13. Drug Testing

Drug tests may simply detect or actually measure the concentrations of drugs in blood, serum or urine. Drugs in tissues, hair and CSF are also assayed. The tests can be broadly categorized into two groups qualitative and quantitative. There are far too many tests and testing methods to cover so we will just look at a few of the major types. Three major issues that crop up are does the assay accurately identify a drug, drug quantitation and when and where was the sample taken relative to the exposure period. Testing in urine is very common however the lag from dose to significant quantities in urine is substantial and variable.

![Drug Detection Times in Different Matrices](image)

**Major Reasons for Testing**

1. **TDM (Therapeutic Drug Monitoring).** Here the levels of a drug in blood are determined to guide further dosing (Dixogin, Busulfan, Vancomycin). Levels are usually determined by a clinical laboratory which also may provide an estimate of a dose change based on exposure. Urine testing is rare in TDM.
Example

See BUAUC on the mayo site. Note the following things. (1) Regimented drug administration (2 hr iv infusion followed by 4 serum data points. (2) Brief discussion of overall test [http://www.mayomedicallaboratories.com/articles/hottopics/2009-6a-busulfan.html](http://www.mayomedicallaboratories.com/articles/hottopics/2009-6a-busulfan.html) 3. A quick read of the Clinical/Interpretive Tab. (4) The means of quantification under performance tab (liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS is probably the most sensitive and specific method of identification and quantitation of a drug. We will look at some others later on.

The Mayo site has gathered all their TDM’s under one heading on this page. Also see section on drugs of abuse testing.


2. **POC Drug Screening:** These are screening studies that focus on identification of drugs, particularly drugs of abuse. Screening for metabolites also occurs and metabolites and drugs may interfere with each other. These tests are typically qualitative, have a fairly high threshold for detection and usually test for drugs in urine. Used in various types of clinics and ERs.
3. **Screening in the Workplace:** Usually urine or saliva. “Last year, U.S. workers peed into one drug testing company’s cups (Quest Diagnostics) about 9.1 million times”. 3.8% were positive in government mandated testing. Marijuana (0.71%), Amphetamines (0.54%) Cocaine (0.25%) Opiates (0.18%). A negative test is considered final. A positive test is usually followed by a laboratory assessment.

4. **Home Screening:** Widely available. Customer counseling may be important in some cases around use, false positives, false neg etc. Most offer back-up clinical testing (for a fee) with positive results. These tests rarely test for a single compound.

In general screening tests a qualitative and do not provide absolute confirmation of the structure. Rather they tend to be responsive to classes of drugs and their metabolites. Almost always, structural assignments must be confirmed in clinical laboratories that employ more sophisticated test platforms, primarily chromatography coupled with mass spectrometry or other specific detection instruments. These
qualitative tests may provide some relative concentration data however for the most part they have a threshold of detection. Threshold of detection varies with type of test, test manufacturer and type of drug detected.

The three most common testing environments are clinics, home and workplace.

**Quantitative Testing** is generally done in a clinical laboratory.

(1) These labs verify structure by various test procedures.

(2) These labs also determine the concentration of a drug by comparison with authentic sample and standard curves.

**A. Chromatographic Methods**

1. **TLC Thin Layer Chromatography**

Drugs extracted from urine or plasma are applied to the bottom of the TLC plate that is coated with cellulose, silica or aluminum oxide. A solvent moves up the plate by capillary action and the drugs move up the plate along with standards in other lanes. Each compound moves different distances ($R_f$ value) during solvent development. Visualization by spray reagents or UV-Vis Lamps may provide additional confirmation. Not used much any more.

**Disadvantages**

1. Chromatographic separation is modest and standards are required.
2. Not quantitative
3. Drug identity usually not confirmed even with a standard.
2. **GC Gas Chromatography**

The sample, after extraction (usually also derivatization), is subjected to gas chromatography using solid phase or capillary columns. Very common in labs for screening of urine and plasma as well as for quantitation of drugs. There are standard sample work-up conditions prior to chromatography. Standards must be run in a separate run so comparison of retention times is the primary method of identification. The compounds are usually detected by flame ionization or electrochemical detectors.

**Advantages**

1. Good separation of components so drug ID is better based on retention time.
2. Quantitative amenable to standard curves.
3. Good to very good sensitivity and accuracy.
4. Sample work up and processing can be automated but not as readily as HPLC.
5. Can be coupled with a mass spectrometer for better detection (Major Mass Spec).
6. Great for Screening Studies particularly where a drug class is suspected.

