10. Drug Level Monitoring

Drug tests either detect or 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. There are far too many tests and testing methods to cover so we will just look at a few of the major types.

Qualitative Testing: These are screening studies primarily focused on the identification of drugs, particularly drugs of abuse. On occasion drug metabolites are also assayed for as well and may interfer. These tests are typically qualitative and usually have some sort of threshold for detection. The simplest screening tests use test strips that you can buy in a pharmacy.

In general screening tests do not provide absolute confirmation of structure. Structural assignment must be determined or confirmed in clinical laboratories that employ more stringent test platforms, primarily mass spectrometry. They also do not provide concentrations of the drugs, instead there is a threshold of detection which can vary from test to test in the case of a given drug.

Samples from qualitative screening can be further tested by

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.

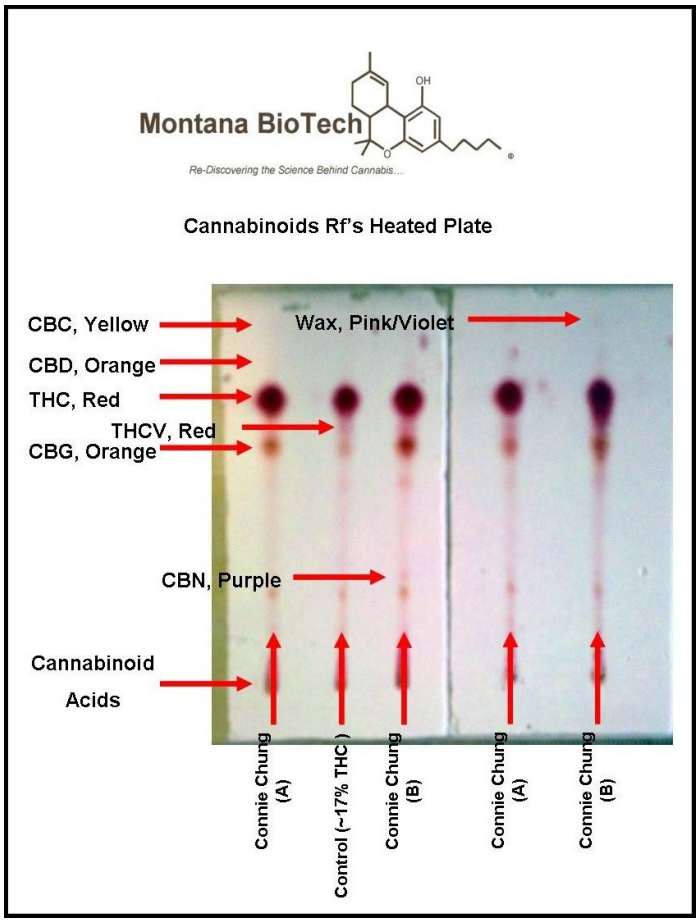
A. Chromatographic Methods

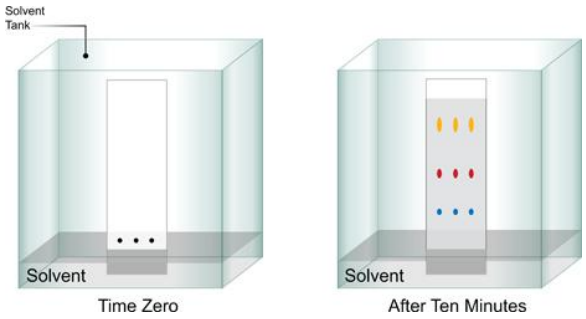
1. **TLC** Thin Layer Chromatography

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. Who is Connie Chung do you suppose?

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. As we will see later a gas chromatograph can be coupled to an alternate detector, a mass spectrometer (MS), to great advantage.

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. Many large polar drugs and all biologics are not volatile and do not chromatograph.
4. Long run-times required for good separation.

