Invited critical review

Saliva specimen: A new laboratory tool for diagnostic and basic investigation

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Abstract

The assay of saliva is an increasing area of research with implications for basic and clinical purposes. Although this biological fluid is easy to manipulate and collect, careful attention must be directed to limit variation in specimen integrity. Recently, the use of saliva has provided a substantial addition to the diagnostic armamentarium as an investigative tool for disease processes and disorders. In addition to its oral indications, the analysis of saliva provides important information about the functioning of various organs within the body. In this respect, endocrine research certainly occupies a central role. The present review considers the laboratory aspects of salivary assays with respect to the different analytes including ions, drugs and various non-protein/protein compounds such as hormones and immunoglobulins. This review also examines the consequences of preanalytical variation with respect to collection strategy and subsequent storage conditions. It is likely that the use of saliva in assays will continue to expand thus providing a new instrument of investigation for physiologic as well as pathophysiologic states.

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Keywords: Saliva; Hormones; Collection; Storage; Stability

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1. Introduction

Saliva in humans is a mouth fluid possessing several functions involved in oral health and homeostasis, with an active protective role in maintaining oral healthiness. Saliva helps bolus formation by moistening food, protects the oral mucosa against mechanical damage and plays a role in the preliminary digestion of food through the presence of α-amylase and other enzymes. It also facilitates taste perception, allowing soluble food-derived molecules to reach the gustative papillae and buffer the acid components of food with the bicarbonates (originating from salivary gland carbonic anhydrase). Saliva also has a role in maintaining teeth enamel mineralization: several proteins (statherin, proline rich proteins – (PRPs) and mucins) allow Ca++ saturation in saliva to be maintained [1]. Saliva has defence functions against pathogen microorganisms, in the presence of defence proteins that react in specific (immunoglobulins) or non-specific (lysozyme, peroxydase, cystatins, lactoferrin, hystatins and others) ways, inhibiting microorganisms growth [2,3].

In humans, oral fluid originates mainly from three pairs of major salivary glands (parotid, sublingual and submandibular) and from a large number of minor salivary glands. Parotid glands are entirely serous glands since their secretion lacks mucin, whereas submandibular and sublingual glands are mixed sero-mucous. Minor salivary glands are mainly Von Ebner glands (entirely serous organs situated in the connective tissue below the circumvallatae papillae) and Blandin-Nühm mucous glands [4].

Salivary composition varies in relation to the serous or mucous component of the glands [5]; the relative contribution of each type of gland to total unstimulated saliva secretion varies from 65%, 23%, 8% to 4% for submandibular, parotid, Von Ebner and sublingual glands respectively [6].

Salivary components have also a non-glandular origin, so oral fluid cannot be considered as the only production of salivary glands, because it also contains fluids originating from oropharingeal mucosae (oral mucosal transudate cells, bacteria, fungi, virus, upper airways secretions, gastrointestinal reflux) [7,8]. Saliva contains also crevicular fluid, an extracellular fluid-derived from the epithelia of the gingival crevice. Crevicular fluid is produced at approximately 2–3 μl/h per tooth and it can be considered as a plasma transudate [9]. Oral fluid may also contain food debris and blood-derived compounds (actively or passively transferred), such as plasmatic proteins, erythrocytes and leucocytes in case of oral inflammation or mucosal lesions [7].

The aim of the present review is to update various aspects related to the laboratory investigation, taking into account the principal compounds present in this body fluid and in particular considering the most important substances. The proteoma and related compounds are described, taking into consideration the matrix effects and therefore the composition of this fluid. The importance of proper collection and storage of the saliva samples is also discussed. Salivary hormones and their connection with the circulating ones, which might be linked, could be an important aspect.

2. Salivary production and composition

Healthy adult subjects normally produce 500–1500 ml of saliva per day, at a rate of approximately 0.5 ml/min [6] but several physiological and pathological conditions can modify salivary production quantitatively and qualitatively, e.g., smell and taste stimulation, chewing, psychological and hormonal status, drugs, age, hereditary influences, oral hygiene [7] and physical exercise [6,10]. Each type of salivary gland secretes a characteristic type of saliva. Differences in the concentration of salts/ions [11] and total proteins [5] among glands can be observed. Salivary proteins are expressed differently among individual glands, like submandibular and sublingual glands. For example cystatin C is secreted by the submandibular gland and MUC5B mucin and calgranulin are secreted by the sublingual gland [5]. Moreover, salivary composition varies, depending upon whether salivary secretion is basal or stimulated [11].

