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Salivary Analysis for Medico-Legal and Forensic Toxicological Purposes

Roberto Scendoni

Abstract

Saliva testing has attracted great interest in the forensic scientific landscape recently, especially among institutions or legal authorities interested in determining drug concentrations (for application in the workplace, drug driving, legal issues associated with drug testing, and pharmacokinetics of selected drugs). Indeed, it has been established that oral fluid is an adequate alternative biological matrix to blood for the determination of xenobiotics and/or drugs of abuse and/or metabolites both in living and deceased individuals. The concentration of a detectable substance in saliva is generally proportional to the free fraction of the drug present in plasma; this measurement therefore makes it possible to correlate the concentration of the substance and its pharmacological effects on the individual. The purpose of this chapter is to examine the main analytical techniques developed thus far in saliva drug testing, from screening to confirmatory analysis, taking into account the interpretation of cut-off levels. Both well-defined and potentially problematic issues are highlighted from medico-legal and toxicological perspectives.

Keywords: salivary analysis, drugs, analytical techniques, legal medicine, forensic toxicology

1. Introduction

Detecting the presence of drugs or their metabolites in biological material requires different approaches and methods, depending on the purpose of the investigation and specific legal requirements. In the forensic toxicology field, multiple biological matrices are commonly used as diagnostic tools (such as blood, urine, keratin matrices, oral fluid, etc.) and the respective results, either alone or in combination with each other, provide useful elements for a correct diagnosis. An investigation may be prompted by various concerns: suitability to drive, professional driver suitability, employee and work suitability, suitability for gun permit, suitability for specific competition and/or contractual rules, diagnosis of use/abuse (also in the contexts of custody of minors and international adoptions), diagnosis of drug addiction, and diagnosis of intoxication in living or dead people.

Technical choices are based on these premises and purposes. For example, urine testing can typically determine the “recent” consumption of substances of abuse (with a temporal detection window of hours or even days depending on the pharmacokinetic characteristics of the substance in question). This sample can also

be used to determine chronic drug use if the analysis is extended to several samples collected on different days and “by surprise” (i.e. with the shortest possible notice given to the interested party, not exceeding 24 hours). Chronic use, as well as previous patterns of use/abuse, can be verified by analysis of the hair matrix too.

In cases where it is necessary to quickly evaluate degree of substance intoxication (for example in an emergency situation) blood testing is particularly useful. Even so, over the past few years oral fluid has been increasingly studied as an alternative matrix of choice, and a number of reviews and papers have recently focused on various aspects of drug testing using oral fluid, although it has a shorter detection window than blood (**Figure 1**). Consideration should be given to the importance of oral fluid as a clinical diagnostic [1] and forensic tool and its relevance for a range of applications including workplace drug testing [2], drug driving [3], legal issues associated with drug testing [4], pharmacokinetics of selected drugs [5], and therapeutic drug monitoring (TDM) [6].

Regarding its composition, saliva is a very dilute fluid. Its major constituent is water (> 97%); other components include electrolytes, immunoglobins, enzymes and proteins. In normal conditions, healthy adults produce approximately 500–1500 mL saliva in 24 hours through the submandibular gland (about 65%), the parotid gland (23%) and the sublingual gland (4%), along with many other small glands distributed in the oral cavity (about 8%). Products of the salivary glands can be classified into four major components with different functions: mucus that serves as a lubricant; amylase, an enzyme that initiates the digestion of starch; lingual lipase, an enzyme that begins the fat digestion process; and a slightly alkaline electrolyte solution that moistens food so that it can be swallowed easily.

The most abundant salivary electrolytes are sodium, potassium, chloride and bicarbonate, while calcium, magnesium and phosphate are present in lesser concentrations. Other salivary constituents include substances transported from the blood through the gland into saliva [7].

Salivation can be stimulated or reduced by several factors. Electrolyte concentrations and volume of saliva produced are influenced by the time of day and type of salivation stimulus. In fact, the volume and composition of oral fluid can vary during the day and over time in each individual. Therefore, it can be said that its composition varies continuously, both quantitatively and qualitatively [8]. When salivary constituents need to be identified, it should be emphasised that the results will depend on the subject's cooperation, psychological status, medication use, method of sampling and time of day.

Saliva has a slightly more acidic pH (6 to 7) than that of blood, and therefore all lipophilic psychoactive substances, with a weak basic nature, low molecular weight and blood protein binding of less than 50%, are preferentially excreted in saliva by passive diffusion of the free fraction of the substance in its ionised form. Moreover, the pH of saliva can change from being slightly acidic at rest, to basic (pH 8) at

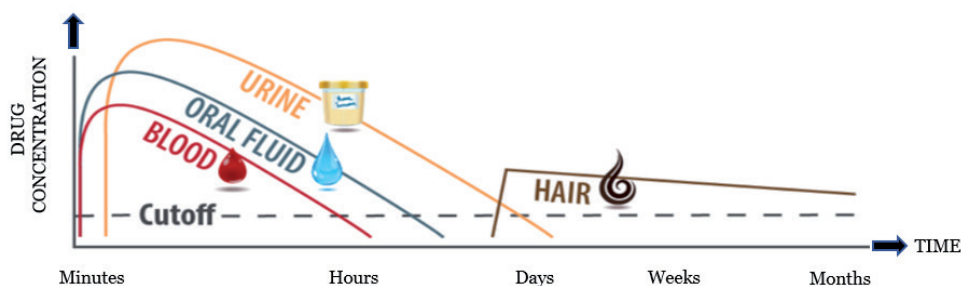


Figure 1.
Drug detection times in different matrices.

ultimate stimulation. Amylase and mucus also increase in concentration after stimulation [8].