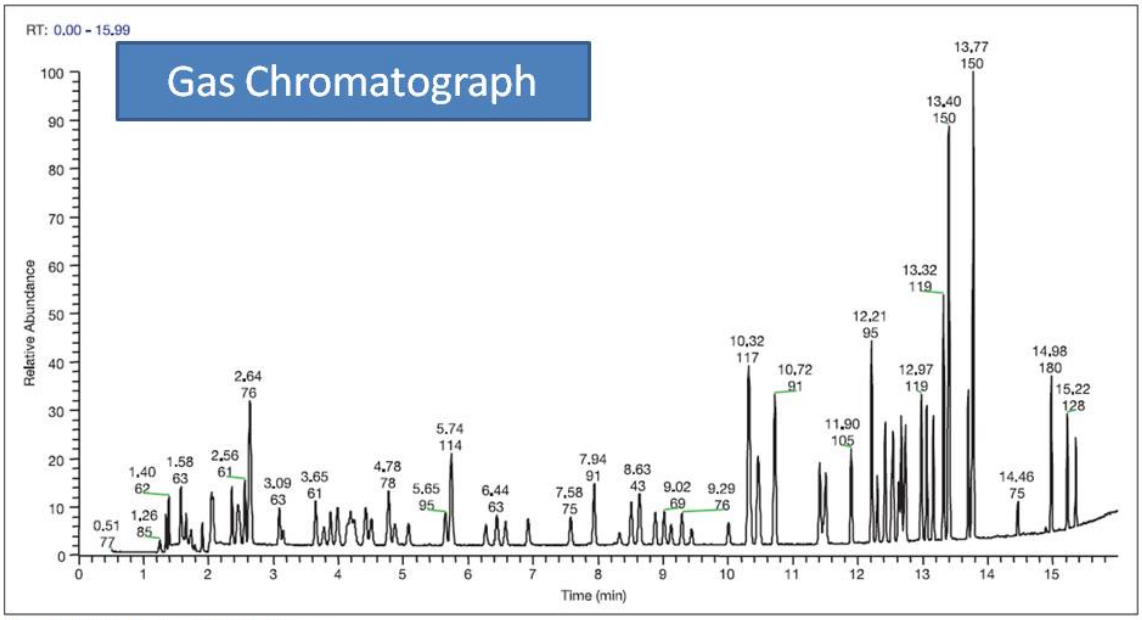


Figure 2: TIC of a 2 µg/L standard in full scan

3. **HPLC** High Pressure Liquid Chromatography

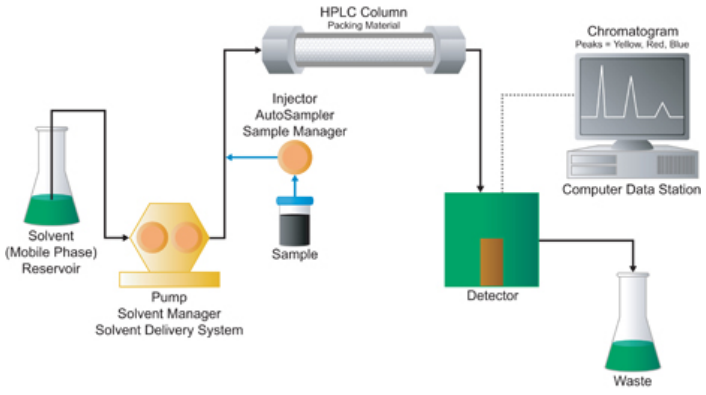
The sample is subjected to liquid chromatography using columns packed with micro-particulate materials that are silica based. The column eluent is assayed by UV-Vis or fluorescence spectrophotometry. Great for screening studies. Different columns types and solvents can be used to firm up structure assignments