Salivary output and composition depend on the activity of the autonomic nervous system: the serous part of the glands is under the control of the sympathetic system and the mucous part of both parasympathetic and sympathetic systems. The α–β adrenergic and cholinergic stimuli (neural or pharmacological) can modify the quantity; viscosity and ionic and protein concentrations can vary. Parasympathetic stimulation results in a high flow of saliva containing low levels of organic and inorganic compounds. Sympathetic stimulation produces a low volume of protein-rich and K+-rich saliva [7]. The presence of food in the mouth can affect salivary composition as a stimulus for selective protein release; after a meal an increase of total proteins and of α-amylase in saliva has been shown [12].

The clearance of compounds from plasma into saliva may involve several processes:

(a) ultrafiltration through gap junctions between cells of secretory units (intercellular nexus). Only molecules with MW <1900 Da are involved (water, ions, hormones such as catecholamines and steroids) and their salivary concentration is 300–3000 times lower in saliva than in plasma [13].

(b) transudation of plasma compounds into oral cavity, from crevicular fluid or directly from oral mucosa. The presence in the saliva of some typical plasmatic molecules, like albumin, depends on this mechanism [13].
2.1. Inorganic compounds

Whole saliva contains mainly water, strong and weak ions (Na⁺, K⁺, Mg²⁺, Ca²⁺, Cl⁻, HCO₃⁻, HPO₄²⁻) which can generate buffer capacity. The primary secretion from salivary glands is plasma ultrafiltrate (isotonic compared to plasma) but in salivary ducts there is energy-dependent reabsorption of Na⁺ and Cl⁻ resulting in a hypotonic fluid secretion, with a lower ion concentration compared to plasma. In salivary gland ducts mineralocorticoid receptors are present, so salivary glands are mineralocorticoid-responsive [16]: for this reason salivary K⁺ concentration is higher than the plasma concentration (25 vs. 4 mmol/l) and Na⁺ concentration is lower in saliva compared with that in plasma (2 vs. 145 mmol/l) [7]. Several factors may modify the salivary ionic concentration, furthermore, the composition of unstimulated saliva is different from stimulated saliva [17] (Table 1). Similar to other biological fluids, salivary ionic concentration is usually measured by ion selective electrodes, atomic absorption (or emission) [18] and the traditional spectrophotometric methods [19,20].

2.2. Organic compounds (non-protein and lipids)

Small amounts of organic non-protein compounds can be detected in saliva. Uric acid is one of the most important antioxidant compounds in saliva [21,22], bilirubin and creatinine are also detectable [8,23]. Saliva moreover contains glucose, amino acids, lipids like cholesterol and mono/diglycerides of fatty acids [13,14,24,25]. In saliva amines, such as putrescine, cadaverine and indole, are also detectable [26].

Fatty acids, too, are measurable in saliva and in particular α-linoleic acid and arachidonic acid can be measured in human saliva: their concentration seems to correlate with a dietary fatty acid intake [14]. In saliva lactate is also measurable and its concentration shows a high correlation with capillary blood lactate concentration [6].

2.3. Protein/Polypeptide compounds

The salivary levels of total protein increase also through β-sympathetic activity in the salivary glands, since saliva secretion is mainly evoked by the action of adrenergic mediators [6]. Saliva contains a large number of protein compounds, of which the structure and function have been studied with traditional biochemical techniques, including liquid chromatography, gel electrophoresis, capillary electrophoresis (CE), nuclear magnetic resonance, mass spectrometry, immunoassays (RIA, IRMA, EIA, ELISA) and lectin probe analysis [27,28]. Most of these analyses were performed on the investigation of a specific group of salivary proteins, but some efforts were made to obtain a complete pattern of all salivary proteins with proteomic techniques.

