SALIVA AS AN ANALYTICAL TOOL IN TOXICOLOGY

Karin M. Höld, B.S.; Douwe de Boer, Ph.D.; Jan Zuidema, Ph.D.;Robert A.A. Maes, Ph.D.
Utrecht Institute of Pharmaceutical Sciences (UIPS), Utrecht University, Department of Analysis and Toxicology, Sorbonnelaan 16, 3584 CA Utrecht, the Netherlands

Address for correspondence and requests for reprints:
Mrs. K.M. Höld, Department of Analysis and Toxicology, Utrecht University,
Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands. Telephone +31 302537305, Fax +31 302537387, E-mail K.M.Hold@far.ruu.nl

Contents

Abstract

In drug analysis, research involving the use of saliva sampling as non-invasive qualitative and quantitative techniques have become increasingly important. Being readily accessible and collectible, saliva may show many advantages over 'classical' biological fluids such as blood and urine. Because of the growing interest in non-invasive procedures, this up-dated review evaluates the use of saliva in drug analysis and in therapeutic and toxicological monitoring. New techniques for the collection and analysis of saliva as well as for identifying the components affecting drug concentrations in saliva are discussed in order to clearly identify its role as a diagnostic medium. Due to our present incomplete knowledge of saliva as a biological specimen, saliva drug levels should be used concomitantly with recorded drug concentrations in other fluids, e.g. plasma, to contribute to a more ideal interpretation of drug concentrations in clinical and forensic case studies.

1. Introduction

In recent years saliva has attracted much attention, in particular among people interested in the determination of drug concentrations, who suggest that saliva might be substituted for plasma in the areas of pharmacokinetic studies and drug monitoring. The traditional biological samples for the qualitative and quantitative measurement of most drugs are blood, plasma and urine. Many substances and their metabolites are present in different concentrations in these media. Blood or plasma provide an estimate of the current circulating concentration of the analyte of interest. Urine permits measurement of the accumulated concentration of analytes since the last void of the bladder. Previous publications have made it clear that for many drugs the monitoring of saliva is a real alternative for determining plasma levels because saliva lacks "the drama of blood, the sincerity of sweat and the emotional appeal of tears" (Mandel, 1990). However, a clear interpretation of the quantitative significance of saliva drug concentrations to explain the mechanisms of drug secretion into saliva has not been achieved. Therefore, it is important to use saliva concentrations in conjunction with concentrations recorded from paired plasma samples.

This review focuses on drug transport from blood to saliva. First, brief information is given on the salivary gland. After a historical overview, attention is focused on its anatomy and physiology. Subsequently, the existing techniques for the collection and analysis of saliva are reviewed. Mechanisms of drug transfer from blood to saliva are discussed. Finally, available information on the detection of doping drugs in saliva is summarized.

2. The salivary gland: anatomy and physiology

Properly speaking, the term "salivary gland" should be taken to include any tissue that normally discharges a secretory product into the oral cavity (Young and Van Lennep, 1979). The functions of such secretions are: 1. to moisten the mucous membranes of the upper aerodigestive tract, to facilitate speech and to control the bacterial flora of the mouth; 2. to supply enzymes destinated to play an important role in preparing food for digestion; 3. to produce hormones and other pharmacologically active compounds; 4. to wet the fur of animals with saliva in response to heat stress, thereby obtaining the same cooling possibility available to man by sweating; 5. to defense and kill. The only mammalian saliva known to be toxic is that of the American short-tailed shrew (Martinez-Madrigal and Micheau, 1989; Young and Van Lennep, 1978, 1979).

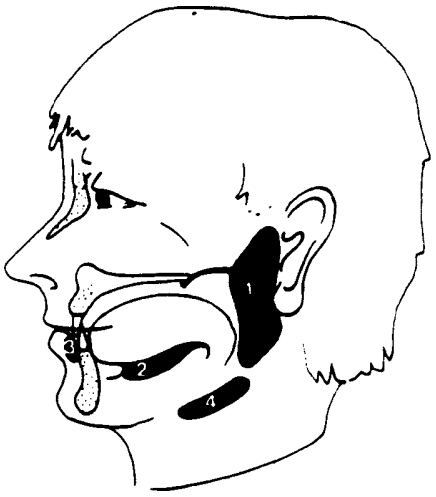
In the late seventeenth century, Antonius Nuck pioneered the injection of marker substances into the salivary glands, not only via their ducts but also via their blood vessels, and he introduced the new word "sialography" for the illustration of his results (Nuck, 1690). However, it was not until the experiments of Claude Bernard in 1856 that any attempts were made to measure the movement of marker solutes from blood to saliva and vice versa (Bernard, 1856). Bernard realized that permeability barriers exist in the glands, because some substances passed readily into the saliva whereas other substances were held back. Some years later Langley observed that methylene blue could pass from blood into saliva (Langley, 1898). Krause studied the transfer of indigo carmine from blood to saliva in submandibular glands of dogs by microscopy and

produced colored illustrations to support his results. He considered that most of the transport occurred via the secretory canaliculi (Krause, 1902). In the 1930's Amberson and Höber showed that for non-ionized solutes there was a relationship between lipid solubility and permeability into saliva (Amberson and Höber, 1932). Straus pioneered the use of the protein marker horseradish peroxidase in the kidney (Straus, 1957). A few years later Garrett and coworkers injected horseradish peroxidase into the arteries supplying the rabbit and dog submandibular gland and studied the barrier function. Like Bernard, they found certain permeability barriers in the glands (Garrett, 1980).

2.1. Morphological and anatomical basis of the saliva formation

Saliva is a complex fluid produced by a number of specialized glands which discharge into the oral cavity of the glands of mammalian vertebrates. Most of the saliva is produced by the major salivary glands (parotid, submandibular, and sublingual), but a small contribution is made by the numerous small labial, buccal, and palatal glands which line the mouth (Van Dam and Van Loenen, 1978; Vining and McGinley, 1985) (Figure 1).

Figure 1. Topography of the salivary glands: 1. glandula parotis; 2. glandula sublingualis; 3. glandula labialis; 4. glandula submandibularis. (From ref. Van Dam and Van Loenen, 1978)



The detailed morphology of the salivary glands has been reviewed by Young and Van Lennep (1978, 1979). The glandular tissue is comprised of acinar cells, specialized groups of cells arranged as endpieces surrounding a small central lumen that opens into a narrow intercalated duct, that leads from the secretory endpieces to the striated ducts, that in turn drain into the secretory ducts to form a single main secretory duct which drains into the oral cavity (Figure 2). Glands are lobulated: the parenchyma consists of discrete lobules of closely packed secretory endpieces and intralobular ducts. The lobules are separated by interlobular connective tissue septa. Secretory endpieces are usually supported by a group of cells thought to be contractile in nature and to contain filaments of actomyosin. They are called myoepithelial cells. Since these cells lie within

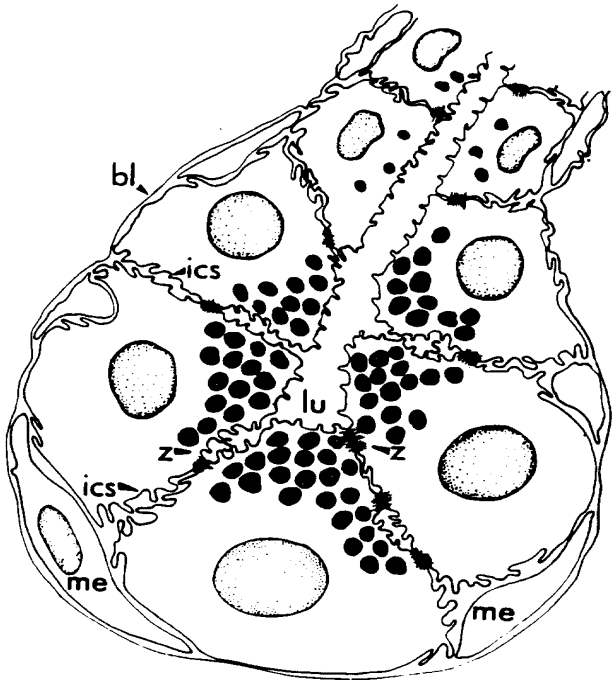
the basement lamina that envelops the endpiece, they should be considered part of the endpiece. These cells have important, albeit indirect, roles to play in salivary transport. They provide support for the endpiece that otherwise would be distended during the secretory process and, in special cases, they provide a propulsive force to aid in the expulsion of especially viscous secretions (Young and Van Lennep, 1978). The volume of saliva secreted appears to be entirely a function of the activity of the secretory endpiece; the ductal system neither reabsorbs nor adds further water (Young and Van Lennep, 1979).

The parotid glands are "serous" glands, for their acinar cells contain only serous-secreting cells, whose secretions are devoid of mucin compared to that of the submandibular and sublingual glands, which contain both serous- and mucin-secreting cells (Davenport, 1977). The viscosity of the submandibular saliva usually decreases with increasing flow rate since the serous cells have a greater response to stimulation than do the mucin-secreting cells. The sublingual gland contains predominantly mucin-secreting cells and thus their secretion has a thick, viscous nature (Vining and McGinley, 1985).

Salivary glands have a high blood flow (Haeckel, 1990). The external carotid arteries enter the submandibular and sublingual glands along with the main ducts and nerves, thereby creating a hilum, although this hilum is not as clearly defined as in larger organs such as the kidney. Within the glands, the vessels follow the subdivision of the secretory duct tree so that each lobule has a distinct and separate blood supply (Young and Van Lennep, 1978). The direction of the blood flow is countercurrent to the direction of the salivary flow (Davenport, 1977).

Clearly, before any drug circulating in plasma can be discharged into the salivary duct it must pass through the capillary wall, the basement membrane and the membrane of the glandular epithelial cells. The rate-determining step for this transportation is the passage of the drug through the lipophilic layer of the epithelial membrane. Physicochemical principles dictate that for such a passage to occur, drug must show a degree of lipophilicity. However, saliva is not a simple ultrafiltrate of plasma, as has sometimes been suggested, but rather a complex fluid formed by different mechanisms: by a passive diffusion process, by an active process against a concentration gradient, by ultrafiltration through pores in the membrane, or by pinocytosis (Caddy, 1984). An active transport mechanism clearly operates for many electrolytes and for some proteins such as IgA. An active transport mechanism has also been suggested for some drugs. However, most mechanisms of active transport are not well understood.

Figure 2. Schematic reconstruction of an endpiece of a typically serous gland such as the parotid, showing secretory canaliculi (S) opening into the lumen (lu). The canaliculi abut tight junctions (z) that separate them from the lateral intercellular spaces (ics).



The canaliculi, in contrast to the intercellular spaces, do not surround the cell on all sides and are seen only occasionally in any particular section. The adjacent acinar cells are coupled by gap junctions (not shown) that permit transcellular exchange of ions and small molecules, including cyclic nucleotides. The functional and electrical unit is therefore the acinus rather than the individual acinar cells. In additions to the secretory cells, myoepithelial cells (me) are shown (from ref. Young and Van Lennep, 1978).

Small molecules can be transported via the ultrafiltration route. The zonula occludens, a tight junction that resides at the apical pole of the cell, separates the apical and basolateral plasma membranes. Within this zonula occludens, the lateral membranes of adjacent cells closely approximate one another. These sites of intimate contact, colloquially termed membrane "kisses", are thought to represent the physical sites at which diffusion of molecules across the zonula occludens is impeded (Madara, 1988). Simson and Bank (1984) used both freeze-fracture and lead-ion tracer techniques to investigate junctional morphology and permeability in the three main epithelial segments of the rat parotid gland. Lead tracer deposition patterns indicated that junctions between acinar cells are highly permeable, those between intercalated

duct cells are less permeable, and junctions between striated duct cells are essentially impermeable. In the same year Mazariegos and coworkers (1984) also investigated the tight junctions. They found indications that the tight junctions in the resting rat parotid gland are impermeable to tracers of molecular weight ³1,900; the behavior of horseradish peroxidase in the rat parotid gland was therefore inconsistent with his molecular weight. Cell membrane damage due to the enzymatic activity or binding of the tracer was given as an explanation for the observed distribution.