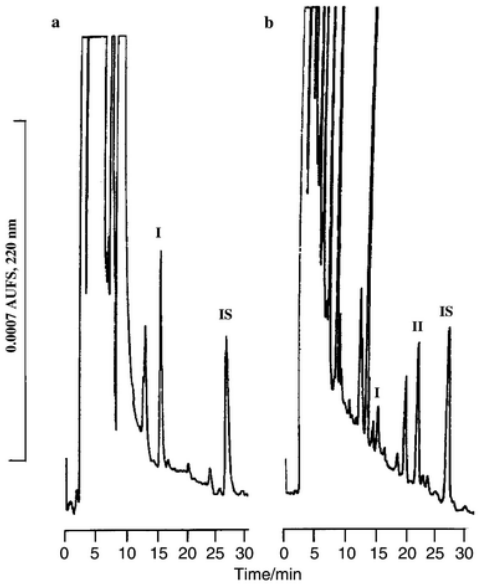
Advantages

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

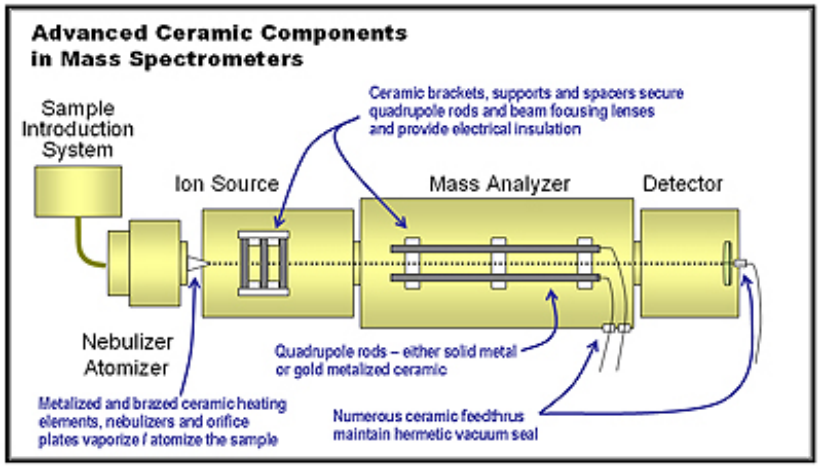
1. Does not confirm drug structure except by retention time.
2. Long run-times required for good separation.
3. Lots of interfering peaks due to contaminants from the biological samples.





4. MS Mass Spectrometric Detection

Here the analytes from the GC or HPLC column separation are directly transferred via an interface into the source of a mass spectrometer. The analytes are ionized in the source and the ions ejected in a focused beam into a mass analyzer. The magnetic or quadrapole mass analyzer separates the ions by mass before detection. 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 it's own "finger print" of ion fragments. This method allows from separate identification of quantitation of compounds that co-chromatograph.



Advantages

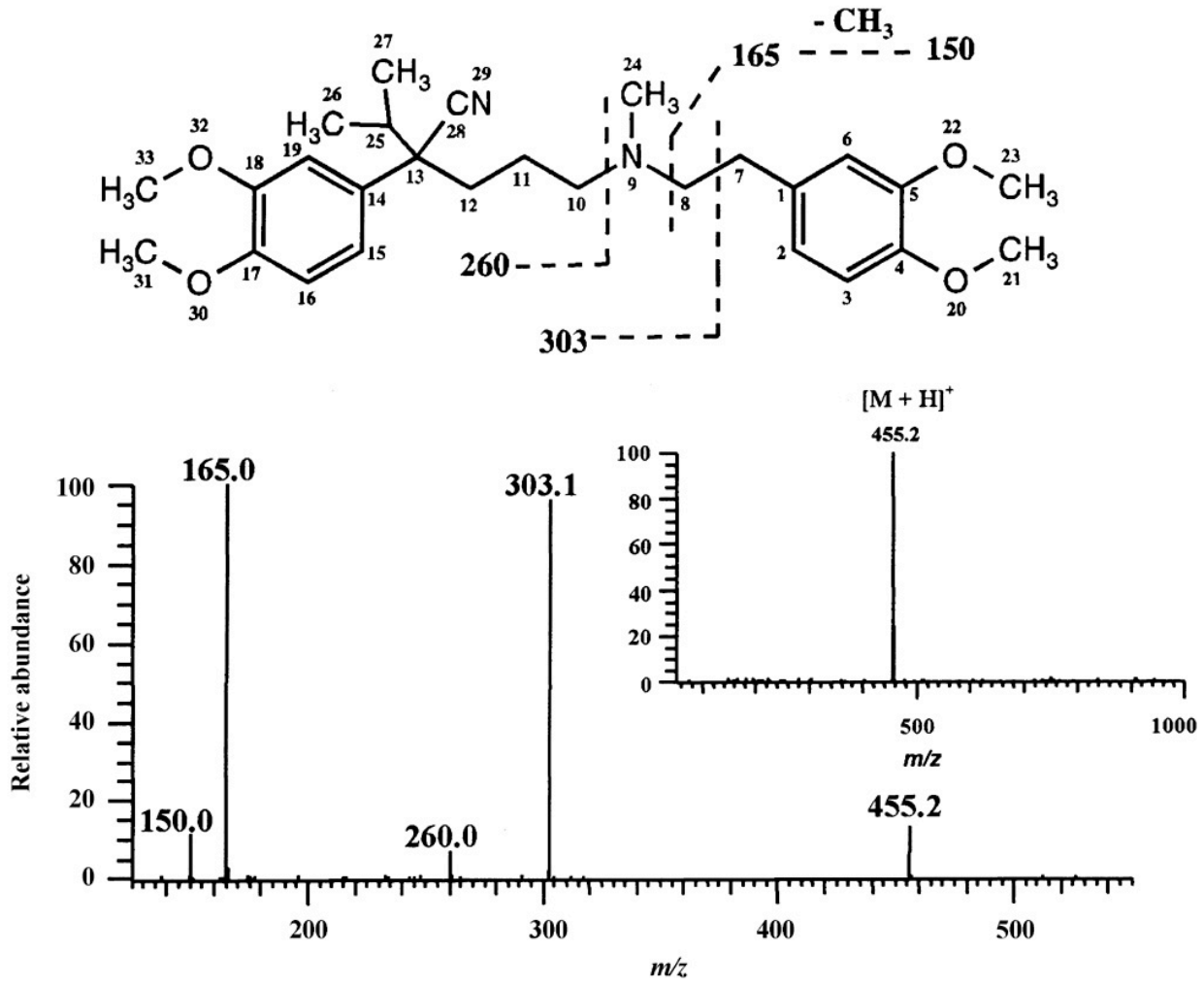
1. Provide the molecular weight of the analyte and high sensitivity and accuracy.
2. The mass spectrometers can detect multiple compounds in what would be a single chromatographic peak.
3. Provides signature fragmentation patterns as the high energy ions in the gas phase decompose to smaller fragments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