Usually, in a proteomic experiment, proteins are first separated with a two dimension electrophoresis (2-DE) and then detected by a suitable stain. For example, to specifically identify a saliva protein, a spot obtained by 2-DE is excised and digested by tryptic enzymes. The resulting fragments are analysed by MALDI-MS [Matrix Assisted Laser Desorption–Mass Spectrometry] to measure molecular masses of peptides [27]. With this method about 40 proteins were originally identified from human whole saliva but, adding a shotgun proteomics method as a complementary technique, about 300 salivary proteins have now been identified [27]. Recently Guo et al. studied the salivary proteome using a capillary isoelectricfocusing coupled with electrospray-ionization tandem mass spectrometry [29]. Hannig et al. obtained a chromatographic pattern of salivary proteins using reverse phase HPLC and the presence of 5 typical salivary proteins was demonstrated [30]. Tanaka et al. used a microchip CE for separation of salivary compounds with fluorescein isothiocyanate derivatization [31]. Comparing the results performed with the different techniques mentioned, low overlaps are obtained because often, in proteomic experiments, a false-positive identification of peptides sequence in proteomic databases occurs. By using these different techniques it has been possible to recognize the most abundant salivary proteins (Table 2) [32–35].

Among the salivary proteins obtained by classical protein analysis methods, those deriving from salivary gland production can be recognized (of which the most abundant are amylase, PRPs secretory IgA (s-IgA) and carbonic anhydrase). Other proteins can be derived from plasma leakage (albumin, transferrin, IgG) [36].

Human saliva proteins can have a wide range of functional properties. They can be related to the immune response and oral

<table>
<thead>
<tr>
<th>Inorganic compounds (mmol/l)</th>
<th>Whole unstimulated saliva</th>
<th>Whole stimulated saliva</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>5</td>
<td>20–80</td>
<td>145</td>
</tr>
<tr>
<td>K⁺</td>
<td>22</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>15</td>
<td>30–100</td>
<td>120</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1–4</td>
<td>1–4</td>
<td>2.2</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>5</td>
<td>15–80</td>
<td>25</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.2</td>
<td>0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>NH₃</td>
<td>6</td>
<td>3</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Adapted from Aps JKM and Martens LC [7].
defence, like lysozyme, lactoferrin, lactoperoxidase, immunoglobulins, agglutinins, chitinases and mucins which in saliva participate in the protection of the oral tissues; other proteins possess bacteria-killing properties, like histatins and defensins.

The salivary immunoglobulins are mainly s-IgA (75-85%) and are produced directly by the B lymphocytes present near the salivary glands. s-IgA is secreted in the interstitial fluid, taken up by the acinar and ductal cells of the salivary glands and subsequently secreted into the saliva. The remaining 5-15% of salivary immunoglobulins are mainly IgG and IgM, derived from crevicular fluid or from plasma leakage [2]. All these proteins and peptides have a broad spectrum of antimicrobial activity, so their functions overlap, but the meaning of this redundancy is still not clear. The physiological conditions under which saliva is collected can have a significant influence on the concentration of immunoglobulins; furthermore, Gleason’s review underlines the immune response and risk of illness in healthy subjects [37]. Other proteins are also present: for example, enzymes and enzyme inhibitors, hormones such as growth factors, and cytokines such as interleukin-8 [38,39].

Further functional properties of human salivary proteins/peptides are:

1. Inhibition of calcium precipitation: PRPs and statherin promote remineralization of the enamel by the binding of calcium ions [1].
2. Taste perception (carbonic anhydrase) [1].
3. Digestion (amylase, involved in digestion of starch, Von Ebner gland proteins with endonuclease activity) [1].
4. Inhibition of proteinase (cystatin, inhibitor of serine proteinases, tissue inhibitors of metalloproteinases) [1].
5. Other functions: transcription, cell proliferation, signal transduction, chemotaxis and cell motility.

2.4. Hormones

Some hormones commonly measured in plasma, such as steroids, non-steroids, peptide and protein hormones, can be detected in the oral fluid. Before any significance can be attached to the measurement of a hormone in saliva, the existence of a connection between circulating levels and mouth areas must be defined. Where a hormone both originates and is active in the mouth, it possesses a very specific role. It may derive also from the circulation by passive diffusion or active transport, or originate partly from both sources.

This information about the salivary hormone origin allows a definition of its role and connection with pathological and physiological states to enable a suitable analysis. At first sight, it appears to be a useful index of systemic bioactivity; the quantitative measurements of hormones in saliva must be studied to prove a correlation with levels in serum. Thus the connection between the free and the total circulating fractions, together with the more or less specific binding proteins, should be considered. Also the correlation of the saliva levels with the free bioactive circulating form, if present, is of great importance.