The first guidelines for the analysis of substances of abuse in saliva were proposed in 2004 in the United States by the “Substance Abuse and Mental Health Service Administration” (SAMHSA) [9] and were mainly intended for analyses carried out in the workplace to determine the possible use of substances. Subsequently, the “European Workplace Drug Testing Society” (EWDTS) [10] also drafted European guidelines, again oriented to analyses in workplaces. SAMHSA published its final Mandatory Guidelines for Federal Workplace Drug Testing Programs using Oral Fluid on October 25, 2019 in the Federal Register [11]. The new regulations only apply to federal workplaces, at the time of writing, but the impact is sure to reach beyond the initial scope of these regulations.

2. Drug transfer from blood to saliva

The most common routes for a drug to migrate to saliva are passive transcellular diffusion, ultrafiltration, active transport and passive diffusion.

- a. **Passive transcellular diffusion:** highly lipid-soluble substances may pass through the capillary wall, basement membrane and acinar cell of the secretory end-piece, with the lipid layer of the epithelial cell wall providing the rate-limiting barrier. The same mechanism would probably enable these molecules 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 [12].
- b. **Ultrafiltration (or paracellular transport):** small polar molecules such as glycerol and sucrose enter saliva. The saliva/plasma (S/P) ratios of several small polar, lipid-insoluble compounds are plotted as a function of their molecular weight (MW). This mechanism is restricted to compounds with a MW of less than about 300 Da, and even those with a MW of about 150 Da are only filtered to a minimal extent. Furthermore, the flow rate of saliva should not affect S/P ratios if diffusion is rapid and passive.
- c. **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 [13]. Borzelleca (1965) [14] investigated whether penicillin and tetracycline were secreted in saliva. The secretion of these antibiotics in saliva appeared to be dependent on 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.
- d. **Passive diffusion process:** is characterised 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

RELATING TO DRUG
Lipid-solubility
Acidic or basic, and the pKa
Molecular weight and spatial configuration
Charged or neutral
RELATING TO SALIVA
Saliva pH
Saliva flow rate
Saliva-binding proteins - usually minimal
Enzymes in saliva capable of metabolising the drug
RELATING TO THE CIRCULATING DRUG LEVEL IN THE FREE (NONPROTEIN-BOUND) FORM
Dose and clearance of drug
Nonprotein-bound blood level

Table 1.
Factors influencing passive diffusion of a drug from blood to saliva.

constant that depends on the physico-chemical properties of each drug [15]. The variables which influence this type of transport are listed in **Table 1** (Landon and Mahmood, 1982) [16].

Salivary secretion is a reflex response controlled by both parasympathetic and sympathetic secretomotor nerves. This is an important factor influencing oral fluid availability and potential drug concentrations. Taking medication which affects either the central nervous system or the peripheral nervous system (or medication which mimics the latter as a side effect) alters salivary composition and salivary volume. Therefore, patients suffering from systemic diseases may show alterations in salivary gland secretion and electrolyte concentrations. Finally, diet and age also have an impact on composition and volume of saliva [8].

3. Methods and techniques

3.1 The sampling

It is essential to prepare Standard Operating Procedures (SOPs) relating to the collection and storage of the oral fluid sample, as well as the training of personnel assigned to take and ship the sample to the laboratory where the toxicological analysis will be carried out. It follows that it is essential to document:

- respect for the privacy and security of the person undergoing analytical assessment;
- the identity of the person undergoing analytical assessment;
- the location where the sample of oral fluid has been collected;
- that no falsification or tampering of the sample has taken place;

- that the informed consent form has been completed in its entirety by the person undergoing the analytical assessment (unless there is a formal mandate from the Legal Authority);
- the use of particular medicines that may interfere with the analytical results;
- the traceability of the sample through appropriate records of its movement, from the place of sampling to the laboratory that receives it, including the identity records of the personnel authorised to handle it.

Neat oral fluid can be collected from expectoration (or spitting), but this is relatively viscous and can therefore be challenging to work with and analyse in the laboratory. It may also be contaminated with food and oral debris, which makes centrifugation essential. In addition, sensitive detection techniques are required, because the volume collected will often be less than 1 mL. Normally, the absorbent foam swab or pad used to collect the oral fluid is added to a diluent. After mixing, the solution is ready for drug analysis. Other devices involve squeezing absorbed oral fluid from a pad or foam directly onto the drug-detection device, a process that can take one to three minutes. A number of devices incorporate some form of indicator to show when an adequate amount of oral fluid has been collected [17].

A number of drugs affect the secretion of oral fluid [8], mostly cannabis and amphetamines, including designer drugs such as MDMA. Other drugs include the sedating antihistamines, antipsychotic drugs, anticholinergic drugs and several antidepressants. Less commonly used drugs increase saliva flow and these include clonidine, pilocarpine and beta-2 stimulants (salbutamol, terbutaline, etc). Overall, there is significant intra- and inter-subject variation in relation to drug concentrations depending on the technique used, the physiology of the person and the factors affecting drug concentration in oral fluid.

3.2 Analytical techniques

An important aspect to consider is the choice of analytical technique used for the detection of drugs and metabolites in saliva. A fundamental element is the certainty and reliability of the results, from both qualitative and quantitative perspectives. The results of quantitative determination, though, are not easy to interpret as the information that makes it possible to trace the metabolic process is often unavailable (e.g. time the drug was taken, amount of active ingredient, and route of administration).

In many forensic contexts, oral fluid is analysed with screening methods. The semiquantitative results obtained must be validated by confirmatory techniques, such as liquid chromatography combined with mass spectrometry [18]. Oral fluids (OF) have been recently introduced as a biological matrix useful for roadside testing to determine illicit drug use because the time course of drugs in oral fluid may resemble that of plasma. Moreover, OF can be considered a valid alternative specimen for confirmation testing because drugs are excreted in saliva mainly as parent compounds [19–21]. In fact police officers, without medical supervision, are not authorised to employ invasive methods but they can collect OF samples. A very comprehensive review of the analysis of drugs of abuse in OF was conducted by Reinstadler et al. [22]. Other studies [23] have highlighted the importance of both the sample treatment process and the use of hyphenated instruments in obtaining analytical performances that satisfy current regulations in terms of sensitivity, selectivity and fast confirmatory analysis.