Salivary secretion is a reflex response controlled by both parasympathetic and sympathetic secretomotor nerves. Stimulation of sympathetic fibers to all glands causes vasoconstriction; in man, stimulation of the sympathetic trunk in the neck or injection of epinephrine causes secretion by the submaxillary but not by the parotid glands. Parasympathomimetic drugs cause high saliva flow rates and enlargement of the tight junctions of the secretory endpieces (Davenport, 1977; Mazariegos, Tice and Hand, 1984).

Relatively few studies have been published on the internal lymphatic drainage of the major salivary glands. In general, there seems to be an extensively intercommunicating lymph capillary system that runs along both the gland ducts and the blood vessels (Young and Van Lennep, 1978).

2.2. Functional development of the salivary gland

During embryologic life, the parotid gland, the first of the three major glands to appear, is seen by the 6th week. It derives from the ectoderm as a primitive oral epithelium. In the submaxillary gland, the primordia appear at the end of the 6th week and, unlike the parotid, are probably of endodermal origin. The sublingual glands are the last of the three major salivary glands to appear. They are, like the submandibular gland, probably endodermal in origin. Some researchers compare the mechanisms of the salivary secretion and urinary excretion of drugs. Although saliva secretion and urinary excretion involve the same mechanisms to transport drugs, the glands involved do not develop from the same germ layer. The kidneys develop from the ureteric bud and the metanephric mesoderm. Both primordia are of mesodermal origin (Moore, 1982).

As the primordia of the salivary glands grow, they ramify into a brushlike system surrounded by mesenchymal tissue. The basal lamina of this mesenchyme play an important role in the lobular organization of the gland and in vascular and neural development. By the 7th week, the primitive gland moves in a dorsal and lateral direction and reaches the preauricular region. The facial nerve development divides the gland by about the 10th week into superficial and deep portions. By the 3rd month, the gland has attained its general pattern of organization. The epithelial structures are arranged in lobules fixed by a capsule of loose connective tissue. Cell differentiation starts in the secretory ducts with the progressive transformation of ciliated cells into columnar, squamous, and goblet cells. Intralobular ducts and acinar differentiation, including myoepithelial cell formation, begins about the 8th month. Saliva production starts at this time as a mucinous liquid; however, several studies in rodents suggest that full maturation is completed only after birth (Martinez-Madrigal and Micheau, 1989).

2.3. Formation and secretion of saliva

Saliva, like other body fluids, is a dilute aqueous fluid containing both electrolytes and protein with an osmolality less than or equal to that of plasma (Paxton, 1979). Also present in saliva is a certain amount of cell debris arising from the epithelial cells of the mouth together with food residues (Caddy, 1984). The osmolality is principally determined by the type of gland and by secretory activity, whose degree is affected by many factors including sex, age, nutritional or emotional state, season of the year (Mandel, 1974; Shannon, Suddick and Dowd jr, 1974), darkness (Shannon and Suddick, 1973), and a variety of diseases and many pharmacological agents. Circadian variations have been shown in unstimulated and stimulated saliva for flow-rate, pH and some salivary constituents (Dawes, 1972; Ferguson and Fort, 1974; Ferguson, Fort, Elliott and Potts, 1973). Opinions differ about the nature of these circadian variations. Are these variations of endogenous, such as innervation or exogenous nature? Diminished salivary flow, resulting in a dry mouth, is observed with some tricyclic antidepressants, due to their anticholinergic properties (Gelenberg et al., 1990; Jeffrey and Turner, 1978). Therapeutic doses of either the non-selective (propranolol) or the $ß_1$-selective (atenolol) adrenoceptor antagonist did alter the electrolyte composition of resting as well as stimulated whole saliva, without affecting secretion rates (Nederfors and Dahlöf, 1992). A possible explanation for the increased chloride concentration is a reduced reabsorption. However, at present there is no obvious explanation for the other electrolyte changes.

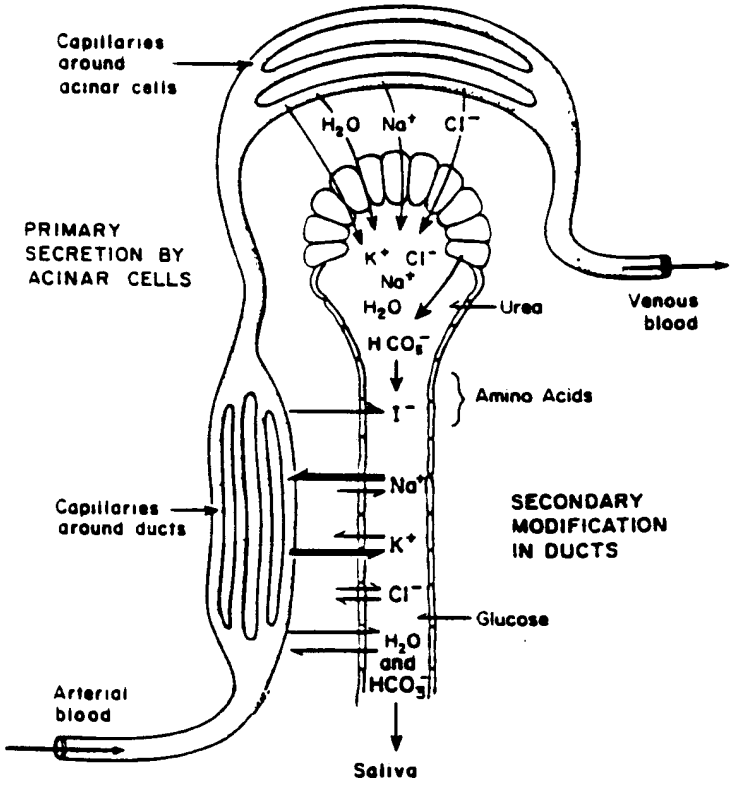
The total volume of saliva produced each day in adults is 500 to 1500 ml (Lentner, 1981). Mixed saliva, which is the most accessible and most frequently used for drug analysis, consists mainly of the secretions of submandibular (65%), parotid (23%), and sublingual (4%) glands, the remaining 8% being provided by the minor numerous glands (Caddy, 1984). These proportions are a function of the type, intensity and duration of stimulation. In patients with alcoholic cirrhosis a reduction was observed in mean basal parotid saliva flow rate in comparison with nonalcoholic control subjects (Dutta, Dukehart, Narang and Latham, 1989). The important stimulus for secretion is the presentation and ingestion of food; the quantity and quality of the secretion vary with the nature of nutrition. A comparison of the compositions of saliva and plasma is given in Table 1 (Ritschel and Thompson, 1983). The large variations in some constituents of saliva are the result of different collection techniques, devices and flow rates.

2.3.1. Inorganic compounds

In general, saliva contains the usual electrolytes of the bodyfluids, the principal ions being sodium, potassium, chloride and bicarbonate. Figure 3 illustrates the current two-stage theory of saliva formation, enunciated by Thaysen and his colleagues in 1954 (Thaysen, Thorn and Schwartz, 1954). The acinar cells forming the secretory endpiece of the salivary gland actively pump sodium ions from the blood into the lumen of the endpiece. The resulting osmotic pressure difference between the blood and the fluid in the endpiece causes water to flow from the blood, through the tight junctions between the acinar cells, and into the lumen of the endpiece. Thus, the primary secretion (as it leaves the endpiece) is thought to be almost isotonic with plasma. As this initial fluid moves down the ductal system of the salivary gland, an energy-dependent transport

process reabsorbs sodium and chloride. Potassium, bicarbonate and lithium ions are actively secreted into saliva. However, the ductal membranes are relatively impervious to water, so the resulting saliva becomes increasingly hypotonic as it moves down the ductal system (Davenport, 1977; Jacobson, 1981; Vining and McGinley, 1982).

Figure 3. A scheme of electrolyte exchanges during secretion of saliva by the parotid gland.



(From ref. Davenport, 1977)

Table 1. Parametric correlation of saliva to plasma (Ritschel and Thompson, 1983).

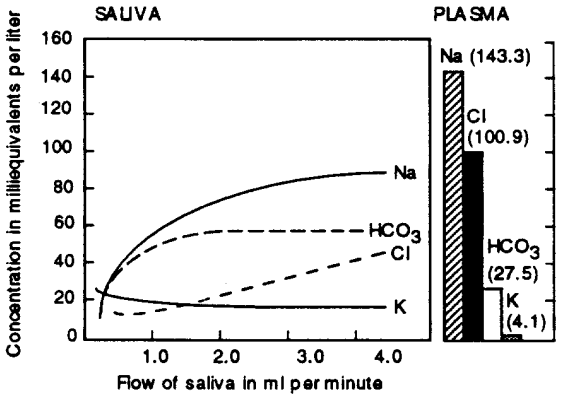
Parameter	Mixed Saliva	Plasma
Volume	500-1500 ml/day	4.3% of BW*
Rate of flow	0.6(0.1-1.8) ml/min	-
pH	6.7(5.6-7.9)	7.4
Water [%]	98(97-99.5)	91.5(90-93)
Total protein [g/100 ml]	0.3(0.15-0.64)	7.3 (6-8)
Albumin [g/100 ml]	-	4.5(4-5)
Mucin [g/100 ml]	0.27(0.08-0.6)	-
Amino acids [mg/100 ml)	0.1-40	0.98
Electrolytes [mMol/l]		
Potassium	8-40	3.5-5.5
Sodium	5-100	135-155
Calcium	1.5-2	4.5-5.2
Phospate	5.5-14	1.2-2.2
Chloride	5-70	100-106
Cholesterol [mg/100 ml]	7.5(3-15)	150-300
Dry Substance [g/l]	6(3.8)	80

*BW = Body Weight

The cells lining the ducts have a limited capacity to pump the sodium out of saliva and this capacity does not increase proportionally as saliva flow rate increases. Therefore, as the saliva flow rate increases so does the sodium ion concentration (Figure 4). Potassium, chloride, and bicarbonate ion concentrations also show a marked dependence upon flow rate. The potassium concentration of resting saliva is normally considerably higher than plasma but drops a little as the saliva flow rate increases. This effect is thought to be due to a constant rate of potassium secretion into the precursor saliva by the cells lining the ducts (Shannon, Suddick and Dowd jr, 1974). The bicarbonate concentration of saliva is highly dependent upon the gland type, nature of the stimulation, and flow rate; it may be larger than or less than the plasma concentration. As a result of a concomitant increase in bicarbonate concentration, the salivary pH rises with increasing rates of secretion. Saliva pH can range from 6.2 to 7.4, with the higher pH exhibited upon increased secretion (Drobitch and Svensson, 1992). Spring and Spirtes (1969) determined that lithium was actively secreted into the saliva of the submaxillary gland of the cat at concentrations far above serum levels in an inverse relationship to the rate of flow of saliva. However, salivary lithium concentrations never decreased to serum level, indicating an active transport of lithium into the saliva. There was no evidence of lithium storage by the gland since salivary concentrations were responsive to changes in serum concentration. Ductal secretion of lithium was related to

an enzyme resembling Na^+, K^+-ATPase because it was both $HgCl_2$ and ouabain sensitive. Salivary secretion of lithium did not resemble natrium secretion in any respect but was most similar to potassium secretion.