Catecholamines can be recognized in saliva ranging from 250 to 800 pg/ml [13], but their source is still uncertain. They seem to originate by diffusion from serum, but there is also an amount of salivary catecholamines derived by direct release from sympathetic nervous terminations, so their concentration is poorly correlated with that of plasma. The increase in plasma catecholamines after physical exercise is not accompanied by a salivary catecholamine elevation. Salivary catecholamine is therefore not considered a useful index of general sympathetic tone. The concentration of catecholamine metabolites (like dihydroxyphenylglycol) instead shows a good correlation with plasma levels [40].

There is little information about thyroxin and triiodothyronine levels in saliva: in preliminary studies they were detected in saliva and their levels (1.10 ± 0.07 nmol/l for T4 in healthy subjects) seem to correlate with plasma levels [41].

Steroid detection is perhaps the most interesting application in salivary hormonal studies. Steroids have often been studied because salivary-free steroid hormones can give good information on serum-free levels [42] (for further details: see Section 6). The most commonly assayed biomarkers in saliva are cortisol, testosterone, dehydroepiandrosterone (DHEA) [43–46], 17-hydroxyprogesterone, progesterone [46,47] and aldosterone [48]. Table 3 outlines steroid hormones measurable in saliva specimens.

Salivary cortisol measurement is today a widely accepted alternative to the determination in plasma or serum: since the adrenal cortex is responsive to stress, venipuncture for blood collection can lead to an iatrogenic increase of plasma gluco-
To collect saliva for analysis, it is important to ensure that any blood contamination is minimal. This is because proteins and polypeptide hormones are often present in saliva, which can affect the results if not properly accounted for.

The protein polypeptide hormones, such as prolactin, insulin-like growth factor I (IGF-I), and melatonin, have been detected in saliva. These hormones, along with others such as cortisol and aldosterone, can be analyzed in saliva samples. However, the concentrations of these hormones are typically much lower in saliva compared to plasma, making saliva a less desirable medium for hormone measurement.

The standardization of salivary collection has a great importance in saliva analysis, because several factors may affect salivary flux and composition. Whole saliva, glandular-duct saliva, crevicular fluid, and mucosal transudate are all achievable specimens for which specially designed collecting methods are possible. Today several methods and devices are available. Among them, the easiest and the most feasible method is the collection of whole oral fluid.

A more specific specimen can be obtained with less easy collection procedures. The following procedures are available to collect a single constituent of the whole saliva:

(a) Saliva produced by a single salivary gland can be collected by cannulation of a single salivary duct [53], or by a metal or acrylic cup placed over the Stenson’s duct for the collection of pure parotid saliva [5].

(b) Crevicular fluid can be collected inserting directly in the gingival crevice an appropriate device [9], such as filter paper, a commercial micropipette or a thin tube. It provides excellent sampling of a serum transudate but it is unlikely that commercial tests would be developed for this fluid due to the difficulty in applying the collector and the small volume of fluid collected [55,56].

(c) Pipette suction from under the tongue [57].

(d) Pipette suction from the paragingival gutter [8,57].

(e) Saliva from submandibular glands can be collected by placing the tip of a collection device at the orifice of the Wharton’s duct, after placing sterile cotton sponges in the floor of the mouth and over the buccal mucosal areas to occlude the parotid and sublingual ducts [5].

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These methods allow differences in composition among salivary gland secretions and other sources of oral fluid constituent to be studied but, from a practical point of view, tests based on this type of sampling could not be widely marketed because of the high degree of training required for the specialist collecting the specimen. For these reasons these sampling methods are not commonly used [58].

Whole saliva (or whole oral fluid) is easy to collect, is more representative of the oral milieu, and collection does not need to be performed by a trained specialist but can be made by the patient or study participant.

Unstimulated whole saliva can be collected with several oral fluid collector devices and commercial devices are available.
1. Passive drooling (no oral movements): allowing saliva to drain off the lower lip into a plastic vial [57,59,60].
2. Spitting directly into a collector vial: specimens collected by spitting contain up to 14 times more bacteria than those collected by drooling and this can affect storage and further analysis of several compounds [57].