3.2.1 On-site screening test

Recent data have shown improvements in the effectiveness of on-site drug testing using oral fluid, and significant progress has been made in terms of sample collection and accuracy of analysis [24].

A number of field drug testing devices are available and used in many countries to perform on-site testing on oral fluids in the context of Driving Under the Influence of Drugs (DUID) [25]. For example, DrugWipe® is an immunochromatographic test strip, based on the Frontline urine test strip from Boehringer Mannheim. A pink colour in the test window indicates the presence of the analyte in question, but different devices are normally required to detect the various classes of drugs of abuse. However, a recent version of this device, DrugWipe 5A, is capable of indicating the simultaneous use of cannabis, amphetamine, methamphetamine, ecstasy, cocaine, and opiates [26]. A recent study investigated the reliability of DrugWipe 5A in establishing exposure to principal drugs of abuse (cannabis, amphetamines, cocaine, and opiates) using oral fluid specimens by comparing the on-site results with headspace solid-phase microextraction (HS-SPME) gas chromatography–mass spectrometry (GC–MS) analyses on extractions from the sample collection pad [27].

Another point of collection test, Rapid STAT®, has broken new ground by combining the convenience of oral fluid collection, surface wipe testing or pure substance measurements with the sensitivity, accuracy and precision of a laboratory based test, with speedy results (in a few minutes).

Table 2 shows the recommended minimum detectable concentrations of drugs in oral fluid according to SAMHSA and European Union roadside assessment testing study (ROSITA) cut-off levels [28].

3.2.2 Laboratory screening test

The enzyme-linked immunosorbent assay (ELISA) is a sensitive and versatile test used in many fields to detect and measure substances in biological samples (**Figure 2**). For almost 50 years it has remained a trusted testing technique for everything from food allergen detection to medical screening for various illnesses. For the toxicology market specifically, ELISA is an excellent and cost-effective solution which meets high-throughput screening (HTS) needs. The procedure is simple and easily automated or it can be conducted by a laboratory technician. It basically works around the principle of competition between two substances in a given sample: an enzyme conjugate such as horseradish peroxidase (HRP) is used to compete with a target substance for a limited number of specific binding sites on a precoated microplate.

Drug	SAMHSA cut-offs (ng/mL)	ROSITA cut-offs (ng/mL)
Cocaine	00	5–10
Morphine	40	—
6-AM	4	10
Methamphetamine/Amphetamine/MDMA	50	70–90
THC	CM	1.9

6-AM = 6-Acetylmorphine, MDMA = methylenedioxymethamphetamine, THC = Δ^9 -tetrahydrocannabinol.

Table 2.
Recommended minimum detectable concentrations of drugs in oral fluid – Instrumental devices field testing.

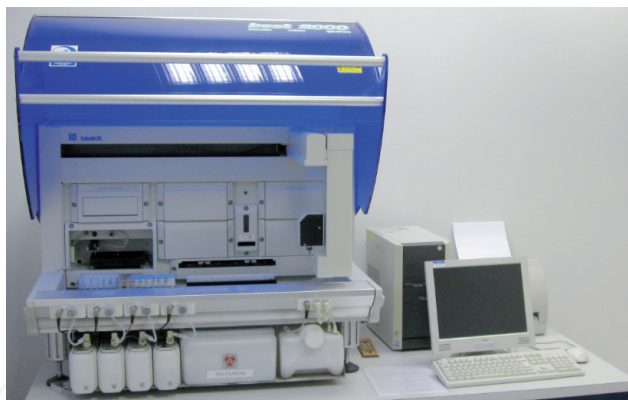


Figure 2.
Fully automated Elisa analyser.

The different available types of ELISAs provide a reliable means for screening oral fluid. In general these work adequately for amphetamines [29], buprenorphine, cocaine [30], methadone [31], and other opioids [32]. Cannabis may pose more difficulties, particularly if the immunoassay has little cross-reactivity to *tetrahydrocannabinol* (THC), the main psychoactive component of the drug. Even so, enzyme immunoassay has been successfully used for cannabis; the same applies to benzodiazepines despite their low oral fluid concentrations [33].

3.2.3 Confirmatory analysis

Confirmatory techniques for drugs in oral fluid [20] are mostly adapted from those used in the analyses of blood or plasma/serum specimens. Recovery of drugs is not typically a limiting factor, considering the higher water content and lower protein levels of oral fluid compared to blood. However, the sample volume of oral fluid will be smaller, with potentially lower concentrations, which means that more adjustments are required to analytical techniques. Indeed, in saliva analysis the detection or quantification limit for drugs is very much determined by the type of screening test and its application. The confirmation method must be able to produce an analytical result that is optimally independent from that of the screening. Therefore, it must be based on different physico-chemical principles and have superior analytical selectivity and sensitivity. In this regard, a quantitative confirmatory method capable of reaching a lower limit of quantification (LLOQ) equal to at least half the cut-off of the screening method is considered acceptable. The use of a confirmatory method which is based on the measurement of a similar analytical signal is not acceptable since it is highly correlated to that of the screening (e.g. confirmation of a given immunochemical with another immunochemical method). The use of an identical chromatographic technique to confirm a set of data obtained by chromatography is acceptable if the detection technique combined with chromatography changes.

