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Oral fluid for workplace drug testing: Laboratory implementation

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As oral fluid increases in popularity for workplace testing, due to its easy and observed collection, the ability to adapt existing laboratory instrumentation without further capital investment will allow more facilities to test oral fluid. The European Workplace Drug Testing Society (EWDTS) guidelines for oral fluid testing outline the maximum cut-off concentrations acceptable under the workplace drug testing programme. The recommended cut-off values may be subject to change as advances in technology or other considerations warrant identification of these substances at different concentrations; however, the instrumentation currently exists for routine screening using immunoassay and confirmation by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography with tandem mass spectral detection (LC-MS/MS) so laboratories can easily implement oral fluid analysis in their current systems. Immunoassays for the detection of the drug classes at recommended levels have been developed using various collection devices and different formats: liquid reagent chemistries and enzyme-linked immunosorbent assay (ELISA) platforms. Immunoassays provide faster turnaround than mass spectral methods particularly when the number of specimens increases. Since the guidelines state that positive immunoassay results should not be reported without confirmation, fully validated methods using LC-MS/MS and/or GC-MS for all drugs are also widely available. All proposed concentrations are easily achievable using MS instruments currently in testing laboratories; however, the likelihood of a low number of positive specimens in workplace populations allows the test facility to screen specimens in a cost-effective manner using immunoassay, while ensuring scientific credibility and defensibility by confirming the positive results with a second test.

Keywords: Oral fluid; Workplace drug testing; Laboratory implementation

Introduction

Workplace drug testing is intended to deter employees from using drugs and/or alcohol at work; or from being under their influence during work hours in order to reduce accidents and promote a safe environment for all. The vision of various scientific organizations including the European Workplace Drug Testing Society (EWDTS) to propose testing guidelines for alternative matrices to urine has promoted the development of immunoassays and mass spectral methods targeted at specific drug classes and concentrations in oral fluid. In January 2011, the Quest Diagnostics Drug Testing Index reported that the annual positivity rate in the general US workforce population for the months between January and June 2010 was 4.2% for urine drug tests and 4.4% for oral fluid. The report noted that oral fluid was a significantly better matrix for the detection of heroin use (0.91%) compared to urine 0.18%. Marijuana accounted for 2.1% of the positives; cocaine for 0.23%; while oral fluid analysis produced slightly higher positive rates with 2.9% for marijuana and 0.47% for cocaine. However, the striking discrepancy between routine workplace testing and actual numbers of drug users becomes apparent when various research studies are considered. For example, the Substance Abuse and Mental Health Services Administration (SAMHSA) report that nearly 7% of employed adults currently use illegal drugs – almost double the detection rate. The 2007 National Roadside Survey provided the first nationally representative estimate of the prevalence of drugs in drivers in the USA using oral fluid collected with the Quantisal™ device as a test matrix. The drivers were stopped randomly and most importantly not 'for cause'. Overall, 16.3% of drivers tested positive for drugs, with just over half of those showing the presence of marijuana (8.6%); cocaine was the second most prevalent drug detected in 3.9% of cases. So, the gap between a standard workplace drug test and the real numbers of drug users in the workplace seems destined to grow unless changes to the current programmes are implemented.

Oral fluid is being increasingly used in many areas of drug analysis, particularly random testing, due to its ease of collection, difficulty of adulteration, and reflection of recent drug intake.[1–3] From a laboratory perspective, the addition of oral fluid to an already existing urine panel may seem daunting, particularly in light of much lower drug concentrations and the difficulty of sample manipulation. However, in recent years, both immunoassay and mass spectrometry(MS) manufacturers have developed assays for the analysis of drugs in oral fluid which are easily implemented into routine testing.[4] There are numerous publications on the analysis of oral fluid for drugs, for both immunoassays[5,6] and gas chromatography-mass spectrometry (GC-MS);[7] however the utility of liquid chromatography with tandem mass spectral detection (LC-MS/MS) is increasing.[8,9]

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Screening procedures

Immunoassays

The initial screening of biological specimens with immunoassays allows an inexpensive manner in which negative samples can be identified and discarded. The inclusion of an immunoassay screening process becomes beneficial, operationally and financially, when (1) sample volume reaches a level where LC-MS/MS systems are overloaded by shear number of tests; and/or (2) the expected number of negative specimens is high (for example, workplace drug testing, after-school programmes).