Stimulated whole saliva can be obtained with oral movements such as gentle mastication or with the use of citric acid. Citric acid has the potential to stimulate salivary secretion and it lowers the sample pH (<3.0), but it may also affect analysis results in hormonal immunoassay by interfering with antibody binding [61]. Citric acid may also interfere with the measurement of some analytes, such as testosterone [62]. The most commonly used devices are sterile cotton dental rolls as Salivette (Sarsted, Newton, NC). The cotton roll is placed into the subject’s mouth and is gently chewed for about 1–2 min, then placed into the vial. Saliva is obtained by expressing the saturated cotton using a needleless syringe or better by centrifugation. Cotton wool sampling may induce variations in salivary immunoassays: testosterone, DHEA, estradiol, 17-OH hydroxyprogesterone [63] may be artificially high and s-IgA levels may be artificially low. Salivary levels of other steroid hormones (cortisol, DHEA-S and cotinine) do not seem to be affected by cotton wool sampling [64], but our laboratory data (unpublished) demonstrate cortisol binding to cotton wool does occur.

To avoid this type of analyte interference, non-cotton based sampling are available:

1. Polystyrene foam swabs [60].
2. Rayon balls, such as Orapette (Trinity Biotech, Dublin, Ireland) [63].
3. Polyester Salivette (Sarsted, Newton, NC) [64].

However, polystyrene and polyethylene vials and tubes were found to bind and remove up to 87% of the progesterone from saliva [65]. Stimulated saliva may also be obtained using non absorbing methods:

1. Chewing a piece of paraffin wax of standardized size [66,67].
2. Chewing neutral gum base [59].
3. Chewing parafilm or rubber bands [20].
4. Keeping in mouth powdered drink crystals [68] or other foodstuff containing citric acid [17].

4. Saliva storage

Saliva specimens, after collection, should preferably be kept on ice, aliquoted and frozen as soon as possible to maintain the sample integrity. The refrigeration prevents the degradation of some molecules in saliva and, when necessary, bacterial growth must also be prevented. Moreover, saliva contains bacterial protease enzymes which can degrade several salivary proteins: this can affect protein compound investigation. Nurkka’s et al. investigation suggested that s-IgA can be degraded at room temperature from bacterial proteases [57]. Ng et al. reported a decrease of 10% in s-IgA levels after 8 months of storage at −30 °C of plain saliva [34]. Morris et al. observed that IgG levels measured by antibody capture ELISA in saliva samples, did not decrease stored at room temperature (10–20 °C) for a week [69]. Other saliva substances, such as catecholamines, can have a short biological half-life and a rapid degradation [40]. The method of specimen storage can therefore influence the concentration of some compounds modifying their levels.

With regard to saliva storage and cortisol, this hormone seems to decrease its concentration by 9.2% per month at room temperature, but at 5 °C no effect on salivary cortisol concentration for up to 3 months was demonstrated [70]. Furthermore, Garde and Hansen demonstrated that repeated cycles of freezing and thawing did not affect the concentration of salivary cortisol [70]. Saliva progesterone concentration was stable for 3 months at room temperature [65].

Storage procedure and time from the collection mainly affect the analysis of the biochemical variables characterized by temperature instability and bacterial growth. Some salivary compounds can have a very short half-life so the sample to be analysed needs a narrow range of time after collection; other substances can remain stable in saliva for a longer time and may be detected and quantified after a long time [57]. For this reason, the choice of different storage procedure before the analysis depends on the type of molecule, taking into account its stability.

We propose, as a general approach to avoid degradation of salivary compounds, that the specimens should be stored taking into account the following outline:

1. Immediately store saliva aliquots without any processing. Specimens can often be stored at room temperature (when analysis is carried out immediately or in 30–90 min from collection), at +4 °C (when analysis is carried out in 3–6 h from collection), at −20 °C and better at −80 °C (when analysis is carried out days to months after collection).
2. Snap-freezing of saliva in liquid nitrogen: mix each saliva aliquot with an equal volume of 80% glycerol in H2O, then dip the sample in liquid nitrogen. This storage procedure aims to inhibit the bacterial protease activity degrading some salivary protein compounds, such as s-IgA [57].
3. Inhibition of the enzyme activity present in saliva: mix each saliva aliquot with enzyme inhibitors 10:1 (leupeptin, aprotinin and 4-[2-aminoethyl] benzenesulfonyl fluoride) [57]. In our laboratory a mixture of protease inhibitors and stabilizing substances is used (aprotinin, leupeptin, antipain, pepstatin A, phenyl methyl sulfonyl fluoride, EDTA and thimerosal).
4. Addition of sodium azide (NaN3) to saliva specimens in attempt to retard bacterial growth. The use of sodium azide does not influence the measurement of salivary markers when serum-based immunoradioassays are modified for saliva, not even if these methods involve separation or extraction steps. But the possible interference of sodium-azide with horseradish peroxidase, a common component of enzyme immunoassays, must be taken into account [45].
5. Addition of trifluor acetate at 10% water solution, to denature salivary enzymes that could degrade several salivary compounds, such as proteins and steroid hormones [49].