The use of a chromatographic technique to confirm screening data obtained by chromatography with the same detection system is allowed only if the two separation techniques produce poorly correlated results (for example, two series of significantly different retention times, with the use of columns of different polarity or selectivity, etc). However, in the forensic toxicological field, chromatographic separation is always necessary in a confirmatory method; the general consensus of the international scientific community is that mass spectrometry (MS) with its many methodological possibilities can be combined with a chromatographic separation technique such as gas chromatography (GC), high pressure liquid chromatography (HPLC) or capillary electrophoresis (EC) for confirmatory analysis (**Figure 3**). Many methods

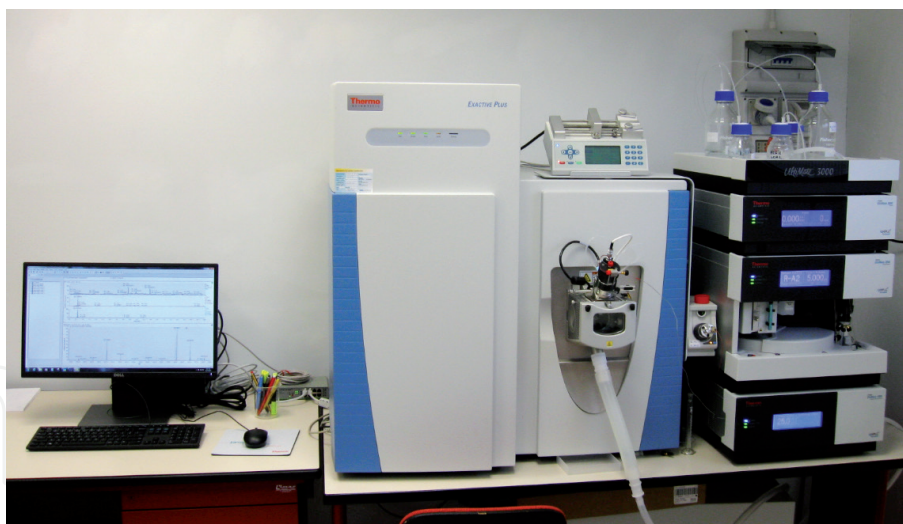


Figure 3.
Ultra-high performance liquid chromatography.

use LC–MS as distinct from GC–MS to cater for the lower sample volumes and low detection limits, although a number of GC–MS techniques have exhibited adequate sensitivity [34].

4. Medico-legal and toxicological issues

4.1 Saliva versus blood

Intra-individual variability of the S/P ratio has been demonstrated for a number of drugs administered orally or intravenously [35]. Following the uptake of an orally applied substance in the intestine, arterial blood has a higher concentration than venous blood (positive arteriovenous difference). If the substance is completely absorbed but not significantly metabolised in a particular organ, the situation is reversed: the substance rediffuses from the cells into the blood (negative arteriovenous difference in the elimination phase). The various organs can be classified into two groups: those with a high blood flow (e.g. liver, kidney, brain, salivary glands), and those with a relatively low blood flow (e.g. skin, resting skeletal muscle, fat). In pharmacokinetics the first group of highly perfused organs is included in the central compartment, while the second group of less perfused organs belongs to the peripheral compartment. This must be taken into account when saliva concentrations are compared with blood concentrations from cubital veins in the peripheral compartment. In any case, salivary glands have a high blood flow, which means that the arteriovenous difference of freely diffusible substances is relatively small, with a ratio close to 1.0. Poor correlations between the two compartments have been documented in the literature, but neglect of the phenomenon described can only partly account for this [36].

Some comparative studies [37, 38] have found that drug concentrations in oral fluid cannot be used to accurately estimate drug concentrations in blood. A positive result in an oral fluid test may certainly confirm recent drug use, but it may only provide a semiquantitative assessment of the drug concentration in the blood (and only for some drugs). For psychiatric patients, oral fluid testing may be used as a non-invasive technique for evaluating substance use. In the case of drivers suspected of driving under the influence of drugs, oral fluid may be used for initial on-site screening tests (afterwards, it may be decided that a blood sample should be taken for forensic drug analysis).

Wille et al. (2009) [37] analysed blood and saliva samples by gas chromatography–mass spectrometry (GC–MS) or liquid chromatography–mass spectrometry (LC–MS). Scatter plots and trend lines of the blood and oral fluid concentrations were created and the median, mean, range, and standard deviation (SD) of the oral fluid to blood (OF/B) ratios were calculated for different classes of drugs, including amphetamines, benzodiazepines, cocaine, opiates, and delta⁹-2 tetrahydrocannabinol. The ratios found in this study were in line with previously published results, but the range was wider. The OF/B ratios of drugs of abuse such as amphetamines, cocaine, and opiates were > 1 [amphetamine: median (range) 13 (0.5-182), methylenedioxyamphetamine: 4 (1-15), methylenedioxymethamphetamine: 6 (0.9-88), methamphetamine: 5 (2-23), cocaine: 22 (4-119), benzoylecgonine: 1 (0.2-11), morphine: 2 (0.8-6), and codeine: 10 (0.8-39)]. Unsurprisingly, the ratios for benzodiazepines were considerably lower: given their high protein binding and weak acidity, benzodiazepines typically have low oral fluid concentrations [diazepam: 0.02 (0.01-0.15), nordiazepam: 0.04 (0.01-0.23), oxazepam: 0.05 (0.03-0.14), and temazepam: 0.1 (0.06-0.54)]. For tetrahydrocannabinol, an OF/B ratio of 15 was found (range 0.01-569). The variability of the OF/B ratios in suspected drugged drivers was clearly mirrored in the data. Be that as it may, blood concentrations could not be reliably calculated from oral fluid concentrations, due to the wide range of ratios.

Gjerde H et al. (2010) [38] analysed 90 pairs of blood and oral fluid specimens from patients undergoing acute psychiatric treatment and 22 pairs of blood and oral fluid specimens from suspected drugged drivers, with the aim of comparing drug concentrations between the two biological matrices. The median oral fluid/blood drug concentration ratios for the most prevalent drugs were 0.036 diazepam, 0.027 nordiazepam, 7.1 amphetamine, 2.9 methamphetamine, 5.4 codeine, 1.9 morphine, and 4.7 tetrahydrocannabinol. For the six most prevalent drugs, the correlation coefficients between drug concentrations in oral fluid and blood ranged from 0.15 to 0.96. The results, therefore, showed large interindividual variations in drug concentration ratios between oral fluid and blood. This wide distribution of OF/B ratios indicated that drug concentrations in oral fluid may not be used to reliably estimate drug concentrations in blood.