For years, workplace drug screening using urine has been carried out with immunoassay followed by confirmation with GC-MS or LC-MS/MS, and this model should not be different because the matrix may change. The dual confidence of a screen followed by confirmation using a technique based on a different chemical principle has been the root of forensic testing for decades and still remains a recommendation by the Society of Forensic Toxicologists and the National Safety Council in the USA.[10]

In the recent past, the required sensitivity for relevant oral fluid testing could only be achieved using enzyme-linked immunosorbent assay (ELISA) formats which while providing a wide range of sensitive assays were also somewhat slow, relative to liquid reagent chemistry analyzers using screens for urine, such as enzyme multiplied immunoassay technique (EMIT), cloned enzyme donor immunoassays (CEDIA), or kinetic interaction of micro-particles in solution (KIMS).

Now there are numerous chemistry analyzers which can be used for the analysis of oral fluid, as long as they have the capability of reading at 340 nm, adding two reagents, and maintaining a constant temperature of 37 °C.

Several manufacturers have 'ready to use' liquid reagent chemistries for oral fluid using different collection devices. All have advantages and disadvantages associated with the collection device itself, laboratory operations in the test facility, and/or immunoassay performance, but the range of oral fluid applications continues to increase.

As with all techniques, there are certain disadvantages to immunoassay testing. As a general rule, immunoassays operate in qualitative mode, giving a positive/negative result rather than a quantitative value; secondly, the degree of cross-reactivity with other structurally related compounds is likely to be different between manufacturers depending on the design of the assay. Thirdly, interferences from either related or non-drug-related substances can cause positive results which subsequently do not confirm.[11,12] While most publications have studied these interactions in urine or blood, it can be inferred that oral fluid would have the same limitations. Immunoassays should always be considered as a preliminary test, and any positive result must be confirmed by a secondary analysis based on a separate chemical principle.

The implementation of immunoassays in an existing urine testing facility should be seamless; however, there are critical points which need to be observed:

1. Since oral fluid concentrations are so much lower than urine levels, chemistry analyzers must be kept clean to allow the span of the assay to be consistent. It is preferable, although not essential, that a separate instrument be used for oral fluid.
2. Most immunoassays require the negative or 'blank' to be run using either a synthetic matrix (usually supplied with the kit) or the transportation buffer present in the collection device, since the presence of preservatives and other anti-microbial agents may cause fluctuation if water or neat oral fluid is used as the negative. Similarly, the use of calibrators and/or controls prepared in one matrix followed by sample dilution with a different buffer is not recommended, since the assay spans will be affected.
3. Both calibration- and test-specific parameters should be set under the same 'matrix type'. Since there is usually an option of urine/serum/other, both measurements should be the same. Daily calibration is also recommended.

In general, commercially available immunoassays operate with a high degree of precision and control differentiation. The availability of screening assays targeted at the recommended cut-off concentrations for chemistry analyzers in particular will help urine laboratories move efficiently into oral fluid testing.

Mass spectrometry

Some facilities use GC-MS, or more likely LC-MS/MS as a screening tool. This approach has certain advantages, in that many compounds can be effectively 'screened' with one injection; however, the converse issue is that such screening procedures limit optimization of specific drugs, and in many cases the sensitivity required may be compromised.

There are already published validated procedures for many of the drug classes recommended in the EWDTS guidelines using LC-MS/MS procedures at the cut-off concentrations (Table 1), so implementation of these regulations should be straightforward.[13]

Gunnar *et al*.[7] used GC-MS to screen 30 drugs of abuse in 0.25 ml of expectorated oral fluid using sequential elution from solid-phase extraction (SPE) columns and separate derivatization methods. The authors note that 'GC-MS is a convenient choice over LC-MS/MS because of the lower purchasing and maintenance costs, improved separation efficiency, typically sharper and more symmetrical peak shapes and the absence of matrix-dependent ion suppression.' This last point is important for laboratories considering the injection of either neat expectorated oral fluid, diluted oral fluid, or oral fluid collected in buffer directly into an LC-MS/MS system.