Figure 4. Relation between the concentrations (mEq/l) of sodium, potassium, chloride, and bicarbonate in the saliva and the rate of salivary flow (ml/min). (From ref. Thaysen, Thorn and Schwartz, 1954)



After secretion, saliva becomes more alkaline as the dissolved carbon dioxide is lost. Young studied the electrolyte secretion in the intact perfused rabbit submandibular gland (Young, Case, Conigrave, Novak and Thompson, 1980). He concluded that the behavior of the gland in vitro is comparable to that seen in vivo and that there was no evidence to indicate that handling of water and electrolytes was in any way abnormal.

2.3.2. Organic compounds

Previously it was noted that saliva supplies enzymes for digestion. These enzymes and other proteins, including saliva-specific glycoproteins, are synthesized by the acinar cells. The transport of proteins into saliva has been reviewed by Young (1979). Almost all of the organic compounds of plasma, such as hormones, immunologlobulines, enzymes, DNA and viruses may be detected in saliva in trace amounts (Vining and McGinley, 1985). The total protein concentration in saliva is negligible because this concentration is less than 1% of that in plasma (Breimer and Danhof, 1980). It seems likely that a major source of these trace amounts originates from the gingival crevicular fluid (from the tooth/gum margin) (Cimasoni, 1974).

Potential health problems for technicians involved in the assay of saliva samples would appear to be no greater than those associated with blood or urine samples except that saliva samples which are contaminated by sputum (very rarely) may be associated with a risk of infection. Blood samples must be drawn by trained staff, and particular care must be taken with samples from drug addicts given the high prevalence of hepatitis B and the human immunodeficiency virus (HIV) infections in this population. As a noninvasive technique, the collection and analysis of saliva would appear to be

particularly attractive for a high-risk patient population where the routine collection of blood is often made difficult because of bruised or thrombosed veins. In addition, the use of saliva to monitor methadone-prescribing practices would be beneficial to both the patient (blood sampling would be reduced) and to those who handle patient samples (clinic and laboratory staff) because saliva can inhibit HIV infectivity (Wolff and Hay, 1991). The antiviral component from saliva is not yet identified, but it is possible that IgA plays a role in this process (Archibald, Zon, Groopman, McLane and Essex, 1986; Fox, Wolff, Yeh, Atkinson and Baum, 1988). It is worth noting that saliva does not contain broad spectrum antiviral activity. For example, cytomegalovirus, Epstein-Barr virus, and hepatitis B virus survive in mixed saliva (Fox, Wolff, Yeh, Atkinson and Baum, 1988). Saliva samples are already used for testing antibodies to HIV. However, until further study salivary HIV testing should only be used for epidemiological studies (Grau, 1992; Morris, 1992; Mortimer and Parry, 1992; Skidmore and Morris, 1992; Turner, Eglin, Woodward and Porter, 1992), because this test has a false positive rate of 0.1% (Turner, Eglin, Woodward and Porter, 1992).

It is generally assumed that steroids in blood plasma which are bound to steroid binding proteins are unable to pass from the blood to saliva. Therefore, attention had been focused on measurement of steroid hormones in saliva, because people are more interested in measuring the nonprotein-bound fraction. But a most disturbing finding one that may result in a re-evaluation of the current concepts in salivary steroid assay, was the detection of specific steroid binding proteins in saliva not contaminated with gingival fluid: corticosteroid binding globulin, sex hormone binding globulin (SHBG), and aldosterone binding globulin (Chu and Ekins, 1988; Hammond and Langley, 1986). Selby et al. (1988) suggest that SHBG and the albumin present in saliva are from contamination by either traces of blood or gingival fluid. Interpretation of measurements of saliva hormones is also complicated by enzyme activity in the salivary gland. The salivary gland contains 11 ß-hydroxysteroid dehydrogenase, which converts most of the free cortisol in blood plasma to cortisone as the hormone passes through the gland (Wade and Haegele, 1991). Other researchers explained higher concentrations of salivary testosterone by assuming in vivo conversion of androstenedione into its reduced metabolite testosterone by 17-hydroxysteroid oxidoreductase activity in the salivary gland (Swinkels, Hoof van, Ross, Smals and Benraad, 1991, 1992).

Saliva is also an adequate source of DNA for analysis and for DNA typing in certain forensic settings (Walsh et al., 1992). The DNA banding patterns obtained from saliva were indistinguishable from the patterns obtained from blood or hair from the same individual.

3. The collection and analysis of saliva

3.1. Techniques for the collection of saliva

Many of the advantages of measuring drugs in saliva relate to the noninvasive nature of the easy collection procedure. For the collection of samples on a patient basis, mixed whole saliva is the only practical alternative. Therefore, if the measurement of a drug level in saliva is to be of general clinical value it will need to be done on mixed (whole) saliva. Several methods have been described for the collection of mixed saliva.

Without stimulation the normal human salivary glands do not secrete saliva. However, many different stimuli will cause salivation and even during sleep there is usually sufficient stimulation to elicit a very small flow of saliva (typically 0.05 ml/min). In many studies claiming to have utilized unstimulated whole saliva, the subjects have usually been asked to spit directly into a collection tube. This spitting itself is usually a sufficient stimulus to elicit a flow of about 0.5 ml/min.

In healthy subjects, gingival crevicular fluid (from the tooth/gum margin) may constitute up to 0.5% of the volume of mixed saliva: this proportion may be markedly increase in patients with gingivitis (Cimasoni, 1974). Plasma exudate from minor abrasions in the mouth may also contribute to saliva. The protein content of gingival fluid is similar to that of plasma and thus it provides a potential route for the entry of many drugs into saliva. Therefore, it is usually recommended that subjects should not brush their teeth or practice any other methods of oral hygiene for several hours before collecting a saliva sample.

Although most patients prefer donating saliva rather than blood, a substantial social barrier exists to "spitting". For this and other reasons, subjects often experience decreased salivary secretion (dry mouth) if asked to provide a sample. Many researchers have found it advantageous to further stimulate salivation and a number of stimuli have been used.

Chewing paraffin wax, parafilm®, rubber bands, pieces of Teflon or chewing gum (Dawes and Macpherson, 1992) will usually elicit a flow of 1 to 3 ml/min. Mucklow (1982) recommends that, when these types of stimuli are used, the subject should allow saliva to accumulate in the mouth until the desire to swallow occurs, at which time the fluid can be expelled smoothly into a vessel. Repeated expectorations should be avoided since this introduces bubbles, which may result in changes in pH leading to errors in interpretation of the saliva/plasma concentration ratio (S/P ratio). The use of acid lemon drops or a few drops of 0.5 mol/l citric acid are among the most potent of taste stimuli and will generally induce a maximal secretion of 5 to 10 ml/min (Vining and McGinley, 1985). In general, the secretion rate increases with the size of the bolus and the pressure required to chew it. If chewing is unilateral, then the glands on the active side may secrete copiously while those on the inactive site secrete very little. For studies requiring high saliva flow rates for extended periods of time, secretion stimulating drugs, such as the parasympathomimetic drug pilocarpine, have sometimes been used either orally, subcutaneously, or intravenously. However, in doses sufficient to produce very high flow rates, parasympathomimetic drugs have undesirable side effects such as flushing, palpitations, colicky abdominal pains, and an urgent desire to micturate. In addition, they appear to cause an enlargement of the tight junctions of the secretory endpieces and thus result in the appearance in the saliva of compounds of higher molecular weight than would normally be expected. For example, stimulation of the rat parotid gland with isoproterenol resulted in a transient increase in junctional permeability, that allowed passage of tracers of molecular weight £34,500 (Mazariegos, Tice and Hand, 1984).

Welch et al. (1975) accurately determined the elimination half-life of antipyrine in

rats by the serial determination of salivary antipyrine concentrations. It was necessary to administer 2.0 mg/kg pilocarpine to some rats in order to collect (by capillary tube) 5 ml of saliva per sample.

There are several advantages of stimulating salivary flow. 1. Large volumes of saliva can be obtained within a short time; 2. The pH of stimulated saliva mostly lies within a narrow range around the value of 7.4, whereas the pH of unstimulated saliva shows a larger variability, that may be of importance for the salivary secretion of weak acidic and basic compounds (Feller and le Petit, 1977; Ritschel and Thompson, 1983). It is apparently not possible to alter saliva pH significantly by acute administration of a systemic antacid (Levy and Lampman, 1975); 3. The intersubject variability in the S/P ratio may be diminished when stimulated saliva is used, as has been reported for digoxin. On the other hand, it is possible that the concentration of drugs in saliva is affected by stimulating salivary flow. For instance, any physical or chemical stimulus used during the collection of saliva must not absorb or modify the compounds to be measured, nor must it introduce interfering factors into the assay procedure (Danhof and Breimer, 1978). In particular, Parafilm® has been shown to absorb highly lipophilic molecules, leading to an apparent reduction in drug level (Chang, 1976; Taylor, Kaspi and Turner, 1978). Gustatory stimulants (e.g., citric acid) can result in changes in salivary pH that can consequently lead to erroneous results when calculating S/P ratios. Speirs (1977) and Paton (1986) also mentioned the interference by citric acid in some immunoassays.

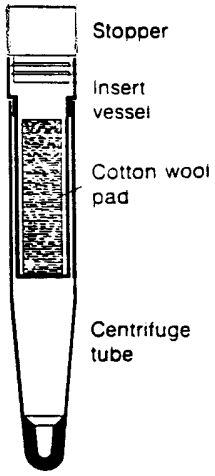
3.2. Special devices

Within the last few years, much research has been done to develop a method that solves many of the existing problems in using saliva for the quantitative determination of the "free" (=non-protein bounded) component of drugs. Cooper et al. (1981) and May et al. (1978) were the first to use a dental cotton roll to collect saliva in order to monitor desipramine. Over the years their method has undergone some improvements, and the dental cotton roll is nowadays available as the Salivette®. The procedure for collection is as follows: the Salivette® is used to absorb the saliva into a dental cotton roll after chewing for 30-45 seconds with or without stimulation. After being soaked with saliva, the dental roll is placed in a container, that is closed with a plastic stopper. The container fits into a polystyrol tube that is then centrifuged for 3 minutes at about 1000 g. During centrifugation the saliva passes from the cotton roll into the lower part of the tube. The container is then taken out of the tube and the clear saliva is poured out of the tube. Cellular particles are retained at the bottom of the tube in a small sink compartment (Figure 5) (Haeckel, 1989). A disadvantage of the Salivette® is that the dental cotton roll interferes with several hormone and drug assays, such as that for testosterone. When assayed, the cotton from the Salivette® contains something that mimics the effect of testosterone (Dabbs, 1991). However, the cotton can be replaced by other absorbing materials. So far, no absorbing material has been found to be inert that can serve for all purposes (Haeckel, 1990). The advantage of the Salivette® over many other sampling devices is that it reliably absorbs a relatively large volume of saliva (1.5 ml) in a short time (Höld, De Boer, Zuidema and Maes, 1995). The OraSure®, another collection device, absorbs only 1.0 ml and, moreover, collects a mixture of gingival crevicular fluid and saliva rather than saliva alone, since the pad is placed

between cheek and gums. The term "oral sample" is used rather than saliva when the OraSure® device is used (Thieme, Yoshihara, Piacentini and Beller, 1992).