**Disadvantages**

1. Does not confirm drug structure.
2. Interference by endogenous compounds like fatty acids.
3. Restricted to certain types of drugs. Many large polar drugs and all biologics are not volatile and do not chromatograph.
**HPLC High Pressure Liquid Chromatography**

The sample is subjected to liquid chromatography using columns packed with micro-particulate materials that are silica based. The column eluent is assayed by UV-Vis or fluorescence spectrophotometry. Great for screening studies. Different columns types and solvents can be used to firm up structure assignments

**Advantages**

1. Sample work up and processing can be readily automated.
2. Good separation of components so drug ID based on retention time is good but not definitive.
3. Quantitative with many alternative column packing materials and solvent systems.
4. Good sensitivity and accuracy
5. Large polar drugs and biologics will chromatograph.
6. Can be coupled with a mass spectrometer for better detection.

**Disadvantages**

1. Does not confirm drug structure except by retention time.
2. Long run-times required for good separation.
3. Lots of interfering peaks due to contaminants from the biological samples.
4. **MS** Mass Spectrometric Detection

Here the analytes from the GC or HPLC column separation are directly transferred to a mass spectrometer as they appear off the end of the column. The analytes are ionized in the source and the ions ejected in a focused beam into a mass analyzer. This technique provides the molecular weight of the analyte. These hybrid instruments are referred to as LC/MS and GC/MS respectively. Mass spectrometric detection provides much improved selectivity and sensitivity for analytes. Each compound has its own “finger print” of ion fragments. This method allows from separate identification of quantitation of compounds that co-chromatograph. Hybrid multi sector instruments (LC/MS/MS) are even more sensitive and selective.

![Advanced Ceramic Components in Mass Spectrometers](image)

**Advantages**

1. Provide the molecular weight of the analyte and high sensitivity and accuracy.
2. The mass spectrometers can detect multiple compounds in what would be a single chromatographic peak.
3. Provides signature, compound specific, fragmentation patterns as the high energy ions in the gas phase decompose to smaller fragments to aid in the identity.
4. Very high sensitivity and selectivity virtually assured when linked to a chromatograph.
5. Shorter run times.
7. **Usually the gold-standard for identification and quantitation**

**Disadvantage:**

1. Cost and maintenance of the instruments.
Here we see a mass chromatogram of verapamil which shows the parent ion and diagnostic fragments which help confirm structure.

1. While many compounds might provide a mass of 455 amu the likelihood that it would also provide the same fragment fingerprint is very unlikely.
2. There are huge searchable data bases that contain fragment information for drugs, drug metabolites and other compounds including common biological contaminants like steroids, fatty acids, sugars, amino acids and hormones.
3. Very useful in screening.

B. Immunochemical Quantitative Methods (Blood)

The methods all require that an antibody has been created that recognizes the drug and a drug conjugated to another antibody, enzyme or protein. Thus the antibody selectivity
is reasonably good however metabolites of drugs and close isomers may give false positives and overestimates of amounts of the analyte of interest. There are a number of ways to detect the concentration of drug bound by the antibody. Some are direct while others are indirect. These types of assays are run for TDM.

1. RIA Radioimmunoassays are sensitive direct assays. RIAs are being replaced with other immunoassays that don’t require the use of radioactive isotopes. The radiolabeled drug is mixed with a selective antibody raised against the drug. The sample containing unlabeled drug (cold Ag in the diagram below) is added which causes a portion of the radiolabeled drug to be released to solution as the labeled and unlabeled drug compete for the antibody. The antibody is isolated by a variety of methods and assayed for radioactivity. In the diagram below the assay uses the addition of a secondary antibody to precipitate the complex. The reduction in radioactivity bound to the antibody in the final complex vs control assays is measured which provides the unlabeled drug concentration in the sample. The higher the drug concentration the lower the bound radioactivity signal.

**Advantage:**
1. Sensitive and very good for proteins and enzymes as well as drugs or drug metabolites.
2. Easy to train technician as all assays follow the same procedure.

Disadvantages:

1. Radioactivity and radioactivity bound to antibody must be completely separated from free radioactivity prior to scintillation counting of radioactive antibody complex.
2. As with all immunoassays, specificity not assured so this type of assay may under-estimate drug concentration.
3. Each assay is designed for a single compound so screening is not common.