5. Chemical and biochemical laboratory analyses

Saliva is a biological matrix still less used than plasma in clinical setting, even though it possesses several advantages mainly regarding the collection and storage steps.

Whole oral fluid compounds have been examined with a large number of methods/techniques: colorimetric/spectrophotometric, solid phase extraction and HPLC or CE with UV detection and immunoassays. In effect salivary analyses are more often performed with the aim to study a group of molecules (e.g., proteins) or to detect and measure a single compound in oral fluid. Most reports do not use the suggested procedure with the use of commercially available reagents, but frequently adapt the commercial kits, designed for serum or plasma assay, modifying the procedures/techniques to obtain a method suitable for saliva. In these cases the analysis results should be well defined with method performance data and reference ranges. Moreover, in some cases, the oral fluid analysis is to be preferred having a better performance and efficiency than the plasma analysis.

Among immunoassays, to date, several commercial kits are available for plasma, serum or urine analysis of a wide range of compounds, and they may be adapted for saliva analyses with slight modifications [34,49,51]. Recently an increasing number of kits especially designed for salivary assays have become available [71] such as salivary melatonin and steroids (cortisol, testosterone, estradiol etc.).

5.1. Interferences

Blood contamination could be a problem in saliva assays because, when some blood compounds are present in the oral mucosa, quantitative estimates of salivary molecules may be compromised. This aspect is relevant because the correlation between saliva and serum concentrations is necessary to use oral fluid as an alternative to plasma matrix (for further details: see Section 6). Schwartz et al. proposed salivary transferrin as an index of blood sample contamination [72]. Huang observed that some proteins (hemoglobin β-chain, apolipoprotein A-1, thioredoxin peroxiredoxin B, a protein highly expressed in erythrocytes) increase their levels in whole saliva when oral bleeding occurs. These proteins have also been proposed as possible markers of blood contamination of saliva [28].

Some studies indicate that food products may contain substances which can potentially cross-react with steroid antibodies used in salivary immunoassays for testosterone (e.g., bovine hormones in dairy products) [64], but some other studies did not find any effect of food intake on the measurement of cortisol and 17OH-progesterone and progesterone with RIA methods [49].

As previously described, sodium azide can interfere in EIA and ELISA.

6. Comparison of saliva and circulating concentrations of compounds

The concentration of the biochemical compounds in the circulation is, in general, well documented and, together with the standardized steady-state condition, defines the range of variation and the reference values. Plasma concentrations of the more investigated components for diagnostic purposes define narrow ranges, whereas oral fluid composition exhibits wide variation, both quantitatively and qualitatively [7]. This large variability, with respect to the composition of oral fluid, challenges the development of bioanalytical technologies and the statement of salivary compound reference values in healthy subjects. This is the major difference between oral fluid and plasma. These physiological variations in oral fluid may cause difficulties in the standardization of salivary analyses, making plasma assays preferable to oral fluid because of their better stability.

Many salivary compounds can derive from plasma by passive diffusion or active transport: in this perspective salivary analysis has a great potential diagnostic role, because several biochemical and immunological parameters can be measured both in blood and in saliva (e.g., immunoglobulins). Therefore to set up saliva as an alternative matrix to plasma for various assays of biological quantitative parameter, there must be a high correlation confirmed between plasma and saliva levels. In the case of passive diffusion from blood, a correlation should be found between blood and salivary levels, but the lack of this correlation does not exclude that the measured component has a blood origin. In effect the diffusion of the investigated molecule might only take place partially and an active transport or a reabsorption can affect the correlation between blood and salivary levels of the analyte. In any case, to reflect systemic bioactivity usefully, a quantitative assay for those parameters in saliva should be highly correlated with the levels in serum.