Disadvantage:

1. Cost and maintenance of the instruments.

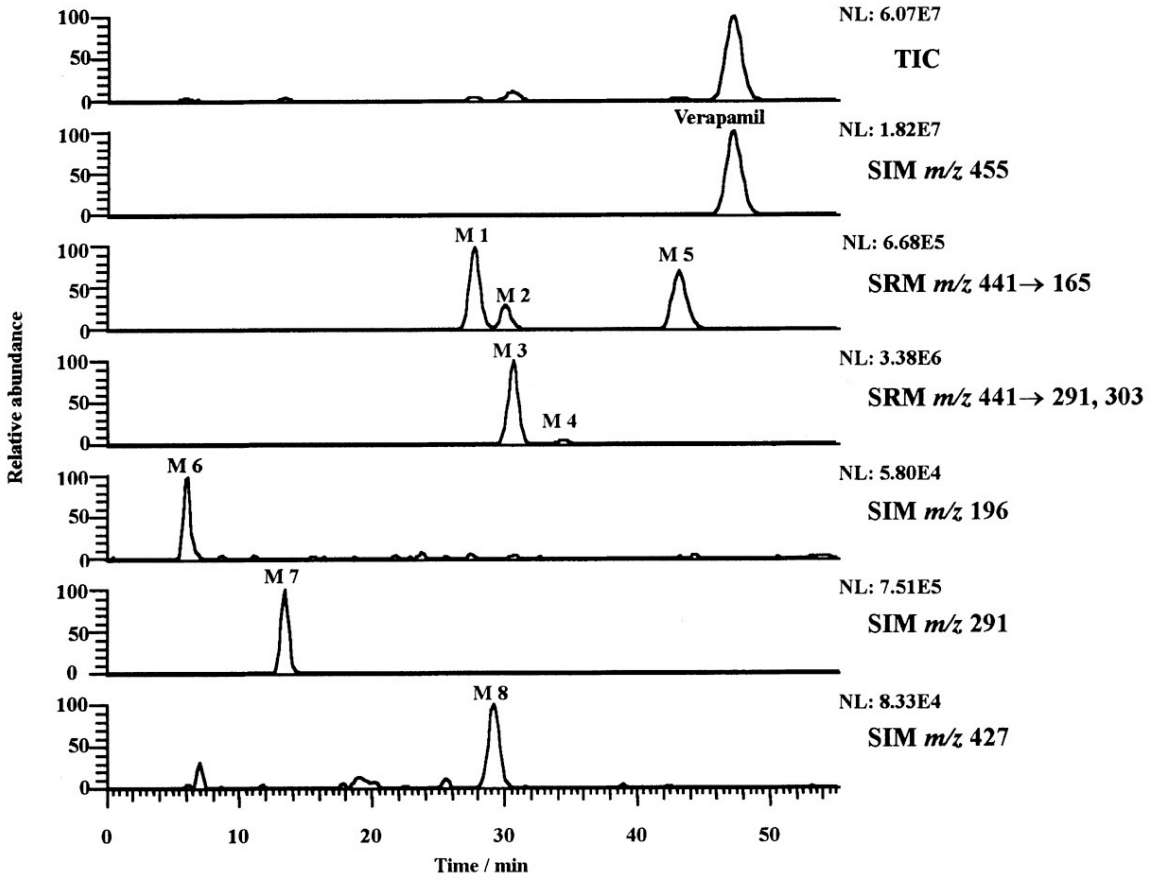
Here we see a mass chromatogram of verapamil which shows the parent ion and diagnostic fragments which help confirm structure.