An Oral-Diffusion-Sink (ODS) device is used for the in situ collection of an ultrafiltrate of saliva (Wade, 1992a; Wade and Haegele, 1991). The ODS device, a variation on an earlier design developed for time-integrated measurement of corticosteroids in interstitial fluid (Wade, 1984), is worn in the mouth and continuously accumulates the compounds of interest as they diffuse into the device along a concentration gradient. A concentration gradient for a particular analyte is maintained by containing inside the device a composition that binds the analyte and thus maintains its free concentration inside the device at a value that is negligible when compared with its concentration in saliva. An antiserum having suitable specificity and binding capacity was used to maintain the concentration gradient for corticosteroids in the device (Wade and Haegele, 1991). In an identical physical package, an insoluble ß-cyclodextrin composition rather than an antiserum can be used to provide an ODS device with broader specificity (Wade, 1992b). Since the ODS can be used to obtain a sample that reflects the availability of hormones in saliva over a defined time interval rather than at a particular moment of sampling, it may prove especially useful in studies of compounds that are secreted episodically and rapidly cleared. Results obtained with the ODS are, in general, highly correlated with more traditional time-integrating methodologies (Gehris and Kathol, 1992; Shipley, Alessi, Wade, Haegele and Helmbold, 1992).

Figure 5. Salivette for saliva sampling. (From ref. Haeckel and Bucklitsch, 1987)



In the same period, Schramm and coworkers (1990) developed a device for the in situ collection of an ultrafiltrate of saliva. The collector is based on the principle of an osmotic pump. A semipermeable membrane (exclusion limit 12,000 Da) encloses an osmotically active substance (sucrose) that within a few minutes of being put into a patient's mouth, draws an ultrafiltrate of saliva into the device (1 ml of ultrafiltrate after 5 min). Large molecules are excluded from the interior of the osmotic pump. The use of the collector eliminates several problems normally encountered in the collection of saliva samples: the expulsion of an excess amount of froth containing little liquid, and

the discomfort of patients that results when strings of viscous saliva form between the mouth and the container. The major advantage in the laboratory for processing the clear ultrafiltrate is that its lower viscosity than regular saliva simplifies processing (no centrifugation, extraction, greater precision). Another advantage of the ultrafiltrate collector is that it eliminates the problem of blood contamination since protein-bound molecules are excluded with this sampling technique (Borzelleca and Cherrick, 1965). However, a major disadvantage of this device is that the density of the liquid after collection has to be determined because the ultrafiltrate contains a high concentration of sucrose that is used as the osmotic driving force. To correct for the actual concentration of analytes in the ultrafiltrate, a correction factor, derived from the density of the solution, was calculated (Schramm, Annesley, Siegel, Sackellares and Smith, 1991). The sampling time from the ultrafiltrate collector is very long in comparison with that of the Salivette® (8 min versus 45s). The use of the collector slightly stimulates salivary flow. Therefore it is not necessary to use mechanical or chemical stimulants, which can interfere with the quantitative determination of analytes either by nonspecific binding or by affecting the immunoassay. In addition, the pH of saliva is stabilized in stimulated saliva. Schramm et al. (1990) used this device for the measurement of progesterone. The ultrafiltrate contained about 20-25% less progesterone than did the whole saliva sample, but this difference was not significant. A possible explanation for the loss of progesterone is the delayed diffusion of the steroid through the semipermeable membrane of the collector. On the other hand, it is possible that the concentration in the ultrafiltrate reflects the truly free fraction of progesterone in saliva.

One year later this research group reported the use of the collector for the determination of phenytoin, carbamazepine (Schramm, Annesley, Siegel, Sackellares and Smith, 1991) and testosterone (Schramm, Paek, Kuo and Yang, 1991). The transfer of phenytoin over time from a bulk solution into the collector was 80% in an in vitro model that simulated closely the conditions encountered in the mouth during collection. Shortly after the sugar crystals had dissolved (3-5 min), the maximal concentration was reached inside the collector. This concentration represents an equilibrium state in which molecules pass from saliva through the membrane and back. The concentrations found in the ultrafiltrate and free in serum were not equivalent, but this is true for most drugs. However, from the ultrafiltrate one can calculate the concentrations in whole saliva and free in serum. This method of sampling can simplify the noninvasive collection of a body fluid to be used for diagnostic evaluation. However, further research is needed to conclude if this method can be used for a wide variety of drugs.

3.3. Analytical methodology for the measurement of drugs in saliva

3.3.1. Pre-analytical manipulations

The usefulness of saliva determinations clearly depends also on the application of analytical procedures adequate for the assay in saliva. Once the samples have been collected, it is important that they be properly stored unless analyses are to be performed immediately. Chen et al. (1992) examined the long-term storage of salivary cortisol samples at room temperature. After 16 weeks of storage samples without preservatives lost >90% of the cortisol. However, the salivary cortisol was stable in the presence of citric acid, 10 g/L, or when preserved in a citric acid-treated Salivette® for as

long as six weeks at room temperature. Opinions differ as to the procedure to be followed, but most workers freeze the sample to -20°C, while some workers recommend centrifugation before freezing, and others recommend centrifugation after thawing and prior to analysis. A problem in saliva analysis is that smokers produce thick, very viscous saliva. By freezing all saliva samples at -40°C for at least 24 h before analysis, cellular components and suspended particles are effectively broken down, leaving a clear liquid that is easier to process (Wolff and Hay, 1991). Meulenberg and Hofman (1990) and Lequin et al. (1986) demonstrated that sonification of saliva yielded significantly higher levels of most steroids than centrifugation of saliva. It was suggested that sonificating the saliva samples disperses some unknown substance(s) which interfere with the analysis. But, another reason for the higher levels is that after centrifugation one loses drug bound to cell debris, particulate matter or mucoprotein (Anavekar et al., 1978). Caddy (1984) mentioned the possibility of correcting this problem with a proteolytic enzyme. In forensic work in which saliva samples have been taken primarily for serological purposes, it is common practice to subject the sample and container to boiling water temperatures for 15-30 minutes prior to freezing. Only in cases in which the toxic material present in saliva is volatile or heat-unstable would this treatment be expected to be deleterious to later analysis of such saliva samples. Another point where opinions differ about is the difference in the point of time in which the pH of the saliva samples should be measured, either immediately after collecting the sample or before analysis of the sample. Probably when the researchers measure pH immediately after collection they use the pH to clarify the transport mechanism, however when pH is measured after thawing, the pH is used for the analytical procedure.

Most authors use the centrifugate, either by extracting the centrifugate with an organic solvent at a desirable pH or by using the centrifugate directly in the analytical process without sample pretreatment (Roth, Beschke, Jauch, Zimmer and Koss, 1981). Caddy (1984) has extensively reviewed the different methods of analysis of saliva. The recent developments in the existing techniques of analyses will subsequently be summarized.

3.3.2. Immunological methods

Immunological methods of analysis have been widely used for monitoring drugs in saliva and other body fluids, mainly because of their relative simplicity of use, requiring little or no extractive operations, their application to large batch analyses, and especially their sensitivity (Caddy, 1984). They do, however, suffer from disadvantages in that they do not always possess the specificity required for distinguishing metabolites from the parent drug (Paxton and Donald, 1980). In 1978, the group from the Tenovus Institute rekindled interest in the determination of hormones in saliva by applying radioimmunoassay (RIA) techniques. Their studies have been followed by a growing number of publications demonstrating the value of RIA methods, especially for the analysis of hormones in saliva, such as estradiol, progesterone, testosterone, cortisol, and cortisone (Evans, 1986; Lequin, Boogaard van den, Vermeulen and Danhof, 1986; Migliardi and Cenderelli, 1988; Rey, Chiodoni, Braillard, Berthod and Lemarchand-Béraud, 1990; Schramm, Paek, Kuo and Yang, 1991; Schramm, Smith, Craig, Paek and Kuo, 1990; Swinkels, Hoof van, Ross, Smals and Benraad, 1992; Wade, 1992b). Other drugs, which have been measured with RIA are for instance, cocaine (Cone and

Weddington, 1989; Inaba, Stewart and Kalow, 1978), cannabinoids (Gross et al., 1985), haloperidol (Yamazumi and Miura, 1981), theophylline (Mally, Keszei and Cserep, 1992) and cotinine (Benkirane, Nicolas, Galteau and Siest, 1991).

The use of the alternative non-radioactive immunological procedure, an enzyme multiplied immunoassay technique (EMIT®), is based on competitive protein binding using an enzyme as a label and an antibody as a specific binding protein. The enzyme activity is related to the amount of drug in the sample and is measured spectrophotometrically. Although this assay is very easy to use and requires no radiochemical facilities, it is not used very often for monitoring saliva levels. Sensitivity may be one major reason for this. However, some anticonvulsant drugs are measured in saliva with EMIT®. Assays of carbamazepine (Paxton and Donald, 1980) and phenytoin (Umstead, Morales and McKercher, 1986) had a lower limit of detection of 0.1 mg/ml with a coefficient of variation for the assay of <10%. Another drug, theophylline (Goldsworthy, Kemp and Warner, 1981; Siegel et al., 1990) was determined in saliva from asthmatic children. The lower limit of sensitivity of the assay was 0.8 mg/ml with a coefficient of variation for the assay of <5% (Siegel et al., 1990). One has to be careful to use citric acid in combination with the EMIT® assay because the enzyme glucose-6-phosphate dehydrogenase (G6PD) used in the EMIT® assay for ethosuximide and phenytoin (Paton and Logan, 1986) is inhibited 38% by citric acid (8 mmol/l).

Another direct immunoassay which is not subject to the disadvantages associated with the use of radioisotopes in RIA is described by De Boever et al. (De Boever, 1990). They developed a chemiluminescence immunoassay (CIA) using isoluminol for the detection of estradiol in saliva. Phenytoin was measured in 1 ml saliva samples by fluorescence polarization (FPIA) (Cai, Zhu and Chen, 1993).

3.3.3. Chromatographic methods

Thin-layer chromatography (TLC) has the advantage of simplicity and allows the simultaneous determination of several samples. However the major limiting factor is the detection of the respective spots on the thin-layer plate at low drug levels in saliva (Drehsen and Rohdewald, 1981). Therefore, this technique is not often used in the drug monitoring of saliva. With the development of high-performance TLC (HPTLC), Drehsen and Rohdewald (1981) tried to reach a higher sensitivity and precision. They monitored salicylic acid, salicylamide, ethoxybenzamide, acetaminophen, and some other weak analgesics in saliva. They were able to detect between 5 and 50 ng of the respective drugs with relative standard deviations ranging from 1 to 6.8% (Drehsen and Rohdewald, 1981). However, this procedure did not found wider application for saliva drug monitoring.

The most popular analytical procedure for the measurement at the nanogram or picogram level is based on gas chromatography (GC), or, in the hyphenated mode, with mass spectrometry (GC/MS). The assay of nitrazepam, determined in saliva by GC after it had been converted into N-butylnitrazepam, showed a detection limit of 0.5 ng/ml (Hart 't and Wilting, 1988). Also clobazam was determined adequately in the saliva of epileptic children by GC (Bardy, Seppala, Salokorpi and Granstrom, 1991). Matin et al. (1977) simultaneously measured d- and l-amphetamine in plasma and saliva after

derivatization the amines with chiral reagents. Another drug of abuse, cocaine, could be detected in saliva with GC/MS for 12 to 36 h after administration (Thompson, Yousefnejad, Kumor, Sherer and Cone, 1987). Gould et al. (1986) developed a GC/MS procedure for the measurement of salivary testosterone in female subjects, a striking application, because of the inefficiency of RIA.