LC-MS/MS is being increasingly used in testing laboratories due to little or no sample preparation, short run-times, and no derivatization compared to GC-MS; and several papers have reviewed the advantage and disadvantages associated with oral fluid analysis.[14–16] Preservatives, anti-microbial agents, and stabilizing compounds are likely to be present in a collection device buffer, which have definite consequences when considering direct injection, so sample preparation is generally required.[17] Sensitivity may also be compromised if drugs are not extracted and concentrated prior to analysis, especially for drugs which are likely to be very low concentration in oral fluid (e.g. benzodiazepines, barbiturates, morphine). However, Concheiro *et al*.[18] published an LC-MS/MS method using only 0.5 ml of oral fluid for the determination of morphine, codeine, 6-AM, methadone, amphetamine, methamphetamine, MDMA, MDA, MDEA, benzoylecgonine, cocaine, delta-9-THC, diphenhydramine, amitriptyline, zolpidem, zopiclone, as well as the benzodiazepines: alprazolam, clonazepam, oxazepam, nordiazepam, lorazepam, flunitrazepam, and diazepam in one specimen.

Allen *et al*.[19] reported on an LC-MS/MS procedure to replace immunoassay in routine measurement of drugs in oral fluid, but their method did not include amphetamines or tetrahydrocannabi-

Table 1. EWDTS recommended maximum cut-off concentrations for screening and confirmation in neat oral fluid

Drug	Screen (ng/mL)	Drug	Confirmation (ng/mL)
Amphetamine group	40	Amphetamine; MDMA; Methamphetamine; Other	30
Benzodiazepines	10	Temazepam; Oxazepam; Desmethyldiazepam; Other	10
Cannabis (THC)	10	Cannabis (THC)	2
Cocaine metabolites	30	Cocaine metabolite	8
Opiates (Morphine)	40	Morphine; Codeine; Dihydrocodeine	40
6-acetylmorphine	4	6-acetylmorphine	4
Methadone or metabolites	50	Methadone or metabolites	20
Buprenorphine or metabolites	5	Buprenorphine or metabolites	5
Propoxyphene or metabolites	40	Propoxyphene or metabolites	40

Note:
1. These recommended cut-off values may be subject to changes as advances in technology or other considerations warrant identification of these substances at other concentrations.
2. Cut-off levels for substances not indicated need to be agreed with the customer taking into account the performance of the assays to be used.
3. Dilution of the sample has to be corrected for when the screen results are interpreted.

nol (THC) which are obviously of great interest in the workplace population.

In another paper by the Allen group,[20] it was shown that there may be some problems in routine work with opioids and opioid metabolites having similar retention times as well as the same MS/MS transitions; Coles *et al*.[21] showed several advantages of using LC-MS/MS including sensitivity, removal of hydrolysis steps for urine, shorter runtimes, and faster instrument maintenance compared to GC-MS, but also noted some increases in concentration due to the presence of other opiates:

> 'Oxymorphone elevated the morphine concentration (to15ng/ml), and norcodeine elevated the hydromorphone concentration (to 60 ng/ml). This observation illustrated the importance of using secondary transitions, even when analyzing samples in MRM mode. Despite multiple adjustments to the LC analysis, these compounds still co-eluted with the opiate analytes. Expected transitions for morphine and hydromorphone were still present in high amounts of oxymorphone and norcodeine, so it was impossible to choose a single transition that would not be affected. Instead secondary transitions were chosen and ion mass ratios (IMR) between the primary and secondary transitions were established so that in the presence of high oxymorphone or norcodeine, the IMR would be out of range. Even though the presence of high amounts of oxymorphone and norcodeine would falsely elevate the concentration of morphine and hydromorphone, respectively, the IMR would be out of range and an indeterminate result would be reported instead of a false positive. . ..'