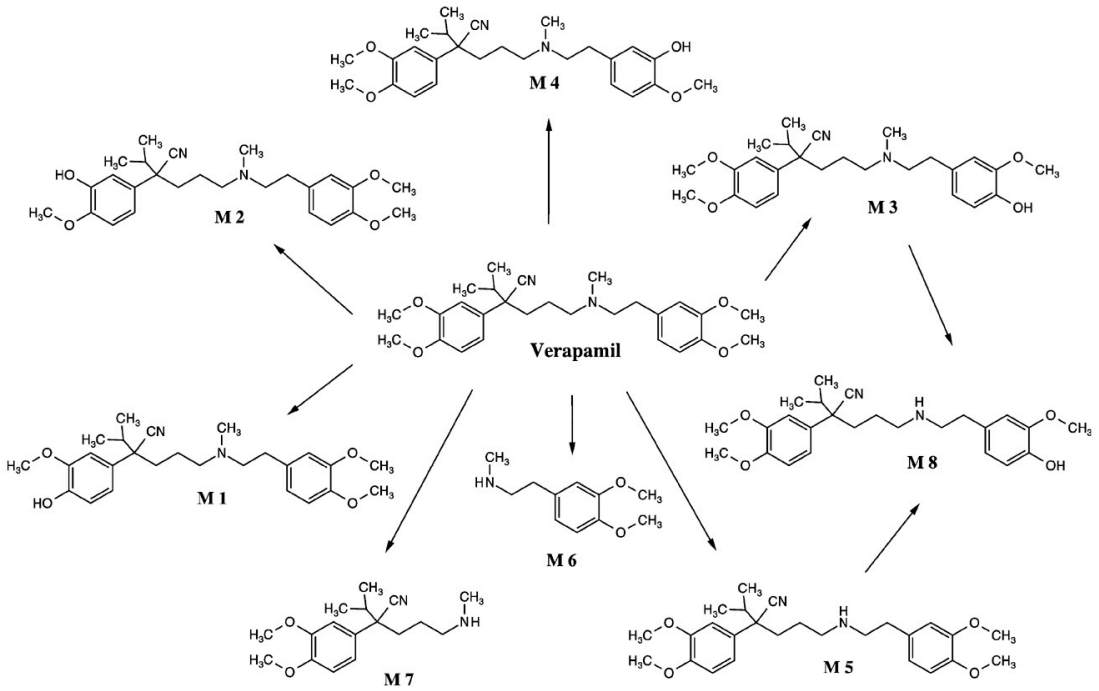
1. While many compounds might provide a mass of 455 amu the likelihood that it would also provide the same fragment fingerprint is very unlikely.
2. There are huge searchable data bases that contain fragment information for drugs, drug metabolites and other compounds including common biological contaminants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



On the next page we see an LCMS chromatogram from a single injection of verapamil and its metabolites where different masses corresponding to the MW (SIM) or fragment (SRM) of each compound are monitored at the same time by the mass spectrometer.

Each trace is a different mass. Note how the mass channels clearly distinguish between compounds that co-chromatograph (M1, M3, M8). Looks like P450 metabolites to me!