Such analytical variability could cause controversy in the judicial field, especially when the values obtained from saliva are only slightly higher than the cut-off levels established by the law of various countries.

<i>Drug</i>	<i>Average oral fluid to blood concentration ratio</i>
Barbiturates [39]	0.3
Ethanol [40]	1.07
Buprenorphine [41]	1
Codeine [42]	4
Methamphetamine [43]	2
MDMA [44]	7
Cocaine [45]	3
Diazepam [46]	0.01–0.02
Methadone [47]	1.6
Morphine [48]	0.8
Δ9- Tetrahydrocannabinol [49]	1.2

Table 3.
Average oral fluid to blood concentration ratios for selected drugs.

Table 3 shows the average values of oral fluid to blood concentration ratios of selected drugs, based on various pharmacokinetic studies; the average ratios change depending on a number of factors, such as pH of oral fluid, protein binding and degree of contamination of the membranes in the oral cavity by recently consumed drug.

4.2 Cut-off levels and analytical interpretation

Interpretation of oral fluid drug test results depends to some extent on the purpose of testing. An employer may decide to implement a workplace drug testing programme primarily to detect drug abuse among employees (or even job applicants), especially regarding safety-sensitive positions or following a safety incident or accident. Random workplace testing could also serve as a deterrent to substance misuse in the general workforce. Drug treatment specialists carry out drug testing to foster drug abstinence and compliance with programme requirements. Numerous factors must be considered when interpreting drug test results. During this process, complex questions may be posed, depending on the nature of the drug-testing programme, and sometimes the answers sought go beyond reasonable scientific certainty. Patterns of metabolic disposition should be understood for each class of drugs. Of course, the interpretation of oral fluid tests requires knowledge of the unique features of this biological matrix, along with a thorough understanding of: the chemical and physiological factors that affect drug transfer into oral fluid; analytical factors; kinetic aspects of drug disposition; drug metabolic patterns; and potential risks of oral contamination and passive exposure. Generally speaking, it has been shown that oral fluid tests are most useful in the detection of recent drug use [50].

The use of a screening method can be justified in a forensic toxicology laboratory when there is a need to analyse a large number of samples in a short time and at low costs, with the advantages of high or total automation. Screening methods usually employ colorimetric, enzymatic, and immunochemical techniques. However, screening methods are characterised by low specificity (qualitative data) and high inaccuracy (quantitative data), particularly when several chemical species can be detected in the sample but not discriminated by the method (e.g. an unchanged compound and its metabolites, or various types of similar species of compounds). Given their intrinsic characteristics, these methods exclusively produce a presumptive result, that is to say the probable negativity (absence) or positivity (presence, better defined as “non-negativity”) of the sample with respect to an analyte, or more often a class of substances, relative to a cut-off value set by the method. In any case, whatever the analytical specificity of the screening method, a positive result obtained through a single screening test cannot have forensic validity. It is therefore essential that this result is verified by a confirmatory analysis on a new sample rate.

The results of a quantitative analysis must be expressed in a uniform unit of measurement, so as to exclude interpretative doubts, directly comparable with any reference values (cut-off) and accepted by the International System of Units (SI). The uncertainty associated with the measurement performed must be indicated; at the same time, the comparison with threshold or reference values must take into account this uncertainty. **Tables 4** and **5** show the recommended cut-off levels of oral fluid tests according to EWDTS and SAMHSA guidelines.

Drugs and metabolites can be detected for a period of several hours to several days following drug exposure. Their concentrations in oral fluid are generally related to content in blood, but may also be present as residual drug in the oral cavity [11].

In what follows, descriptions of the main drugs of abuse are given [50].

Drug	Screening Cut-off (ng/mL)	Confirmation Cut-off (ng/mL)
OPIATES		
Morphine	Opiates (Morphine) 40 Opiates (6-MAM) 4	15
Codeine		15
Norcodeina		2
6-Acetylcodeine		2
Dihydrocodeine		15
6-Monoacetylmorphine		2
METHADONE AND METABOLITES	L-Methadone, 50	20
BUPRENORPHINE AND METABOLITES	5	1
COCAINE AND METABOLITE		
Cocaine	Cocaine + metabolites 30	8
Benzoylcegonine		8
AMPHETAMINE AND CONGENERS		
methamphetamine	Amphetamines 40	15
amphetamine		15
MDMA		15
MDA		15
CANNABINOIDS		
THC	THC 10	2

Table 4.
Recommended maximum screening and confirmation cut-off values for oral fluid tests in the workplace according to EWDTS guidelines [51].

AMPHETAMINE: a synthetic substance related to natural sympathomimetic amines with central nervous stimulant activity. Amphetamine appears rapidly in oral fluid following administration and parallels plasma drug concentrations. Amphetamine is also produced as a metabolite of methamphetamine and from a variety of pharmaceutical products. A positive test result for amphetamine indicates amphetamine use; determination of d/l-isomer ratio should rule out the possibility of mystification with another drug.

METHAMPHETAMINE: a synthetic sympathomimetic amine with central nervous stimulant activity similar to amphetamine but with more lasting effects. It is misused in numerous ways including smoking, snorting, injecting, and oral administration. Methamphetamine and amphetamine appear rapidly in plasma and oral fluid following administration. Determination of d/l-isomer ratio rules out the possibility that methamphetamine presence is due to the metabolism of another drug or use of an over-the-counter nasal inhaler. A positive test result for methamphetamine and amphetamine (methamphetamine < amphetamine) indicates possible combined use of methamphetamine and amphetamine.