Often, high performance liquid chromatography (HPLC) is used for the analysis of drugs in saliva. Thompson et al. (1987) used HPLC with electrochemical detection for the determination of D^9-tetrahydrocannabinol (THC). Unfortunately, ultraviolet detectors are not sufficiently sensitive for the quantitation of THC at human physiological concentrations. Because fluorescence detection is more adequate, derivatization with a fluorescence probe is sometimes used to enhance sensitivity. Recently, mexiletine, an anti-arrhythmic drug was monitored in this way (Katagiri, Nagasako, Hayashibara and Iwamoto, 1991). HPLC was used to optimize the separation of caffeine from its metabolites, theophylline and paraxanthine, in saliva (Moncrieff, 1991). Lam et al. (1993) developed a chlorhexidine assay that required only 200 ml saliva sample. The detection limit of this HPLC assay is 50 ng/ml, which is a significant improvement compared to spectrophotometric detection. Among other drugs which have been routinely measured with HPLC in saliva are quinine and analogue (Salako and Sowunmi, 1992).

4. Mechanisms of drug transfer from blood to saliva

4.1. General considerations

The possible routes which may lead to a drug being present in mixed saliva are passive transcellular diffusion, ultrafiltration, active transport and pinocytosis (Landon and Mahmod, 1982).

Clearly, if a patient has just received a drug orally there may be a spurious elevation of the salivary drug level. Even hard gelatin capsules containing amphetamine have produced oral drug retention (Wan, Matin and Azarnoff, 1978). Usually the problem can be overcome by washing residual drug from the mouth with water prior to sampling, but one has to be careful of dilution effects. Similarly, the handling of drugs by subjects has been suggested as a possible source of saliva contamination. The contribution of gingival crevicular fluid to mixed saliva and the need to refrain from vigorous brushing of the teeth to prevent contamination of the saliva by blood was discussed earlier.

4.2. Mechanisms of salivary drug transport

Of the four known mechanisms whereby a drug could be transferred from the blood into saliva via the salivary gland, there is no evidence that pinocytosis plays any role. However, the other three mechanisms are known to be involved.

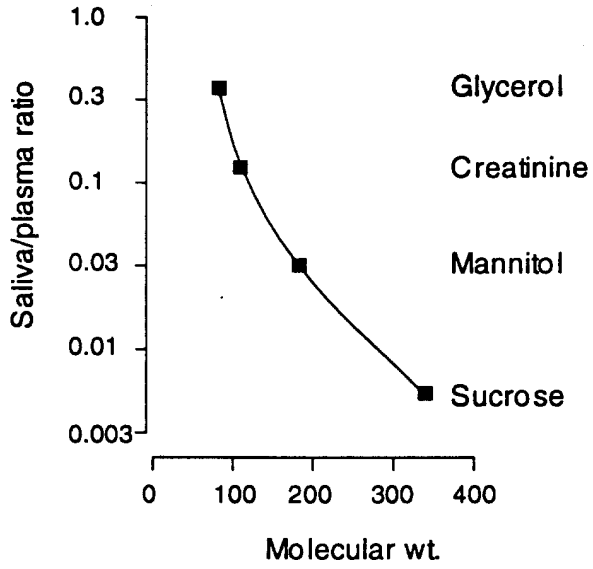
In **passive transcellular diffusion** highly lipid-soluble materials may cross the capillary wall, basement membrane and acinar cell of the secretory endpiece, with the

lipid layer of the epithelial cell wall providing the rate - limiting barrier. The same mechanism would probably enable them to pass through the cells lining the ducts of the gland. The salivary concentrations of the lipid-soluble, unconjugated steroids such as oestriol, cortisol and testosterone approximate the unbound plasma concentrations. But, the concentration of the lipid-insoluble, conjugated steroid dehydroepiandrosterone sulphate is approximately 1% of the unbound plasma concentration (Vining and McGinley, 1982).

By **ultrafiltration (or paracellular transport)** small polar molecules such as glycerol and sucrose enter into saliva. The S/P ratios of several small polar, lipid-insoluble compounds are plotted as a function of their molecular weight (Figure 6). This mechanism is restricted to compounds with a molecular weight (MW) of less than about 300 Da, and even those with a MW of about 150 Da are only filtered to a minimal extent, as evidenced by salivary levels much lower than those in plasma (Burgen, 1956; Martin and Burgen, 1962; Vining and McGinley, 1982). Furthermore, the flow rate of saliva should not affect S/P ratios if diffusion is rapid and passive. Mangos and McSherry (1970) reported that the S/P ratio for urea, a hydrophillic nonionized compound, in the primary saliva of the rat parotid was 0.95 ± 0.20 (SD) at low saliva flow rates and 0.58 ± 0.18 at high flow rates. They concluded that urea is at equilibrium between the primary saliva and the interstitial fluid under resting conditions but that its diffusion coefficient is not large enough for equilibrium to be maintained when the gland is stimulated. This lack of equilibration could be considered the result of a permeability diffusion barrier in the cells of the secretory part of the gland (Burgen, 1956).

An **active transport mechanism** clearly operates for many electrolytes and for some proteins such as IgA. This mechanism has also been proven for some drugs. Lithium (MW = 7 Da) would be expected to appear in saliva by ultrafiltration. However, the findings of a S/P ratio of more than two indicates an active secretory mechanism (Groth, Prellwitz and Jänchen, 1974; Idowu and Caddy, 1981; Landon and Mahmod, 1982). Borzelleca (1965) investigated if penicillin and tetracycline were secreted in saliva. The secretion of these antibiotics in the saliva appeared to be dependent upon the concentration in the blood. Since the secretion of penicillin by the salivary apparatus and by the kidney were both inhibited by probenecid, an inhibitor of the active renal pathway, at least a part of the penicillin secretion in saliva involved an active mechanism (Borzelleca and Cherrick, 1965). These authors were the first who compared the salivary secretion of a drug with the renal excretion. Zuidema and Van Ginneken (1983b) also found an indication of active transport of penicillin. However, probenecid showed no effect on the salivary secretion of diprophylline, whereas the active renal pathway was inhibited by probenecid. These data suggested that the secretory mechanism in the kidney and in the salivary gland is not identical.

Figure 6. Concentration of lipid-insoluble, non-electrolytes in saliva as a function of molecular size. (From ref. Burgen, 1956; Martin and Burgen, 1962)



Most drugs appear to enter saliva by the first mechanism mentioned, a simple **passive diffusion process** which is characterized by the transfer of drug molecules down a concentration gradient with no expenditure of energy. The rate of diffusion of a drug is a function of the concentration gradient, the surface area over which the transfer occurs, the thickness of the membrane, and a diffusion constant that depends on the physico-chemical properties of each drug (Paxton, 1979). The variables which influence this type of transport are listed in Table 2 and will next be summarized (Landon and Mahmod, 1982).

Killmann and Thaysen demonstrated that the S/P ratio was correlated with the pK_a-value for various sulphonamides, as only the un-ionized form of the drug diffused across the epithelium of the salivary gland (Killmann and Thaysen, 1955). Figure 7 shows their results. Since the parotid secretion in ruminants is alkaline, drugs in cow and goat saliva offered an opportunity for additional appraisal of the correlation between ionization and diffusion of drugs. The concentration of sulphonamides and barbiturates in the alkaline saliva was higher than or equal to the concentration in the ultrafiltrate of plasma. The concentration in saliva depended on the pK_a-value and the lipid solubility of the drug (Rasmussen, 1964).

Table 2. Factors influencing passive diffusion of a drug from blood to saliva (Landon and Mahmod, 1982; Vining and McGinley, 1985).

Relating to drug:

- Acidic or basic, and the pK_a
- Lipid-solubility
- Charged or neutral
- Molecular weight and spatial configuration

Relating to the circulating drug level in the free (nonprotein-bound) form:

- Nonprotein-bound blood level
- Dose and clearance of drug

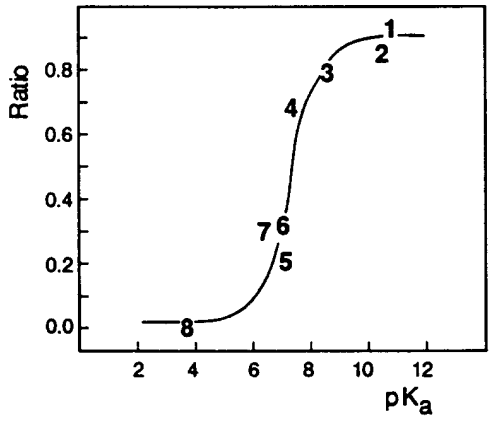
Relating to saliva:

- Saliva flow-rate
- Saliva pH
- Saliva binding proteins - usually minimal
- Enzymes in saliva capable of metabolising the drug

The relative affinity between water and lipid, as for instance expressed by its octanol/water partition coefficient, is an important factor because it determines the ease with which the molecule is able to permeate the lipid membranes of the acinar cells. If a drug is ionized at physiological pH, its effective lipophilicity is substantially lower, because the ionized species are surrounded by water molecules due to ion-dipole interaction that disfavors membrane interaction. It should also be noted that lipid-soluble drugs are usually metabolized to more polar, water-soluble metabolites prior to excretion in the urine. One example is phenytoin, which is metabolized in the liver to parahydroxyphenytoin glucuronide. This inactive metabolite will not appear in saliva because it is partly ionized and water-soluble. Advantage may be taken of that, since an assay that would determine both phenytoin and the inactive metabolite in plasma could be applied to saliva, where only the active drug is present (Landon and Mahmod, 1982). Molecular size is of importance for diffusion rates of drug molecules: theoretical considerations imply a linear relationship between permeability and $P/(MW)^{1/2}$ (P: partition coefficient octanol/water; $MW^{1/2}$: square root of molecular weight), a relationship which could be verified by experimental data (Van Bree, 1990).

Figure 7. Saliva-plasma concentration ratios versus the pK_a of some sulfonamides and p-aminohippuric acid. 1 = methylsulfonamide; 2 = sulfanilamide; 3 = sulfapyridine; 4 = sulfadimidine; 5 = sulfathiazol; 6 = sulfamerazine; 7 = sulfadiazine; 8 = p-aminohippuric acid.

(From ref. Killmann and Thaysen, 1955)



Another variable which also influences the passive diffusion process is the pH of saliva. As the salivary flow rate increases, irrespective of the cause, there is a marked rise in the concentration of bicarbonate with a concomitant increase in pH from as low as 6.0 to as high as 8.0 (Landon and Mahmod, 1982). The pH of saliva affects the S/P ratio of drugs, but there has been relatively little study of this phenomenon. The influence of salivary pH on this transport depends upon the pK_a of the drug. For acidic drugs salivary pH influences the concentration of acidic drugs when pK_a values are less than about 8.5 (Feller and le Petit, 1977), for basic drugs this occurs when pK_a is greater than 5.5.

One of the most useful aspects of monitoring drug saliva levels relates to the significance of the fractions of unbound drug in plasma and saliva (f_p and f_s). For those acidic drugs with $pK_a > 8.5$ and those basic drugs with $pK_a < 5.5$, the S/P ratio is independent of pK_a. This ratio must therefore equal the ratio of f_p to f_s and, since f_s can usually be considered as unity, the S/P ratio is equal to the fraction of unbound drug in plasma. That is of great significance since it is this fraction that is responsible for the pharmacological action of a drug.