Sauvage *et al*.[22] pointed out several potentially false-positive observations when running LC-MS/MS in terms of drugs sharing the same transitions, specifically clomipramine and some phenothiazines; benzoylecgonine transitions similar to atropine; and a metabolite of zolpidem which may have been mistaken for LSD. While some of these were separated on the basis of retention time, the authors recommend other approaches to help prevent similar problems, specifically:

> '[. . .] the use of stable-isotope internal standards when possible, relative retention times, 2 transitions or more per compound when possible, and acceptable relative abundance ratios between transitions, with an experience-based tolerance of +−15% for transitions with a relative abundance >10% and with an extension to +−25% for transitions <10% when the concentration is at the limit of quantification. . . . . .Our results indicate that the specificity of screening procedures is questionable for LC-MS/MS analyses performed in the selected reaction–monitoring mode and involving a large number of compounds with only 1 transition per compound.'

In 2010, Vogeser and Seger published a comprehensive report on various areas where problems may arise in LC-MS/MS screening and stated that disadvantages to the technique must be recognised and addressed.[23] They discussed inaccuracies related to the ionization process, specifically matrix effects having a different impact on each analyte (ion enhancements or suppression). The necessity of using deuterated or carbon labelled internal standards; the need for matrix matched calibration standards and controls; the likelihood of isobaric compounds being present in the specimens; potential issue with 'cross-talk'; and the importance of good chromatography are all covered in the paper. The authors note that: 'Indeed, because of the lack of automation in LC-MS/MS, the risk of (such) human-related gross errors can be assumed to be greater than that associated with the use of automated clinical chemistry analyzers. . .'

In specifically discussing the analysis of drugs in saliva, Mortier *et al*.[24] address the issue of ion suppression, even in a relatively clean sample matrix, such as saliva. They state that the analysis of the specimen with corresponding deuterated internal standards is a viable approach for one compound and its metabolites, but for multiple drug analysis the number of isotopically labelled standards which may be necessary is then a limiting factor. The presence of unexpectedly large quantities of interfering substances can dramatically influence the degree of ionisation. The final conclusion states: 'The data presented once more prove the importance of an adequate sample preparation, even with LC/ESI-MS(/MS).'

So LC-MS/MS while having definite advantages also has some pitfalls in routine use, and laboratories should be aware of possible interference and potential false positive results. A minimum of two transitions should be monitored during analysis and acceptable

criteria established for determination of positivity. All LC-MS/MS validation procedures should assess potential interference from structurally related drugs and metabolites which may produce the same transitional pathways. Extensive sample preparation is highly recommended, as is the incorporation of corresponding labelled internal standards for compounds of interest.

Other considerations

Since oral fluid has difficulties associated with analysis which do not occur with urine testing, laboratories should be aware of other areas where additional method validation may be necessary.

Collection and dilution

The predominant variables in the analysis of oral fluid are:

- Method of collection
- Volume of oral fluid collected
- Amount of diluent to which neat oral fluid is then subjected

There are numerous commercially available collection devices, most of which use some form of pad which is placed into the mouth for sample collection. Examples include the Quantisal™, Intercept®, Salivette®, and StatSure Saliva Sampler™; whereas the Greiner Bio-One saliva collection system calls for a non-toxic extraction solution to be kept in the mouth for about 2 min, then emptied out. The quantity of collected oral fluid in the extracted saliva solution is determined by photometric quantification of the non-toxic dye in the extraction solution.

For a collection device with a pad, both the volume of saliva collected and the amount of drug recovered from the pad are critical to achieving a valid quantitative result. Publications are increasingly reporting the efficiency of saliva collection and drug recovery; but there are two important caveats to testing recovery:

1. The pad must remain in the transportation buffer long enough for the drugs to be removed efficiently; in most cases, a minimum of 4 h should be allowed before analysis; in many publications the drugs are allowed to remain overnight to simulate transportation to a test facility. Failure to allow the drugs time to desorb from the pad gives inaccurate results for drug recovery
2. The concentration at which the recovery is studied should be relevant to either a proposed cut-off or a likely concentration in authentic specimens; overloading the pad will cause low drug recoveries.

An additional advantage to using a system which incorporates a transportation buffer rather than the collection of expectorated oral fluid is that the stability of the drugs is enhanced due to the preservatives present in the buffer.