2. EMIT (Enzyme Multiplied Immunoassay Technique; Siemens and others) This is a very popular, established and widely used technique. In clinical labs the assay is quantitative. The UW system runs quite a few EMIT assays in TDM and other applications.

Here an antibody is prepared that recognizes both the free drug and the drug covalently bound to an enzyme. The enzyme acts as the detector that converts a special substrate to a colored or fluorescent product whose concentration can be determined with a spectrophotometer. When the enzyme-drug conjugate is bound to the antibody the enzyme is inactive. The enzyme-drug conjugate and the free drug compete for the antibody. The higher the drug concentration the higher the concentration of active enzyme and the colored product is made at a higher rate giving a stronger signal. Since the enzyme can make many molecules of product it acts like a multiplier of binding events, unlike RIA.

**EMIT SET-UP**

![EMIT Set-Up Diagram]

- Free Drug in Sample
- Inactive Enzyme Antibody Complex
- Enzyme-Drug Conjugate Active
- Antibody Bound to Free Drug

**Increasing Drug Concentration**
- Shifts Equilibrium to the Right Which Increases Formation of P

**Drug Concentration**
Advantages

1. Fast, simple sensitive and easy to automate.
2. No Radioactivity
3. No chromatographic separation required
4. All reagents standardized and purchased.

Disadvantages

1. Specificity is not assured since any compound that binds to the antibody will elicit a response leading to an over-estimate of drug concentration. For instance metabolites might also bind to the antibody.
2. Expensive for screening and quantitation of multiple drugs.

3. **FPIA (Fluorescence Polarization Assay)** (ADx assay; Abbott)

This is also a competitive assay where an antibody is made that binds to the drug (Antigen) as well as the drug covalently bound to a fluorescent reporter molecule (FP Conjugate). Thus the final mixture contains FP Conjugate free in solution and FP Conjugate that is bound to the antibody. These two species have different fluorescent properties because the free FP conjugate rotates more rapidly in solution and gives a lower signal than the FP-conjugate bound to the antibody. Thus the higher the free drug concentration the lower the signal.

The advantages and disadvantages are the same as EMIT however FPIA is being replaced by EMIT as labs settle on a single assay method in quantitative immunochemistry assays to support TDM. The Mayo Clinic has completely moved to EMIT.

4. **KIMS (Kinetic Interaction of Microparticle in Solution; Roche Diagnostics)** (A similar test from Abbott is call Petinia)

This assay uses an antibody to the drug of interest that also binds to the drug covalently linked to a microparticle. When the antibody binds to the drug-linked microparticle the sample is turbid and absorbs light. When the free drug competes for the antibody the solution is less turbid and absorbs less light. The higher the drug concentration the higher the transmission of light through the sample (less turbid) giving a higher the
response. So the signal strength (light transmission) in KIMS is positively related to the drug concentration while absorption of light is inversely related.

**E. Qualitative Tests (POC and ER’s)**

Qualitative tests are focused on drugs of abuse and toxicants in urine, saliva, hair and serum. They are designed to screen for classes of drugs, individual drugs and drug metabolites. We will look at urine screening tests at home and simple point of care devices.

Most of these devices use a lateral flow immunochromatography. The principle here is similar to ELISA and Colloidal Gold tests but there are some differences. A short description follows

1) A specified aliquot of the sample that may contain the drug (free drug) to be tested for is added to the sample port.

2) The fluid travels across the conjugate pad that contains an antibody to the drug that is tagged with either colloidal gold or a colored latex bead.

3) The solution of this primary antibody conjugate and free drug then travel as a mixture until they reach the capture zone.
4) The capture zone has an immobilized drug that recognizes the primary drug antibody conjugate and will bind to it. This immobilized drug competes with free drug in the sample if present

a) Negative Test: When no drug is present the primary antibody conjugate binds to the immobilized drug conjugate and forms a colored Test Line in the capture zone.

b) Positive Test: When drug is present the free drug and immobilized drug compete for the primary antibody conjugate and no Test Line line appears in the capture zone.

5) The control line contains a secondary antibody that recognizes the primary antibody-drug conjugate which binds and forms the control line. This checks that the antibody-drug conjugate was active through the capture zone.

!!!!!! Note that the positive test for drug is counter-intuitive in that no line appears in the capture zone.

Below see that basic set-up for these POC devices.