Rasmussen (1964) developed an equation based on pH partitioning that describes the S/P ratio and that also considers differences in protein binding and ionization between plasma and saliva as essential factors. For lipid soluble drugs the S/P ratio can be predicted on the assumption of a passive diffusion process by use of equations (1) for acidic and (2) for basic drugs. In the derivation of equations (1) and (2), it is assumed that the diffusion of drugs between plasma and saliva is passive and rapid. Accordingly, there should be a linear relationship between salivary and unbound concentration in plasma (Graham, 1982).

$$S/P = \frac{1 + 10^{(pHs-pKa)} * f_p}{1 + 10^{(pHp-pKa)} * f_s} \quad (1)$$

$$S/P = \frac{1 + 10^{(pKa-pHs)} * f_p}{1 + 10^{(pKa-pHp)} * f_s} \quad (2)$$

where S = concentration of drug in saliva
P = concentration of drug in plasma
pK_a = pK_a of drug
pH_s = Saliva pH
pH_p = Plasma pH
f_p = Free (unbound) fraction of drug in plasma
f_s = Free (unbound) fraction of drug in saliva.

Acidic drugs tend to be lower in concentration and basic drugs to be higher in concentration in the saliva than in plasma. The reverse situation occurs if the salivary pH is higher than the blood pH; for instance, after a strong stimulation of the salivary flow rate, or as usually observed in goats and cows which have a salivary pH of about 8.0 (Rasmussen, 1964). Two variables in equation (1) and (2) are relatively constant and will not be considered further. The first is the pH of plasma, which is assumed to be 7.4 in healthy subjects. The second is the binding of drugs to salivary constituents. That is generally assumed to be negligible (f_s=1), although the absence of significant binding has been demonstrated with only a few drugs, such as sulphathiazole (Killmann and Thaysen, 1955), barbiturates (Rasmussen, 1964), tolbutamide (Matin, Wan and Karam, 1974), propranolol (3.8%) and phenytoin (2.3%) (Mucklow, Bending, Kahn and Dollery, 1978). However, it has been reported that phenytoin is bound to the mucoid sediment of mixed saliva, the concentration in the sediment being approximately three times as great as that in the supernatant (Anavekar et al., 1978). As already mentioned, basic drugs can have a S/P ratio exceeding one, due to pH partitioning. Wan et al. (1978) observed a S/P ratio of 2.76 for the basic drug amphetamine. This ratio was in reasonable agreement with the calculated S/P ratio of 2.21, according to the equation of Rasmussen. Cocaine (pK_a=8.6) (Thompson, Yousefnejad, Kumor, Sherer and Cone, 1987), methadone (pK_a=8.6) (Lynn et al., 1975), and codeine (pK_a=8.2) (Sharp, Wallace, Hindmarsh and Peel, 1983) have a S/P ratio exceeding one. In most studies saliva pH was not measured and therefore the observed S/P ratio could not be compared with the calculated ratio.

In the equations (1) and (2) it is assumed that the diffusion of drugs between plasma and saliva is passive and rapid. However, when the salivary glands remove only a small fraction of the drug presented to them through their blood supply, this transport becomes dependent of the salivary flow. Graham (1982) developed an equation for salivary clearance (CL_s) in which this dependency is processed:

$$CL_s = Q_s * S/P$$

where Q_s is the rate of saliva production and S/P the concentration ratio of the drug. From this equation, it is evident that salivary clearance is directly proportional to the S/P ratio and to the rate of salivary flow. Zuidema and Van Ginneken (1983a) developed a similar equation, but they divided the clearance in an active and a passive component. They characterized substances with a high extraction ratio as high salivary clearance drugs. In the case of good lipophilicity and thus a high extraction ratio, the salivary clearance will be proportional to the blood flow. In the case of low lipophilicity and thus a low extraction ratio, the substance transport across the membrane is rate-determining for its appearance in the saliva. The salivary clearance is then independent of the blood flow. However, the salivary flow remains dependent on the blood flow. The salivary concentration will therefore decrease with increasing blood and salivary flow. In case of an active clearance independent of protein binding and ionization, the passively secreted amount is increased by a constant amount secreted by the saturated active mechanism. This saliva clearance concept is a start in the design of models of salivary secretion of drugs. Future research has to further clarify the mechanisms whereby a drug could be transferred from the blood into saliva.

Intra-individual variability of the S/P ratio of several drugs has been demonstrated after their oral or intravenous administration. Posti (1982) offered a plausible theory based on the phenomenon of varying arterial-venous concentration ratios. Haeckel (1990) described this phenomenon more in detail. After the uptake of an orally applied substance from the intestine, at first the arterial blood has a higher concentration than the venous blood (positive arteriovenous difference). If the absorption is completed and the substance is not metabolized (or only metabolized to a minor extent) in a particular organ, the situation is reversed because the substance rediffuses from the cells into the blood (negative arteriovenous difference in the elimination phase). That results in a fluctuation of the arteriovenous difference, which was shown for caffeine (Haeckel, 1990). The various organs differ with respect to the volume of the blood flow: one group has a high flow (e.g. liver, kidney, brain, salivary glands), and one has a relatively low flow (e.g. skin, resting skeletal muscle, fat). In pharmacokinetics the first group is often considered as a central compartment and the second as a peripheral compartment. This must be considered if saliva concentrations are compared with blood concentrations derived from cubital veins belonging to the peripheral compartment. However, salivary glands have a high blood flow. Therefore, the arteriovenous difference of freely diffusible substances is relatively small and the ratio is close to 1.0. The poor correlations between both compartments which have been reported in the literature may only be partly explained by the neglect of the phenomenon described (Haeckel, 1990).

5. Doping drugs in saliva

The most frequently used biological specimen for the determination of drugs in doping control is urine, since only a non-invasively obtained sample is acceptable for routine collection. Yet, even the acceptability of a urine sample is being disputed in view of the potential invasion of privacy, especially if a directly observed collection is advisable to prevent adulteration or substitution of the sample (Schramm, Smith and Craig, 1992). That happens, for instance, when the athletes try to escape detection by

using urine from someone else. Another major disadvantage of urine is the variability in the renal clearance of drugs and their metabolites, which is largely due to fluctuations in the flow rate and pH of urine. Further, not all drugs are excreted in the urine, for instance, the lipid-soluble ß-blocking drugs tend to be rapidly eliminated by various metabolism systems in the liver (McDevitt, 1987).

Around 1910, the Russian chemist Bukowski developed a method to detect alkaloids in saliva of horses. Two years later this method was used for drug testing in horse racing. At present, saliva is not used as a biological fluid for doping control. Unlike a urine sample, saliva can be obtained under supervision without direct observation of private functions. Although a qualitative doping control mainly depends on the sensitivity of the assay, the usefulness of saliva needs to be explored here further.

6. Conclusions

For the measurement of drugs, saliva was suggested as early as the 1970's as an alternative medium (Gorodetzky and Kullberg, 1974). Since these years, saliva has been used for therapeutic and toxicologic drug monitoring of a variety of drugs. The easy noninvasive, stress-free nature of saliva collection makes it one of the most accessible body fluids to obtain. The major disadvantage of saliva is that many drugs are retained for a shorter period of time than they are in urine. New collecting devices should make physicians more comfortable with using saliva as an alternative to blood or urine. So far, no device could be found to serve for all saliva analyses. Although highly sensitive methods of detection are required, most drugs can be detected in salivary secretions.

Measurements of saliva drug concentrations will usually be of value, only if they accurately reflect the plasma level. Thus, before a useful model of salivary secretion of drugs can be designed one needs to know the relationship between the saliva level of each drug and its plasma level, the mechanisms by which drugs enter the saliva, and also the effect of salivary flow rate, production in the salivary glands, and the nature of any protein binding in the saliva. The factors affecting the drug concentrations in saliva are well examined, but in future research the mechanisms by which drugs enter the saliva have to be clarified more adequately.

7. References

Amberson, W. R., Höber, R. (1932). The permeability of mammalian salivary glands for organic non-electrolytes. *Journal of Cellular and Comparative Physiology*. 2, 201-221.

Anavekar, S. N., Sanders, R. H., Wardell, W. M., Shoulson, I., Emmings, F. G., Cook, C. E., Gringeri, A. J. (1978). Parotid and whole saliva in the prediction of serum total and free phenytoin concentrations. *Clinical Pharmacology and Therapeutics*. 24, 629-637.

Archibald, D. W., Zon, L., Groopman, J. E., McLane, M. F., Essex, M. (1986). Antibodies to human T-lymphotropic virus type III (HTLV-III) in saliva of acquired immunodeficiency syndrome (AIDS) patients and in persons at risk for AIDS. *Blood*. 67, 831-834.

Bardy, A. H., Seppala, T., Salokorpi, T., Granstrom, M. L. (1991). Monitoring of concentrations of clobazam and norclobazam in serum and saliva of children with epilepsy. *Brain & Development*. 13, 174-179.

Benkirane, S., Nicolas, A., Galteau, M. M., Siest, G. (1991). Highly sensitive immunoassays for the determination of cotinine in serum and saliva. Comparison between RIA and an avidin-biotin ELISA. *European Journal of Clinical Chemistry and Clinical Biochemistry*. 29, 405-410.

Bernard, C. (Ed.). (1856). Leçons de physiologie expérimentale (Vol. 11). Paris: Baillière et fils.

De Boever, J. (1990). Direct chemiluminescence immunoassay of estradiol in saliva. *Clinical Chemistry*. 36, 2036-2041.

Borzelleca, J. F., Cherrick, H. M. (1965). The excretion of drugs in saliva. Antibiotics. *Journal of Oral Therapeutics and Pharmacology*. 2, 180-187.

Van Bree, J. B. M. M. (1990). Drug transport across the blood-brain barrier. Unpublished Ph.D. Dissertation, State University Leiden.

Breimer, D. D., Danhof, M. (1980). Saliva: a fluid for measuring drug concentrations. *Pharmacy International*. 1, 9-11.

Burgen, A. S. V. (1956). The secretion of non-electrolytes in the parotid saliva. *Journal of Cellular and Comparative Physiology*. 40, 113-138.

Caddy, B. (1984). Saliva as a specimen for drug analysis. In R. C. Baselt (Ed.), Advances in analytical toxicology (Vol. 1, pp. 198-254). Foster City: Biomedical Publications.

Cai, W. M., Zhu, G. Z., Chen, G. (1993). Free phenytoin monitoring in serum and saliva of epileptic patients in China. *Therapeutic Drug Monitoring*. 15, 31-34.

Chang, K. (1976). Interactions between drugs and saliva-stimulating parafilm and their

implications in measurements of saliva drug levels. *Research Communications in Chemical Pathology and Pharmacology*. 13, 357-360.

Chen, Y. M., Cintron, N. M., Whitson, P. A. (1992). Long-term storage of salivary cortisol samples at room temperature. *Clinical Chemistry*. 38, 304.

Chu, F. W., Ekins, R. P. (1988). Detection of corticosteroid binding globulin in parotid fluids: evidence for the presence of both protein-bound and non-protein-bound (free) steroids in uncontaminated saliva. *Acta Endocrinologica*. 119, 56-60.

Cimasoni, G. (Ed.). (1974). Monographs in oral science. The crevicular fluid (Vol. 3). Basel: Karger,S.

Cone, E. J., Weddington, W. W. (1989). Prolonged occurrence of cocaine in human saliva and urine after chronic use. *Journal of Analytical Toxicology*. 13, 65-68.

Cooper, T. B., Bark, N., Simpson, G. M. (1981). Prediction of steady state plasma and saliva levels of desmetylimipramine using a single dose, single time point procedure. *Psychopharmacology*. 74, 115-121.

Dabbs, J. M. (1991). Salivary testosterone measurements: collecting, storing, and mailing saliva samples. *Physiology and Behavior*. 49, 815-817.

Danhof, M., Breimer, D. D. (1978). Therapeutic drug monitoring in saliva. *Clinical Pharmacokinetics*. 3, 39-57.

Davenport, H. W. (1977). Salivary secretion. In H. W. Davenport (Ed.), Physiology textbook series. Physiology of the digestive tract: an introductory text (Fourth ed., pp. 85-94). Chicago: Year Book Medical Publishers.