A large amount of salivary compounds are produced directly by salivary glands and can hardly be detected in plasma. For example, secretory IgA and lysozyme are present in great amounts in saliva, but less in blood, so they can be considered as originating from saliva. Otherwise the concentration of transferrin, iron, bilirubin, cholesterol, TG, lipoproteins, IgG and IgM in blood is 4–15 times higher than in saliva. Nagler et al. found a high correlation between plasma and salivary levels for blood urea nitrogen, C4 and total bilirubin, suggesting that the presence of these compounds in saliva may derive from passive diffusion from plasma [8].

Saliva is currently used in the measurement of steroid hormones, such as cortisol [73] and cortisone [74], testosterone [75,76], because it is commonly accepted that the salivary level of these steroids reflects the free, unbound circulating fraction. Steroids are also present in urine with their free, unbound fraction, but urinary steroid metabolites may cross-react with antibodies used for the steroid immunoassay analyses. Furthermore, urinary-free steroids are usually measured in the 24-h urine and this way of collection is often not easy.

The majority of steroids in plasma circulates bound to albumin and specific proteins, such as SHBG (sex hormone binding globulin) and CBG (cortisol binding globulin), while
only the free fraction (in equilibrium with the bound steroids) is the bioactive fraction. Free steroids, because of their liposolubility, pass through the cell membrane, and are directly available for the cell receptors. Thus, salivary steroids are thought to reflect the concentration of the physiologically active unbound steroids in serum [66]. Blood leakage into oral fluid (due to periodontal diseases, injury or vigorous cleaning) affects the quantitative estimates of salivary-free steroid concentrations [55].

Also non-steroid hormones can be detected in saliva, such as melatonin [52], and preliminary studies suggest that thyroid hormones can also be measured in saliva [41], but it is not clear if their detection is caused from plasma leakage.

Protein/Polypeptide hormones do not seem to be detectable in saliva unless they are directly produced by salivary glands: for example, leptin seems to be directly produced by salivary glands and it can be detected in saliva even if it is a protein hormone (16 kDa) [76]. Generally, protein plasma hormones cannot be detected in saliva because of their large dimensions: i.e., they cannot pass through the salivary glands by passive diffusion [7]. In this case, their detection in oral fluid may be an index of plasma leakage from oral lesions, but mechanisms other than passive diffusion from plasma, such as an active transport and a primary secretion, might be present. Another example of a polypeptide hormone recently proposed for measurement in human saliva is the insulin-like growth factor 1 [51]. This hormone might be produced in the mouth as a growth factor, but it could be also originated by a diffusion (active or not).

7. Saliva as a diagnostic tool

Recently there has been increasing interest in diagnosis based on saliva analyses, because saliva has a simple and non-invasive collection method. Oral fluid sampling is safe for the operator and the patient, and has easy and low-cost storage. These characteristics make it possible to monitor several biomarkers in infants, children, elderly and non-collaborative subjects, and in many circumstances in which blood and urine sampling is not available. Another reason that makes saliva interesting for diagnostic purposes is the linkage with traditional biochemical parameters which appear in the circulation in various forms.

Saliva analyses have been used mainly in dentistry and for studies in oral diseases to help assess the risk of caries, by measuring saliva buffer capacity and bacterial contents [2]. Oral fluid is mainly utilized for research and diagnostic purposes concerning systemic diseases that involve the salivary glands and oral cavity, such as Sjögren syndrome [11,77], Behçet syndrome, benign and malignant oral tumours [78].

Saliva investigation for diagnostic purposes has been proposed in the following.

7.1. Viral and bacterial infections (genomes and antibodies detection)

Oral fluid contains a small amount of IgG, mainly derived from crevicular fluid and from mucosal transudate. For this reason, oral fluids with a higher content of IgG are suitable for use in the screening of viral infections and immunization. A correlation between salivary and serum antibodies has previously been reported for HIV (human immunodeficiency virus) [79]. Blood contamination improves the HIV antibody detection in saliva [60]: so this phenomenon is not always a negative fact, because it can help the revealing of antibodies, drugs, and other molecules, which are normally detected in higher levels in plasma than in saliva. Correlation between salivary and serum IgG levels is also present in HCV antibodies [80], HAV (hepatitis A virus) [81], EBV (Epstein Barr virus) [82], CMV (Cito Megalo virus) and rubella virus. Salivary antibodies have also been reported after immunization against poliovirus, rotavirus and HAV [80]. For these reasons, salivary testing for specific antibodies can be an important means to evaluate systemic immunity in diseases or to evaluate immunity in response to vaccination. Oral fluid collection devices, designed for this purpose, are commercially available [60].