In the examples below the appearance of a color is due to the fact that the primary antibody to the drug on the pad is also bound to a colored bead.
In some applications there are multiple primary antibodies on the pad that recognize different drugs and have separate capture zone for each one (See the Bio-Rad example).

Below left is a BioRad product that screens for amphetamine, barbiturates, benzodiazepines, cocaine, methamphetamine, methadone, opiates, PCP, TCAs and THC. Below right is an iScreen product that has been developed to show the results of a clean specimen for cocaine, 6-acetyl morphine, THC, Opioids. Urine is applied in the wells and sometimes chased with water. Each manufacturer sells these kits in multiple drug configurations. Some also have a zone for testing for urine that has been diluted or spiked with oxidants in an effort to evade detection.
Issues and Facts With Point of Care Drug Testing:

1) Results are qualitative and each system has different limits of sensitivity. There are standards here for POC devices.

2) Results often do not specify the drug or drug metabolite, only a particular drug class (eg Barbituates below)

3) Cross reactivity with drugs of another class is a problem and may lead to false positives. For instance amphetamine false positive due to chlorpromazine and Sudafed, opiates false positive due to poppy seeds and levofloxacin, and heroin (6-acetylmorphine) with methadone.

4) False Positives and Negatives for 3 test systems are shown below (n=80).

5) If necessary positive values must be confirmed by HPLC, GCMS or LCMS usually by a reference laboratory.

Table 2. Number of False-Positive (FP) and False-Negative (FN) Results in the Three Point-of-Care Tests in the 80 Urine Specimens

<table>
<thead>
<tr>
<th>Compound</th>
<th>TesTcard 9&lt;sup&gt;®&lt;/sup&gt;</th>
<th>Syva RapidTest d.a.u. 10&lt;sup&gt;®&lt;/sup&gt;</th>
<th>Triage TOX Drug Screen&lt;sup&gt;®&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamines/methamphetamine</td>
<td>FP 4</td>
<td>FP 2</td>
<td>FP 0</td>
</tr>
<tr>
<td>Opiates</td>
<td>FN 0</td>
<td>FN 0</td>
<td>FN 0</td>
</tr>
<tr>
<td>Methadone</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cocaine</td>
<td>FN 0</td>
<td>FN 0</td>
<td>FN 0</td>
</tr>
<tr>
<td>Cannabis</td>
<td>FN 0</td>
<td>FN 0</td>
<td>FN 0</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>FN 0</td>
<td>FN 0</td>
<td>FN 0</td>
</tr>
<tr>
<td>Barbiturates</td>
<td>FN 2</td>
<td>FN 0</td>
<td>FN 0</td>
</tr>
<tr>
<td>Tricyclic antidepressants</td>
<td>FN 2</td>
<td>FN 0</td>
<td>FN 1</td>
</tr>
</tbody>
</table>

6) For certain drugs of abuse these tests are designed to detect urinary metabolites which are present at much higher concentrations than the parent drug. For instance heroin is detected in urine a 6-acetylmorphine, cocaine is detected as the metabolite benzoylecgonine and morphine is detected as morphine-6-glucuronide.

F. Home Testing Kits

“The FDA regulates and reviews drugs of abuse tests before they can be sold to consumers or healthcare professional in the United States. In its review, the FDA evaluates the design and performance of tests and sample collection systems to help ensure that they produce accurate results. The FDA also reviews the test instructions and package inserts to help ensure that the end users can understand how to perform the tests easily and successfully.” The FDA site has some useful information.
The major drug chains carry home testing kits. They are also available online. Major brands are At Home, First Check, AccuChek and Teen Savers.

These kits work on the same principle as the point of care devices but when test strips are used instead of cassettes the test strips are dipped in cups with collected urine.

An AccuTest test strip. This strip set tests for the following drugs with low cut-off as shown. Just a 5 second dip will do it.

- Amphetamines - 1000 ng/ml
- Cocaine - 300 ng/ml
- Methamphetamine - 1000 ng/ml
- Morphine (opiates) - 2000 ng/ml
- PCP - 25 ng/ml
- THC (marijuana) - 50 ng/ml

The kits come with instructions which include directions and containers for submitting samples for confirmatory testing by a laboratory.

Some test strips also have lanes reserved for creatinine, pH, artificial oxidants and specific gravity to detect adulterated urine.

The better test companies have information that caution parents about over-interpreting results.

The test systems come in many shapes, sizes but they all have the same readouts.
No line in the test zone means a positive test for the drug.