METHYLENEDIOXYMETHAMPHETAMINE (MDMA): a synthetic, ring-substituted amphetamine derivative. N-demethylation of MDMA yields 3,4-methylenedioxyamphetamine (MDA), an active metabolite exhibiting similar pharmacological properties as the parent drug. O-demethylenation of MDMA and MDA produces 3,4-dihydroxymethamphetamine (HHMA) and

Initial Test Analyte	Screening Cut-off (ng/mL)	Confirmatory Test Analyte	Confirmatory Test Cut-off (ng/mL)
THC (Cannabis)	4	THC	2
Cocaine/Benzoylecgonine	15	Cocaine/Benzoylecgonine	8
			8
Codeine/Morphine	30	Codeine	15
		Morphine	15
Hydrocodone/Hydromorphone	30		15
		Hydrocodone/Hydromorphone	15
Oxycodone/Oxymorphone	30	Oxycodone/Oxymorphone	15
			15
6-Acetylmorphine (heroin)	4	6-Acetylmorphine	2
Phencyclidine (PCP)	10	Phencyclidine	10
Amphetamine/Methamphetamine	50	Amphetamine/Methamphetamine	25
			25
Methylenedioxymethamphatime (MDMA)	50	MDMA MDA	25
			25
Methylenedioxyamphetamine (MDA)			

Table 5.
Cut-off levels of oral fluid testing according to SAMHSA oral fluid guidelines [11] (effective January 1, 2020).

3,4-dihydroxyamphetamine (HHA), respectively. MDMA is typically administered orally and reaches maximal blood concentrations in approximately 2 hours. Oral fluid concentrations of MDMA are highly correlated with plasma MDMA. Oral fluid concentrations of MDMA are an order of magnitude higher than in plasma; this is attributed to the high pKa of MDMA and low plasma-protein binding. A positive test result for MDMA (no MDA) indicates illicit MDMA use; a positive test result for MDMA and MDA suggests illicit MDMA use (presence of MDA probably due to metabolism of MDMA to MDA but if $MDA \geq MDMA$, a combined use of illicit MDMA and illicit MDA is admissible).

3,4-METHYLENEDIOXYAMPHETAMINE (MDA): a synthetic, ring-substituted amphetamine derivative. MDA has been reported to appear in oral fluid following the administration of MDMA in concentrations representing approximately 4–5% of MDMA. Possible sources of MDA: illicit MDA, metabolite of illicit MDMA, metabolite of illicit MDEA. However, the confirmed presence of HHA and/or HMA in oral fluid would be useful to substantiate MDA use.

3,4-METHYLENEDIOXYETHYLAMPHETAMINE (MDEA): a synthetic analogue which is generated when an ethyl group is substituted for the methyl group of MDMA. MDEA is metabolised by O-demethylenation and by N-dealkylation of the ethyl-group. The major metabolite is formed by O-demethylenation to yield N-ethyl-4-hydroxy-3-methoxyamphetamine (HME); N-dealkylation leads to the formation of the active metabolite MDA. A positive test result for MDEA without MDA means illicit MDEA use, otherwise (in co-presence with MDA) combined use or an initiated metabolism of MDEA.

DELTA-9-TETRAHYDROCANNABINOL (THC): a naturally occurring psychoactive constituent of *Cannabis sativa*. THC appears rapidly in plasma following the smoking of cannabis products and is found in oral fluid following smoked and oral ingestion. According to several studies, THC is more highly present in oral fluid than blood, primarily as a result of deposition in the oral cavity. THC tends to decline in a similar manner to plasma concentrations.

COCAINE: a natural stimulant compound made from the leaves of the coca plant. Cocaine has a short half-life (approximately 1 hour) and is rapidly hydrolysed by hepatic esterases to benzoylecgonine (BZE) and ecognine methyl ester (EME). Cocaine and its metabolites appear rapidly in oral fluid following all routes of administration. Cocaine concentrations decrease rapidly within approximately 1 hour; thereafter, oral fluid concentrations appear to decline in parallel with concentrations of the drug in the blood. If cocaine concentration > BZE concentration: cocaine has probably been taken within the past 2–8 hours; cocaine concentration < BZE concentration: cocaine use in the past 12 hours for occasional users and 48 hours for daily users.

HEROIN: a semisynthetic opioid, diacetyl derivative of morphine prepared from opium for the illegal drug trade. Heroin is most commonly administered intravenously and by other parenteral routes, but may also be smoked. Heroin and 6-acetylmorphine appear in oral fluid within 2 minutes of administration. Drug and metabolite concentrations in oral fluid are generally similar to blood concentrations following intravenous administration, but may be substantially higher than blood when smoked. Elevated drug and metabolite concentrations following smoking are probably a consequence of residual drug deposited in the oral cavity. Thirty to sixty minutes after heroin is smoked, concentrations in oral fluid diminish considerably and begin to reflect blood concentrations. If 6-acetylmorphine and morphine are detected, the use of heroin (and not morphine) can be confirmed.

MORPHINE: a natural opiate alkaloid isolated from the plant *Papaver somniferum*; it is also a metabolite of heroin and codeine. Following parenteral administration, morphine appears rapidly in saliva. Cone [48] reported an approximate

45-minute delay in equilibration of morphine concentrations in saliva compared to plasma following intramuscular administration of 10- and 20-mg doses; thereafter, saliva concentrations paralleled plasma concentrations. Morphine can be detected in oral fluid following intravenous administration, the smoking of heroin and poppy seed ingestion. Positive tests for morphine and codeine (with higher codeine concentration) implies codeine use.

