 98%, pellets anhydrous), lactate (TraceCERT, 1000 ± 2 mgL^{-1}), 9-chloromethyl-anthracene (purity ≥ 98%), tetra-*n*-butylammonium bromide (purity ≥ 98%) and triethanolamine (purity ≥ 99%), sodium chloride (purity ≥ 99%), sodium nitrate (purity ≥ 99%), phosphoric acid (purity ≥ 99%) and acetonitrile at HPLC grade were purchased from Sigma Aldrich (Milan, Italy). HPLC grade water was produced by a Millipore milli-Q reagent water system (Bedford, MA, USA).

All the liquid solutions and OF samples were stored in sterile polypropylene containers from Eppendorf (Milan, Italy).

Salivette collection devices (cotton swab, cotton swab impregnated with citric acid and polyester swab) were purchased from Sarstedt (Nümbrecht, Germany).

2.3. Equipment

High performance liquid chromatography (HPLC) analysis was carried out using a Jasco HPLC system (Lecco, Italy) equipped with an AS 2055 autosampler, a PU 2089 quaternary low-pressure gradient pump, an UV 2070 ultraviolet detector and a FP 2020 fluorescence detector. The column temperature was controlled by a RECIPE ClinLab HT 3000 thermostat (Munich, Germany). The HPLC system was controlled using ChromNAV™ software (v. 1.9, Jasco, Japan).

A VELP Scientifica ZX4 Advanced Vortex Mixer (Usmate, Italy) and an Eppendorf Centrifuge 5804 R equipped with an A-4-44 swinging bucket rotor (Milan, Italy) were used for sample vortex-mixing and centrifugation, respectively.

Chromatographic separation of uric acid was carried out with an Agilent (Santa Clara, CA, USA) Zorbax SB-Aq reversed-phase column (250 × 4.6 mm, 5 μm) connected to an Agilent Zorbax SB-Aq guard column (12.5 × 4.6 mm, 5 μm). The lactate-9-chlormethyl-anthracene adduct was separated using an Agilent Poroshell EC-C-18 reversed-phase column (100 × 4.6 mm, 2.7 μm) connected to an Agilent guard column TC-C-18 (12.5 × 4.6 mm, 5 μm).

Absorption spectra of uric acid were recorded by a PerkinElmer Lambda 25 spectrophotometer (Waltham, MA, USA).

A Crison chloride Ion Selective Electrodes (ISE) (Barcelona, Spain) was used to determine the concentration of chloride in oral fluid samples. All the potential measurements were carried out by a Eutech Instrument PC2700 pH/mV/Conductivity/°C/°F meter (Vernon Hills, IL, USA) capable of reading to 0.01 mV.

All data were analysed using GraphPad Prism (v. 6.0, GraphPad Software Inc., La Jolla, CA, USA).

2.4. Oral fluid collection

Oral fluid samples were collected by Salivette roll-shaped polyester swabs between 9 and 11 AM, in order to avoid that variations related to circadian rhythms could affect results [32]. Each volunteer was asked to refrain from eating, drinking, smoking, chewing gum, and oral hygiene practises for at least 1 h prior to oral fluid collection. Subjects placed the swab in the mouth between the gum and cheek and kept it steady for 2 min (procedure A) to collect non-stimulated oral fluid samples. On the contrary, different frequencies of stimulation were obtained by moving the swab in the mouth at 50, 100 and 150 min^{-1} for 1 min (procedure B). Three stimulated oral fluid samples were consecutively collected from a subgroup of 5 volunteers moving

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