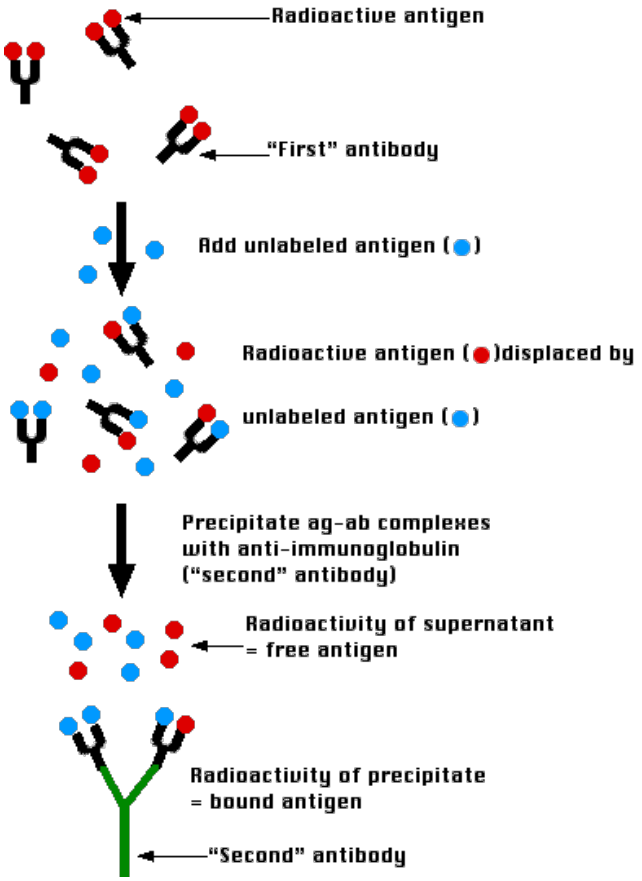


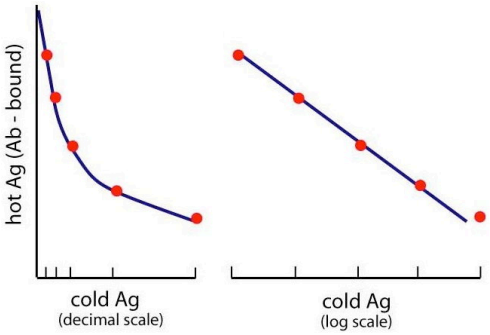


B. Immunochemical Quantitiative Methods (Blood)

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.

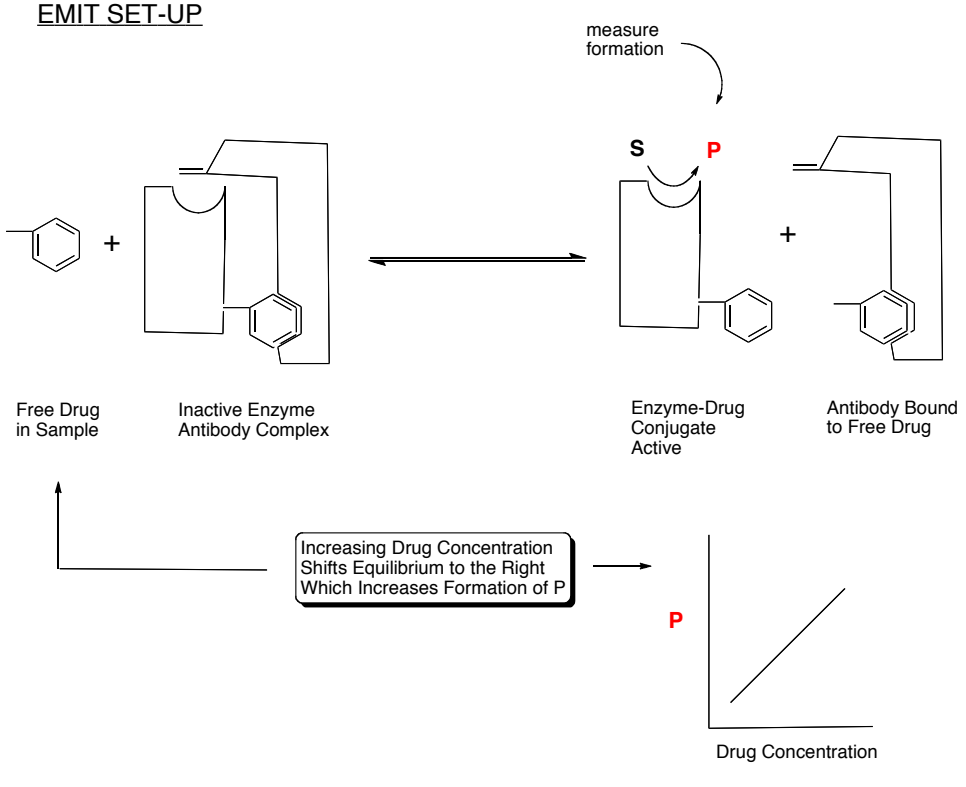
Disadvantages:

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



Advantages

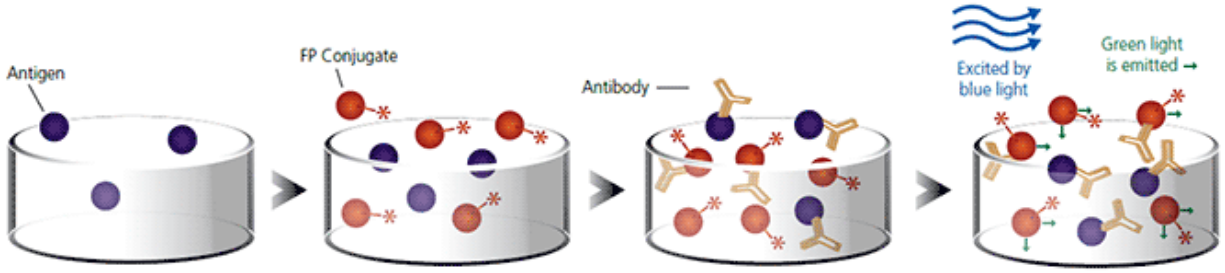
1. Fast, simple sensitive and easy to automate.
2. No Radioactivity
3. No chromatographic separation required
4. All reagents standardized and purchased.

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 low the signal.

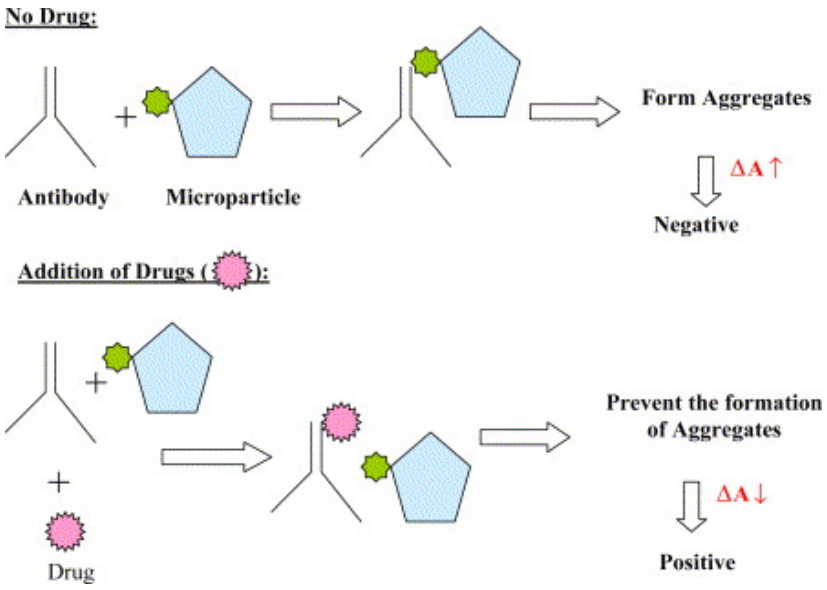


The advantages and disadvantages are the same as EMIT however FPIA is being replaced by EMIT as labs settle on a single assay method in quantitative immunochemistry assays to support TDM. The Mayo Clinic has completely moved to EMIT.

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

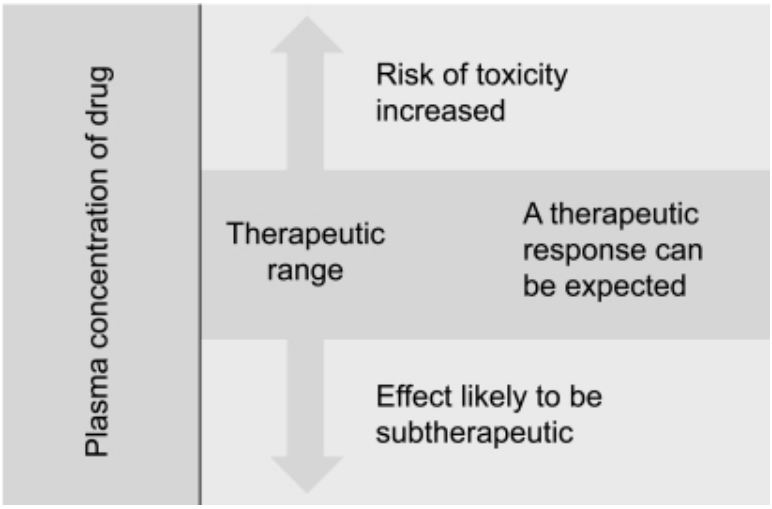
This assay uses an antibody to the drug of interest that also binds to the drug covalently linked to a microparticle. When the antibody binds to the drug-linked microparticle the sample is turbid and absorbs light. When the free drug competes for the antibody the solution is less turbid and absorbs less 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.