CODEINE: a naturally occurring phenanthrene alkaloid and opioid agonist. It appears to be most commonly taken orally. While it is not a metabolite of morphine, it is metabolised by oxidation to morphine and norcodeine and by conjugation. Kim et al. [52] demonstrated that following oral administration of 60 and 120 mg, codeine appeared in oral fluid within an hour and reached maximum concentration in approximately 1.6–1.7 hours. Concentrations in oral fluid correlated significantly with plasma concentration and were three to four times higher in oral fluid than plasma. Codeine could be detected in oral fluid for approximately 21 and 7 hours at cut-off concentrations of 2.5 and 40 ng/mL, respectively. Following intramuscular codeine of 60 and 120 mg, codeine appeared rapidly in oral fluid and reached maximal concentrations in 0.5–0.75 hours. A positive test result for codeine and morphine generally indicates codeine use.

METHADONE: a synthetic opioid used widely as an analgesic as well as in maintenance therapy for persons with opioid dependency. Methadone undergoes extensive metabolism in the liver to form cyclic metabolites, 2-ethylidene-1,5-dimethyl-3, 3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrrolidine (EMDP), and other minor metabolites. Methadone and EDDP appear rapidly in oral fluid and correlate with plasma concentrations. Therefore, the confirmed presence of oxidative metabolites such as EDDP and EMDP in oral fluid would be useful to substantiate use.

BUPRENORPHINE: an orally available, semisynthetic opioid analgesic, used as a pain reliever and in the management of opioid dependence. Following sublingual administration, buprenorphine reaches maximal plasma concentrations in 1.3–1.6 hours. Its main metabolite is norbuprenorphine. Cone [48] reported measurements of buprenorphine in saliva following intramuscular and sublingual administration of single doses of buprenorphine. Drug concentrations in saliva were substantially lower than plasma at all times following intramuscular administration and were substantially higher following sublingual administration. The low S/P ratio following intramuscular administration is probably due to the high fraction of drug that is protein-bound in plasma. Close correspondence between saliva and plasma buprenorphine concentrations was observed in subjects who administered buprenorphine sublingually on a daily or every-other-day basis. If the oral fluid test reveals buprenorphine \leq norbuprenorphine, this suggests chronic buprenorphine use.

4.3 Quality assurance of the analysis

Drug testing laboratories must implement a quality management system that includes all aspects of the testing process, such as sample reception, chain of custody, safety and reporting of results, screening and confirmation tests, certification of calibrators and controls, and validation of analytical procedures.

Hence, the laboratory should remain constantly updated on the evolution of analytical techniques, whether for finding new drugs or responding to requests for investigations concerning narcotic and pharmacological drugs. A highly qualified analytical chemical-toxicological laboratory depends on ISO/IEC 17025 [53] accreditation standards and procedures to demonstrate that it operates competently and generates valid results. These standards are agreed by experts the world over,

thus promoting confidence in the work of accredited laboratories and other bodies, on national and international levels, and facilitating cooperation between them. With ISO certification, results are more widely accepted between countries without the need for further testing, consequently improving international trade.

Of course, the benefits of advanced equipment in the chemical-toxicological laboratory go hand in hand with the expertise of qualified personnel with specific competence and adequate scientific training (not restricted to the analytical chemical field). Only through this combination (state-of-the-art tools plus qualified personnel) will it be possible to develop new analytical methodologies in the field of toxicological-forensic analysis and respond to administrative, criminal and social needs imposed by the legal system.

4.3.1 Validation of an analytical method for the detection of drugs in saliva

The validation of analytical methods includes procedures designed to establish that a particular method, used for the identification and/or quantification of an analyte in a given biological matrix, is reliable and reproducible. It is a question of demonstrating that the performance characteristics of the method meet all the requirements for its intended purpose and application. Any analysis methodology used routinely by the laboratory must be previously validated according to internationally agreed procedures [17].

For the most commonly used screening tests, validation procedures are not usually necessary as the method is validated by the manufacturer. In any case, the analysis kit includes calibrators and controls which are to be inserted into each batch of samples to be analysed in order to verify the accuracy and precision of the analyses (according to predetermined target values). In the event that changes are introduced which deviate from the manufacturer's instructions (for example, the biological matrix used is not the one indicated by the manufacturer, variation of the quantification limit, etc.) the laboratory must carry out a complete validation of the method/modified kit. It is best to fully respect the instructions provided by the manufacturer in the use of a kit, or in any case modifications should only be carried out in cases where it is not possible to use other methods.

The analysis methodology can be used routinely by the laboratory if the calculated validation parameters fall within the limits established by the relevant international directives [54].

The use of a good internal quality programme guarantees the reliability of the analytical results and avoids any random errors that may occur in the analytical and/or pre- or post-analytical phase that may affect the accuracy of the result.

The laboratory must participate in appropriate external quality assessment programmes. Analytical performances outside the criteria established by the External Quality Assessment (EQA) programme must be promptly corrected. The choice of one programme over another must be made on the basis of the best scientific evidence obtainable. Participation may concern the identification of classes of substances or individual substances and quantification in the case of confirmatory analyses according to the legal cut-offs or established by the management body of the programme.

In the case of screening tests, the expression of the results is generally in terms of "positive" or "negative". In the case of confirmatory analysis, it is necessary to provide not only qualitative but also quantitative data, namely the concentration detected according to a given calibration curve for the analyte identified in the saliva sample. The results of participation in the EQA are useful for the laboratory director and staff in helping to gauge the performance of the laboratory. In the event of errors, it is important to identify the causes and implement corrective actions that prevent them from recurring.