While there may be publications on the recovery of specific drug classes, and often there is information available from the manufacturer of the device, the test facility may wish to determine the recovery 'in-house' using their procedure as part of method validation. Several approaches for this purpose have been published, including gravimetric analysis of the collection device,[25] or direct comparison of drug not passed through the collector to drug which has been subjected to the pad/device.[26]

The volume of oral fluid is obviously less than is usually available for blood or urine analysis, so techniques which use very small amounts of specimen while still retaining sensitivity are paramount. Immunoassays use microlitres of diluted specimen, and subsequent decisions on how large an aliquot may be used for confirmation can be based on the extent of inhibition (degree of positivity).

In order to preserve the limited sample volume, other methods may include multi-analyte methods (such as single injection LC-MS/MS); however, a simple 'dilute and shoot' approach for oral fluid specimens collected into transportation buffers will cause significant matrix effects due to the presence of surfactants, preservatives, and anti-microbial components. Direct injection of such buffers into an LC-MS/MS system over time will cause chromatographic and ionization problems, so some form of sample preparation is highly recommended. For oral fluid then, in contrast to urinalysis, solid-phase or liquid-liquid methods which encompass the extraction of multiple drugs should be considered prior to LC-MS/MS for either screening or confirmation.

Matrix matched controls

In many publications, synthetic oral fluid is used for calibration standards and controls, generally because of the lack of a homogeneous authentic oral fluid source. It is advantageous then to select a collection device with a transportation/storage buffer, since any matrix effects from real oral fluid are considerably diluted, minimizing immunoassay fluctuation and ion suppression in LC-MS/MS.

Proficiency programmes

The type of collection device used has implications for proficiency testing. In external programmes, it is not practical to provide each laboratory facility with proficiency specimens collected in the device they use; in fact some testing facilities have validated procedures for multiple collection devices based on their clientele. Therefore, it may be more efficient for an external proficiency programme to provide drugs in neat oral fluid, with the requirement that the laboratory dilute the specimen with the buffer from a specific device prior to analysis. In this way, performance between laboratories can be monitored and compared.

During the European DRUID project (Driving under the influence of Drugs, Alcohol, and Medicines), four rounds of oral fluid proficiency testing were organized between March 2008 and September 2009 and 11 laboratories participated. Taking into account that many of the laboratories had not previously analyzed oral fluid, the results showed significant improvement as the facilities gained testing experience. In the final round, very low rates of false negative (0.9%) and false positives (0.4%) were reported.[27] In addition, the UK offers an oral fluid proficiency testing scheme, UKNEQAS; and an oral fluid program from Italy, OralVeq, has been available for several years.[28]

All reports from proficiency programmes show that experience in testing is the key to excellent analytical performance. Since all the physical collection devices, assays, and instrumentation required are now widely available, proficient oral fluid analysis should be achievable by testing laboratories.

Storage

In general, THC is the least stable analyte, being sensitive to fluorescent light as well as temperature;[29] however, even cocaine will degrade to benzoylecgonine over time and 6-acetylmorphine

(6-AM) may convert to morphine. Even in commercially available specimen collectors, Ventura *et al*.[30] showed oral fluid devices containing 600 ng/ml of 6-AM, cocaine; and 240 ng/ml of THC, carboxy-THC (THC-COOH) showed losses following transportation to test facilities. On average, 9–12% of the 6-AM was converted to morphine and 26–41% of the cocaine degraded to benzoylecgonine. Good recoveries were observed for the THC-COOH in the devices, but THC recovery was poor.

The EWDTS guidelines recommend that specimens not currently undergoing testing (short-term storage) may be kept refrigerated prior to analysis; once analysis is complete, specimens must be stored frozen (long-term storage).

Summary

Since most laboratories already have confirmation equipment (GC-MS or LC-MS/MS), the additional availability of an immunoassay provides options for a laboratory operation. As oral fluid is increasingly the matrix of choice for workplace situations, the use of small aliquots from limited specimen volume, cost-efficient screening and confirmatory options for the laboratory are also expanding.

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