Dawes, C. (1972). Circadian rhythms in human salivary flow rate and composition. *Journal of Physiology*. 220, 529-545.

Dawes, C., Macpherson, L. M. D. (1992). Effects of 9 different chewing-gums and lozenges on salivary flow-rate and pH. *Caries Research*. 26, 176-182.

Drehsen, G., Rohdewald, P. (1981). Rapid high-performance thin-layer chromatography of salicylic acid, salicylamide, ethoxybenzamide and paracetamol in saliva. *Journal of Chromatography*. 223, 479-483.

Drobitch, R. K., Svensson, C. K. (1992). Therapeutic drug monitoring in saliva. An update. *Clinical Pharmacokinetics*. 23, 365-379.

Dutta, S. K., Dukehart, M., Narang, A., Latham, P. S. (1989). Functional and structural changes in parotid glands of alcoholic cirrhotic patients. *Gastroenterology*. 96, 510-518.

Evans, J. J. (1986). Progesterone in saliva does not parallel unbound progesterone in

plasma. *Clinical Chemistry*. 32, 542-544.

Feller, K., le Petit, G. (1977). On the distribution of drugs in saliva and blood plasma. *Int. J. Clin. Pharmacol.* 15, 468-469.

Ferguson, D. B., Fort, A. (1974). Circadian variations in human resting submandibular saliva flow rate and composition. *Archives of Oral Biology*. 19, 47-55.

Ferguson, D. B., Fort, A., Elliott, A. L., Potts, A. J. (1973). Circadian rhythms in human protid saliva flow rate and composition. *Archives of Oral Biology*. 18, 1155-1173.

Fox, P. C., Wolff, A., Yeh, C. K., Atkinson, J. C., Baum, B. J. (1988). Saliva inhibits HIV-1 infectivity. *J. Am. Dental. Assoc.* 116, 635-637.

Garrett, J. R. (1980). Permeability of salivary glands to macromolecules. In T. Zelles (Ed.), Advances in physiological sciences; vol 3. Saliva and salivation: satellite symposium of the 28th international congress of physiological sciences, Hungary (pp. 109-117). Oxford: Pergamon Press.

Gehris, T. L., Kathol, R. G. (1992). Comparison of time-integrated meaurement of salivary corticosteroids by oral diffusion sink technology to plasma-cortisol. *Endocrine Research*. 18, 77-89.

Gelenberg, A. J., Wojcik, J. D., Falk, W. E., Spring, B., Brotman, A. W., Galvin-Nadeau, M. (1990). Clovoxamine in the treatment of depressed outpatients: a double-blind, parallel-group comparison against amitriptyline and placebo. *Comprehensive Psychiatry*. 31, 307-314.

Goldsworthy, S. J., Kemp, M., Warner, J. O. (1981). Salivary and urine theophylline levels in management of childhood asthma. *Journal of the Royal Society of Medicine*. 74, 415-418.

Gorodetzky, C. W., Kullberg, M. P. (1974). Validity of screening methods for drugs of abuse in biological fluids. II. Heroin in plasma and saliva. *Clinical Pharmacology and Therapeutics*. 15, 579-587.

Gould, V. J., Turkes, A. O., Gaskell, S. J. (1986). Gas chromatography-mass spectrometric analysis of salivary testosterone with reference to diethylstilboestrol-treated prostatic cancer patients. *Journal of Steroid Biochemistry*. 24, 563-567.

Graham, G. G. (1982). Noninvasive chemical methods of estimating pharmacokinetic parameters. *Pharmacology*. 18, 333-349.

Grau, J. A. (1992). Salivary testing for HIV-infection. *British Medical Journal*. 305, 1093-1094.

Gross, S. J., Worthy, T. E., Nerder, L., Zimmermann, E. G., Soares, J. R., Lomax, P.

(1985). Detection of recent cannabis use by saliva D9-THC radioimmunoassay. *Journal of Analytical Toxicology*. 9, 1-5.

Groth, U., Prellwitz, W., Jänchen, E. (1974). Estimation of pharmacokinetic parameters of lithium from saliva and urine. *Clinical Pharmacology and Therapeutics*. 16, 490-498.

Haeckel, R. (1989). The application of saliva in laboratory medicine; report on the workshop conference of the German Society for Clinical Chemistry, held under the auspices of the International Federation of Clinical Chemistry, in Bremen, March 3-4, 1988. *Journal of Clinical Chemistry and Clinical Biochemistry*. 27, 221-252.

Haeckel, R. (1990). Relationship between intraindividual variation of the saliva/plasma- and of the arteriovenous concentration ratio as demonstrated by the administration of caffeine. *Journal of Clinical Chemistry and Clinical Biochemistry*. 28, 279-284.

Haeckel, R., Bucklitsch, I. (1987). The comparability of ethanol concentrations in peripheral blood and saliva. The phenomenon of variation in saliva to blood concentration ratios. *Journal of Clinical Chemistry and Clinical Biochemistry*. 25, 199-204.

Hammond, G. L., Langley, M. S. (1986). Identification and measurement of sex hormone binding globulin (SHBG) and corticosteroid binding globulin (CBG) in human saliva. *Acta Endocrinologica*. 112, 603-608.

Hart 't, B. J., Wilting, J. (1988). Sensitive gas chromatographic method for determining nitrazepam in serum and saliva. *Journal of Chromatography Biomedical Applications*. 424, 403-409.

Höld, K. M., De Boer, D., Zuidema, J., Maes, R. A. A. (1995). Evaluation of the Salivette as sampling device for monitoring b-adrenoceptor blocking drugs in saliva. *Journal of Chromatography Biomedical Applications*. 663, 103-110.

Idowu, O. R., Caddy, B. (1981). A review of the use of saliva in the forensic detection of drugs and other chemicals. *Journal of the Forensic Science Society*. 22, 123-135.

Inaba, T., Stewart, D. J., Kalow, W. (1978). Metabolism of cocaine in man. *Clinical Pharmacology and Therapeutics*. 23, 547-552.

Jacobson, E. D. (1981). Salivary secretion. In L. R. Johnson (Ed.), Gastrointestinal physiology (Second ed., pp. 46-54). St. Louis: The C.V. Mosby Company.

Jeffrey, A., Turner, P. (1978). Relationship between plasma and salivary concentration of amitriptyline. *British Journal of Clinical Pharmacology*. 5, 268-269.

Katagiri, Y., Nagasako, S., Hayashibara, M., Iwamoto, K. (1991). Salivary excretion of mexiletine in normal healthy volunteers. *Journal of Pharmacy and Pharmacology*. 43, 513-515.

Killmann, S. A., Thaysen, J. H. (1955). The permeability of the human parotid gland to a series of sulfonamide compounds, paraaminohippurate and inulin. *Scandinavian Journal of Clinical Laboratorium Investigation*. 7, 86-91.

Krause, R. (1902). Beiträge zur histologie der speiceldrüsen. Über die ausscheidung des indig-schwefel-sauren natrons durch die glandula submaxillaris. *Archiv. F. Mikrosk. Anat.* 59, 407-417.

Lam, Y. W. F., Chan, D. C. N., Rodriguez, S. Y., Lintakoon, J. H., Lam, T. H. (1993). Sensitive high-performance liquid chromatographic assay for the determination of chlorhexidine in saliva. *Journal of Chromatography Biomedical Applications*. 612, 166-171.

Landon, J., Mahmod, S. (1982). Distribution of drugs between blood and saliva. In G. F. Read, D. Riad-Fahmy, R. F. Walker, K. Griffiths (Eds.), Immunoassays of steroids in saliva: proceedings of the ninth Tenovus workshop, Cardiff november 1982 (pp. 47-55). Cardiff: Alpha Omega Publishing Limited.

Langley, J. N. (1898). The salivary glands. In E. A. Schaefer (Ed.), Textbook of physiology (Vol. 1, pp. 475-530). Edinburgh: Young J. Pentland.

Lentner, C. (Ed.). (1981). Geigy scientific tabels (Eighth ed., Vol. 1). Basel: Ciba Geigy.

Lequin, R. M., Boogaard van den, A., Vermeulen, J., Danhof, M. (1986). Progesterone in saliva: pitfalls and consequent implications for accuracy of the determination. *Clinical Chemistry*. 32, 831-834.

Levy, G., Lampman, R. (1975). Relationship between pH of saliva and pH of urine. *Journal of Pharmaceutical Sciences*. 64, 890-891.

Lynn, R. K., Olsen, G. D., Leger, R. M., Gordon, W. P., Smith, R. G., Gerber, N. (1975). The secretion of methadone and its major metabolite in the gastric juice of humans. Comparison with blood and salivary concentrations. *Drug Metabolism and Disposition*. 4, 504-509.

Madara, J. L. (1988). Tight junction dynamics: Is paracellular transport regulated? *Cell*. 53, 497-498.

Mally, J., Keszei, C., Cserep, G. (1992). Theophylline concentrations in serum, saliva, and cerebrospinal-fluid in patients with essential tremor. *Therapeutic Drug Monitoring*. 14, 135-137.

Mandel, I. D. (1974). Relation of saliva and plaque to caries. *Journal of Dental Research*. 53, 246-266.

Mandel, I. D. (1990). The diagnostic uses of saliva. *Journal of Oral Pathology & Medicine*. 19, 119-125.

Mangos, J. A., McSherry, N. R. (1970). Micropuncture study of urea excretion in parotid saliva of the rat. *American Journal of Physiology*. 218, 1329-1332.

Martin, K., Burgen, A. S. V. (1962). Changes in the permeability of the salivary gland caused by sympathetic stimulation and by catecholamines. *Journal of General Physiology*. 46, 225-243.

Martinez-Madrigal, F., Micheau, C. (1989). Histology of the major salivary glands. *American Journal of Surgical Pathology*. 13, 879-899.

Matin, S. B., Wan, S. H., Karam, J. H. (1974). Pharmacokinetics of tolbutamide: prediction by concentration in saliva. *Clinical Pharmacology and Therapeutics*. 16, 1052-1058.

Matin, S. B., Wan, S. H., Knight, J. B. (1977). Quantitative determination of enantiomeric compounds. I-Simultaneous measurement of the optical isomers of amphetamine in human plasma and saliva using chemical ionization mass spectrometry. *Biomedical Mass Spectrometry*. 4, 118-121.

May, P. R. A., Van Putten, T., Jenden, D. J., Cho, A. K. (1978). Test dose response in schizophrenia: chlorpromazine blood and saliva levels. *Archives of General Psychiatry*. 35, 1091-1097.

Mazariegos, M. R., Tice, L. W., Hand, A. R. (1984). Alteration of tight junctional permeability in the rat parotid gland after isoproterenol stimulation. *Journal of Cell Biology*. 98, 1865-1877.

McDevitt, D. G. (1987). Comparison of pharmacokinetic properties of beta-adrenoceptor blocking drugs. *European Heart Journal*. 8(Suppl. M), 9-14.

Meulenberg, E. P. M., Hofman, J. A. (1990). The effect of pretreatment of saliva on steroid hormone concentrations. *Journal of Clinical Chemistry and Clinical Biochemistry*. 28, 923-928.

Migliardi, M., Cenderelli, G. (1988). Direct radioimmunoassay of testosterone in human saliva. *Analytica Chimica Acta*. 207, 161-170.

Moncrieff, J. (1991). Determination of theophylline in serum and saliva in the presence of caffeine and its metabolites. *Journal of Chromatography Biomedical Applications*. 568, 177-185.

Moore, K. L. (Ed.). (1982). The developing human (Third ed.). Philadelphia: W.B. Sanders Company.