7.2. Cancer

c-erbB-2 soluble fragments and 15-3 cancer antigen in breast cancer research have been demonstrated in saliva [38]. Also, the profiling of salivary RNA transcript with microarray analysis seems to be able to distinguish several genes exhibiting significantly different expression levels in saliva comparing oral squamous cell carcinoma patients with controls [78].

7.3. Pharmaceutical and abuse drugs

Some pharmaceutical drugs (e.g., lithium, digoxine, phenobarbital and others) have a narrow therapeutical index; for this reason a constant monitoring of their plasma concentrations is necessary to achieve the best therapeutic effect while reducing the risk of adverse effects, especially in patients with hepatic or renal impairment. Therapeutic monitoring of these drugs is often performed in plasma. Recently Guo et al. developed a method with liquid chromatography-electrospray tandem mass spectrometry to measure levetiracetam levels also in saliva [83]. Furthermore, many substances which are commonly abused can be detected in human saliva including alcohol, amphetamines, barbiturates, benzodiazepines, cocaine, lysergic acid diethylamide (LSD), opioids, phencyclidine and cotinine for tobacco smoke [84]. Salivary testing of these substances is particularly useful where a “yes/no” answer is required, because plasma leakage does not affect qualitative analysis [38]. The possibilities offered by saliva for protein compound detection have recently been proposed [51] and this field of research seems to be a new frontier for sport anti-doping test purposes.

7.4. Hormones

Putignano et al. proposed midnight salivary cortisol measurement as a screening procedure for patients with suspected Cushing’s syndrome [85]. Abnormal salivary diurnal cortisol rhythms have been shown to be predictive of disease progression in metastatic breast cancer patients [86]. In this
respect, salivary concentrations may be a better measure of the exposure of target organs to the steroids than the serum concentrations are. The salivary-free estradiol measurement is an FDA-approved test which can predict preterm labour in women at risk [38]. The non-steroid hormone detection and measurement are rarely performed. Also protein polypeptide hormones have been analysed in the saliva, as previously mentioned [28,43–48,51,52] and a patient with hormonal pathologies could be investigated. Therapy monitoring could also be included.

7.5. DNA tests

DNA in cells present in whole saliva could also be investigated. The traditional source of genomic DNA is blood, but recently saliva has increasingly been investigated as a source of DNA deriving from oral cells. It provides a useful source for biomarker profiling and forensic identification [38,87]. DNA tests in saliva could also be carried out for the detection of HIV infection, recognizing viral sequences in total salivary DNA amplifying by polymerase chain reaction a relatively constant region of HIV-1 genome, [88]. In oral fluid are also detectable by PCR several oral pathogens that may cause periodontitis [89].

7.6. Sialochemistry analysis

Sialochemistry for environmental heavy metals (cadmium, lead, mercury) may be useful in monitoring environmental, atmospheric and occupational pollutants: Gonzalez et al. found that saliva can be a good milieu for early monitoring of an exposure to lead and cadmium, since salivary levels of these elements arise from the diffusible fraction of plasma [20]. Therefore, there is an increasing interest on the diagnostic role of saliva but there are only very few studies that utilize oral fluid in the diagnosis of systemic diseases, such as alcoholic cirrhosis and cystic fibrosis [3,8]. Consequently this research area must be further developed, because in saliva the measurements of some markers of systemic diseases, normally detected in serum/plasma or urines, seem to be possible.

8. Conclusions

The saliva matrix is an upcoming area of research for basic and clinical application purposes, with considerable potential for growth and progress. Saliva is a really useful specimen when a qualitative answer is required (for example in toxicology). It is also usable for quantitative measurements of several analytes, particularly when a stable correlation between plasmatic and salivary levels can be achieved. Nevertheless, to date salivary assays are still little used compared with plasma assays, even if it is possible to have a quantitative estimate of hormones and other substances in saliva.

In conclusion, saliva is a biological fluid that offers several opportunities in diagnosis, toxicology and in forensic science. Furthermore, many salivary proteins offer great potential in clinical and epidemiological research, in oral as well as in general health studies.

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