4.3.2 The activity of the toxicology laboratory for forensic purposes

When the presence of drugs is confirmed in oral fluid, the person under investigation may request the counter-analysis of another aliquot (B) of the saliva sample. This second test can be performed at the same laboratory that analysed the first aliquot (A) of the saliva sample or at another laboratory chosen by the subject in question. Aliquot B must be accompanied by a chain of custody form and include information regarding the results of the original analysis and the cut-offs used. Any laboratory that conducts analysis on aliquot B of the saliva sample must have documentation to demonstrate the use of validated analysis methodologies that meet the precision and accuracy criteria appropriate to the required analyses. It is crucial to guarantee the chain of custody [55], a documented procedure designed to ensure the authenticity, integrity and traceability of a sample from the moment of collection to its disposal. Following the proper chain of custody protocol is fundamental in the reconstruction process and ensures that the sample can be located at any point, unequivocally identified, stored correctly under the right conditions, and protected from tampering and voluntary or involuntary adulterations in all phases. Documentation of the chain of custody must also record every movement and manipulation of the sample, on which dates and under whose care. In the judicial field, the chain of custody is deemed broken in any of the following scenarios (these shortcomings will lead to dispute and may even constitute instances of mystification):

- Missing or non-identical barcodes.
- Missing documentation (supposed to be attached).
- Absence of the informed consent of the person subjected to analytical assessment.
- Broken or tampered safety seals on sample containers or transport container.
- Absence of security seals.
- Insufficient sample volume for testing.
- Containers not intact and evident loss of sample.

On the basis of what has been said thus far, it is evident that laboratory staff are required to fulfil many responsibilities, with potential repercussions in the forensic field in the event of proven professional malpractice. It is therefore necessary to:

- define the type of services that can be provided (screening analysis and confirmation analysis), the suitability of resources and the guaranteed level of safety and reliability;
- ensure the availability of sufficient, adequately trained staff with the necessary experience to monitor and conduct the required laboratory tests (specifically, the analysis of substances of abuse on saliva samples);
- assure the competence of laboratory staff, document in-service training, validate the analytical method, and re-evaluate work performance;

- provide the personnel of the laboratory with access to the complete, updated Standard Operating Procedures (SOP) manual;
- maintain an internal quality control programme which ensures that the analyses are performed correctly and that the results of the tests are communicated in compliance with SOPs;
- participate in appropriate External Quality Assessment (EQA) schemes;
- maintain acceptable analytical performance for all analysis methodologies applied in the laboratory;
- guarantee and document the validity, reliability, accuracy, precision and performance characteristics of each analysis and each analysis system;
- ensure that the necessary corrective actions are taken to maintain laboratory operation and performance at satisfactory levels (e.g. when a quality control system indicates non-compliance with performance specifications, or in response to errors in reporting results or in the analysis of the results of an EQA); the analytical results must not be reported until all the appropriate corrective actions have been taken.

In this context, the forensic toxicologist clearly has a fundamental role, with the responsibility of interpreting the analytical results of tests for substances of abuse in oral fluid at the request of a Legal Authority, competent doctor, potential customer or designated expert representative.

5. Conclusion

Oral fluid testing for drugs of abuse offers significant advantages. A saliva sample can be collected under direct observation with reduced risk of adulteration and substitution and in a less embarrassing or unpleasant manner than urine or blood collection. As oral fluid collection is non-invasive, most people find the procedure more acceptable than having to provide other biological matrices, and suitable hygiene conditions can be respected while the donor is under the collector's observation.

By providing an estimate of the actual circulating amount, the measurement of a drug concentration in oral fluid can be used for the determination of intoxication. In fact, measurements of oral fluid drug concentrations will usually be of value only if they accurately reflect the plasma level. Therefore, before designing a useful model for the salivary secretion of drugs, it is necessary to constantly update information about the relationship between the saliva concentration level of each drug and its plasma concentration level, the mechanisms by which drugs enter oral fluid, and also the effect on salivary flow rate, production in the salivary glands, and the nature of any protein binding in the saliva.

It is very useful to know the limitations and possibilities of salivary analysis in forensic and diagnostic fields. Standardisation of the conditions for collection of oral fluid is strictly essential for achieving reliability and interpretation of the data. Furthermore, appropriate cut-off concentrations need to be established in the development of guidelines for oral fluid testing [56]. In future research, the mechanisms by which drugs enter the saliva must be clarified more adequately.

These considerations are matters of ongoing discussion in the scientific community, in particular the proposed initial screening and confirmatory cut-offs.

When an oral fluid test is performed on a corpse, the forensic pathologist must be accompanied by a toxicologist for the interpretation of the analytical data. It should also be noted that significant ethical issues are involved in the study of many licit and illicit drugs that preclude or limit the study of their short- and long-term effects under “real-world use” conditions, which means that some knowledge will always remain inaccessible.

Finally, there are some open questions and limitations to consider in salivary analysis for forensic purposes. Despite a substantial number of clinical studies on drug disposition in oral fluid, many psychoactive drugs have not been studied. Benzodiazepines and barbiturates and some opioid products have received limited or no evaluation in oral fluid; meanwhile, there is a lack of controlled dosing studies of hallucinogens in humans. Furthermore, the dynamic nature of oral fluid, especially its pH, can substantially affect drug concentrations of basic drugs. It follows that, to date, in a forensic context, the result of an oral fluid test remains questionable, not only for reasons strictly connected to pharmacokinetic and metabolic characteristics, but also for purely analytical reasons:

- a. difficulty in applying standardised procedures for sampling;
- b. frequent smallness of the sample compared to conventional matrices (e.g. blood) with consequent limitations in terms of multiclass analyses and sampling for counter-analyses;
- c. variability of the relationship between salivary and blood concentrations as a function of the variability of salivary pH;
- d. possibility of contamination of the oral cavity after ingestion of a substance via intranasal use or inhalation.
- e. laboratory deficiencies and/or incorrect application of the analytical procedures.

Therefore, salivary analysis for forensic purposes, now and in the future, necessarily requires a union between highly qualified personnel (able to apply analytical methods and interpret results in the light of up-to-date scientific knowledge) and toxicological laboratories equipped with state-of-the-art instrumentation.

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Author details

Roberto Scandoni
Department of Law, Institute of Legal Medicine, University of Macerata, Macerata,
Italy

*Address all correspondence to: r.scandoni@unimc.it

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