Morris, D. F. (1992). False positive salivary HIV test. *British Medical Journal*. 305, 834.

Mortimer, P. P., Parry, J. V. (1992). Salivary testing for HIV-infection. *British Medical Journal*. 305, 1093.

Mucklow, J. C. (1982). Review. The use of saliva in therapeutic drug monitoring. *Therapeutic Drug Monitoring*. 4, 229-247.

Mucklow, J. C., Bending, M. R., Kahn, G. C., Dollery, C. T. (1978). Drug concentration in saliva. *Clinical Pharmacology and Therapeutics*. 24, 563-570.

Nederfors, T., Dahlöf, C. (1992). Effects of the b-adrenoceptor antagonists atenolol and proranolol on human whole saliva flow-rate and composition. *Archives of Oral Biology*. 37, 579-584.

Nuck, A. (1690). Sialographia et ductum aquosorum anatome nova. Leyden: .

Paton, R. D., Logan, R. W. (1986). Salivary drug measurement: a cautionary tale. *Lancet*. December 6, 1340.

Paxton, J. W. (1979). Measurement of drugs in saliva: A review. *Methods and Findings in Experimental and Clinical Pharmacology*. 1, 11-21.

Paxton, J. W., Donald, R. A. (1980). Concentrations and kinetics of carbamazepine in whole saliva, parotid saliva, serum ultrafiltrate, and serum. *Clinical Pharmacology and Therapeutics*. 28, 695-702.

Posti, J. (1982). Saliva-plasma drug concentration ratios during absorption: theoretical considerations and pharmacokinetic implications. *Pharmaceutica Acta Helvetiae*. 57, 83-92.

Rasmussen, F. (1964). Salivary excretion of sulphonamides and barbiturates by cows and goats. *Acta Pharmacologica et Toxicologica*. 21, 11-19.

Rey, F., Chiodoni, G., Braillard, K., Berthod, C., Lemarchand-Béraud, T. (1990). Free testosterone levels in plasma and saliva as determined by a direct solid-phase radioimmunoassay: a critical evaluation. *Clinica Chimica Acta*. 191, 21-30.

Ritschel, W. A., Thompson, G. A. (1983). Monitoring of drug concentrations in saliva: a non-invasive pharmacokinetic procedure. *Methods and Findings in Experimental and Clinical Pharmacology*. 5, 511-525.

Roth, W., Beschke, K., Jauch, R., Zimmer, A., Koss, F. W. (1981). Fully automated high-performance liquid chromatography - a new chromatograph for pharmacokinitic drug monitoring by direct injection of body fluids. *Journal of Chromatography*. 222, 13-22.

Salako, L. A., Sowunmi, A. (1992). Disposition of quinine in plasma, red-blood-cells and saliva after oral and intravenous administration to healthy adult africans. *European Journal of Clinical Pharmacology*. 42, 171-174.

Schramm, W., Annesley, T. M., Siegel, G. J., Sackellares, J. C., Smith, R. H. (1991).

Measurement of phenytoin and carbamazepine in an ultrafiltrate of saliva. *Therapeutic Drug Monitoring*. 13, 452-460.

Schramm, W., Paek, S. H., Kuo, H. H., Yang, T. (1991). Ultrafiltrate of saliva collected in situ for the measurement of testosterone. *Analytica Chimica Acta*. 248, 517-528.

Schramm, W., Smith, R. H., Craig, P. A. (1992). Drugs of abuse in saliva: a review. *Journal of Analytical Toxicology*. 16, 1-9.

Schramm, W., Smith, R. H., Craig, P. A., Paek, S., Kuo, H. (1990). Determination of free progesterone in an ultrafiltrate of saliva collected in situ. *Clinical Chemistry*. 36, 1488-1493.

Selby, C., Lobb, P. A., Jeffcoate, W. J. (1988). Sex hormone binding globulin in saliva. *Clinical Endocrinology*. 28, 19-24.

Shannon, I. L., Suddick, R. P. (1973). Effects of light and darkness on human parotid salivary flow rate and chemical composition. *Archives of Oral Biology*. 18, 601-608.

Shannon, I. L., Suddick, R. P., Dowd jr, F. J. (1974). Monographs in oral science. Saliva: composition and secretion (Vol. 2). Basel: Karger,S.

Sharp, M. E., Wallace, S. M., Hindmarsh, K. W., Peel, H. W. (1983). Monitoring saliva concentrations of methaqualone, codeine, secobarbital, diphenhydramine and diazepam after single oral doses. *Journal of Analytical Toxicology*. 7, 11-14.

Shipley, J. E., Alessi, N. E., Wade, S. E., Haegele, A. D., Helmbold, B. (1992). Utility of an oral diffusion sink (ODS) device for quantification of saliva corticosteroids in human subjects. *Journal of Clinical Endocrinology and Metabolism*. 74, 698-700.

Siegel, I. A., Ben-Aryeh, H., Gozal, D., Colin, A. A., Szargel, R., Laufer, D. (1990). Comparison of unbound and total serum theophylline concentrations with those of stimulated and unstimulated saliva in asthmatic children. *Therapeutic Drug Monitoring*. 12, 460-464.

Simson, J. A. V., Bank, H. L. (1984). Freeze-fracture and lead ion tracer evidence for a paracellular fluid secretory pathway in rat parotid glands. *Anatomical Record*. 208, 69-80.

Skidmore, S. J., Morris, C. A. (1992). Salivary testing for HIV-infection. *British Medical Journal*. 305, 1094.

Speirs, C. F. (1977). Oral absorption and secretion of drugs. *British Journal of Clinical Pharmacology*. 4, 97-100.

Spring, K. R., Spirtes, M. A. (1969). Salivary excretion of lithium: II. Functional analysis. *Journal of Dental Research*. 48, 550-553.

Straus, W. (1957). Segregation of an intravenously injected protein by "droplets" of the cells of rat kidney. *J. Biophys. Biochem. Cytol.* 3, 1037-1040.

Swinkels, L. M. J. W., Hoof van, H. J. C., Ross, H. A., Smals, A. G. H., Benraad, T. J. (1991). Concentrations of salivary testosterone and plasma total, non-sex-hormone-binding globulin-bound, and free testosterone in normal and hirsute women during administration of dexamethasone synthetic corticotropin. *Clinical Chemistry*. 37, 180-185.

Swinkels, L. M. J. W., Hoof van, H. J. C., Ross, H. A., Smals, A. G. H., Benraad, T. J. (1992). Low ratio of androstenedione to testosterone in plasma and saliva of hirsute women. *Clinical Chemistry*. 38, 1819-1823.

Taylor, E. A., Kaspi, T. L., Turner, P. (1978). The pH dependent absorption of propranolol and indomethacin by Parafilm, a stimulant of salivary secretion. *Journal of Pharmacy and Pharmacology*. 30, 813-814.

Thaysen, J. H., Thorn, N. A., Schwartz, I. L. (1954). Excretion of sodium, potassium, chloride and carbon dioxide in human parotid saliva. *American Journal of Physiology*. 178, 155-159.

Thieme, T., Yoshihara, P., Piacentini, S., Beller, M. (1992). Clinical evaluation of oral fluid samples for diganosis of viral hepatitis. *Journal of Clinical Microbiology*. 30, 1076-1079.

Thompson, L. K., Cone, E. J. (1987). Determination of D9-tetrahydrocannabionol in human blood and saliva by high-performance liquid chromatography with amperometric detection. *Journal of Chromatography Biomedical Applications*. 421, 91-97.

Thompson, L. K., Yousefnejad, D., Kumor, K., Sherer, M., Cone, E. J. (1987). Confirmation of cocaine in human saliva after intravenous use. *Journal of Analytical Toxicology*. 11, 36-38.

Turner, P. C., Eglin, R. E., Woodward, C. G., Porter, G. S. (1992). Salivary testing for HIV-infection. *British Medical Journal*. 305, 1093.

Umstead, G. S., Morales, M., McKercher, P. L. (1986). Comparison of total, free, and salivary phenytoin concentrations in geriatric patients. *Clinical Pharmacy*. 5, 59-62.

Van Dam, J. G., Van Loenen, A. C. (1978). De aanwezigheid van geneesmiddelen in speeksel. *Pharmaceutisch Weekblad*. 113, 65-74.

Vining, R. F., McGinley, R. A. (1982). Transport of steroids from blood to saliva. In G. F. Read, D. Riad-Fahmy, R. F. Walker, K. Griffiths (Eds.), Immunoassays of steroids in saliva; proceedings of the ninth Tenovus workshop, Cardiff november 1982 (pp. 56-63). Cardiff: Alpha Omega Publishing Limited.

Vining, R. F., McGinley, R. A. (1985). Hormones in saliva. *Critical Reviews in Clinical*

Laboratory Sciences. 23, 95-146.

Wade, S. E. (1984). A direct method for determination of the time integral of corticosterone - availability in interstitial fluid of rats. *Analytical Biochemistry*. 138, 365-371.

Wade, S. E. (1992a). Less-invasive measurement of tissue availability of hormones and drugs: diffusion-sink sampling. *Clinical Chemistry*. 38, 1639-1644.

Wade, S. E. (1992b). An Oral-Diffusion-Sink device for extended sampling of multiple steroid hormones from saliva. *Clinical Chemistry*. 38, 1878-1882.

Wade, S. E., Haegele, A. D. (1991). Time-integrated measurement of corticosteroids in saliva by Oral Diffusion Sink technology. *Clinical Chemistry*. 37, 1166-1172.

Walsh, D. J., Corey, A. C., Cotton, R. W., Forman, L., Herrin, G. L., Word, C. J., Garner, D. D. (1992). Isolation of deoxyribonucleic acid (DNA) from saliva and forensic science samples containing saliva. *Journal of Forensic Sciences*. 37, 387-395.

Wan, S. H., Matin, S. B., Azarnoff, D. L. (1978). Kinetics, salivary excretion of amphetamine isomers, and effect of urinary pH. *Clinical Pharmacology and Therapeutics*. 23, 585-590.

Welch, R. M., DeAngelis, R. L., Wingfield, M., Farmer, T. W. (1975). Elimination of antipyrine from saliva as a measure of metabolism in man. *Clinical Pharmacology and Therapeutics*. 18, 249-258.

Wolff, K., Hay, A. (1991). Methadone in saliva. *Clinical Chemistry*. 37, 1297-1298.

Yamazumi, S., Miura, S. (1981). Haloperidol concentrations in saliva and serum: determination by the radioimmunoassay method. *Intern. Pharmacopsychiat.* 16, 174-183.

Young, J. A., Case, R. M., Conigrave, A. D., Novak, I., Thompson, C. H. (1980). Secretory processes in the perfused rabbit mandibular gland. In T. Zelles (Ed.), Advances in physiological sciences; vol 28. Saliva and salivation: satellite symposium of the 28th international congress of physiological sciences, Hungary (pp. 35-46). Oxford: Pergamon Press.

Young, J. A., Van Lennep, E. W. (Eds.). (1978). The morphology of salivary glands. London: Academic Press.

Young, J. A., Van Lennep, E. W. (1979). Transport in salivary and salt glands. In G. Giebisch, D. C. Tosteson, H. H. Ussing (Eds.), Membrane transport in biology (pp. 563-674). Berlin: Springer-Verlag.

Zuidema, J., Van Ginneken, C. A. M. (1983a). Clearance concept in salivary drug excretion, part I: theory. *Pharmaceutica Acta Helvetiae*. 58, 88-93.

Zuidema, J., Van Ginneken, C. A. M. (1983b). Clearance concept in salivary drug excretion, part II: experiments. *Pharmaceutica Acta Helvetiae*. 58, 136-143.