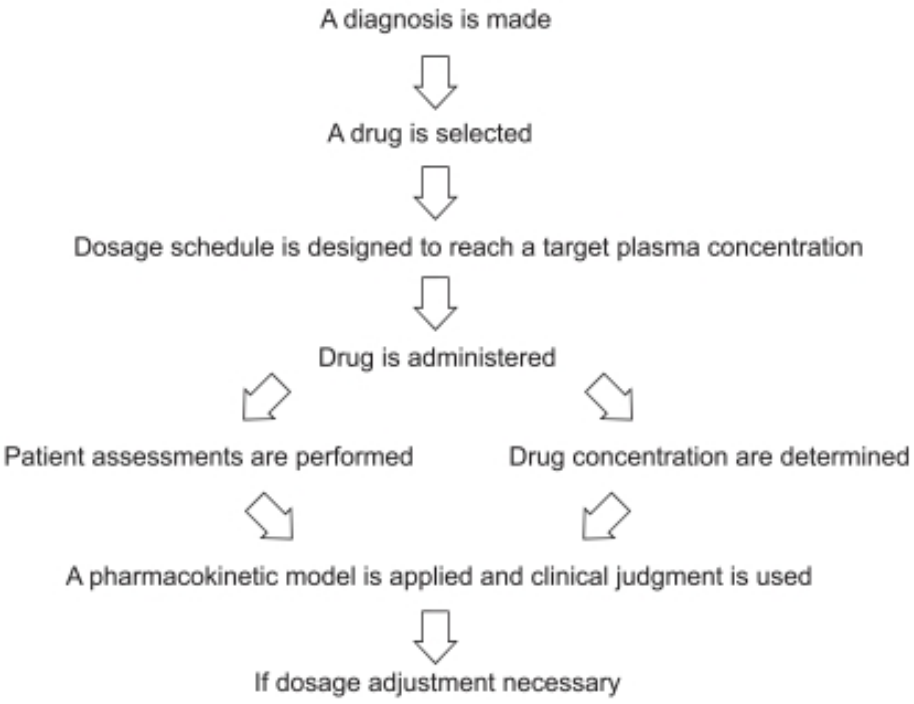


C. Chromatatography Based Systems GCMS, LCMS and LCMS/MS assays are more expensive to run but in some cases they are the only reliable method where the compound of interest can be assayed with no interference from contaminants, other drugs or drug metabolites and with high sensitivity. LCMS/MS is even more selective and sensitive than LCMS and is used for large compounds like tacrolimus and cyclosporine.

D. TDM (Therapeutic Drug Monitoring)



The UW provides testing of blood levels for over 30 drugs and more are available from labs like the Mayo Clinic. TDM is becoming more and more common as studies are generated for a drug or drug class that demonstrate improvement in care. Full TDM includes monitoring and dose adjustments based on an understanding of the pharmacokinetics of the drug. For instance high dose methotrexate therapy usually requires leucovorin rescue which is managed by monitoring MTX levels. In some rarer cases total and free drug in serum is monitored. Most drugs subject to TDM have narrow therapeutic indexes.



The Mayo Clinic has a good site if you want to look up a particular test and how it is run. Some UW samples are sent there

http://www.mayomedicallaboratories.com/index.html

The UW site is a little less user friendly

http://depts.washington.edu/labweb/referencelab/index.htm

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. There are also more automated systems that only produce qualitative detection. Another big area is employee drug testing. These tests are almost always qualitative and have detection limits.

Most of these devices use a lateral flow immunochromatography. The principle here is exactly the same as ELISA and Colloidal Gold tests that Carlos talked about at the start of this class. This is different from dipsticks that are typically used in home testing kits although the basic principle is also the same. A short description follows

1) A specified aliquot of the sample containing the free drug is added to the sample port.

2) The fluid travels across the conjugate pad that contains the drug-primary antibody conjugated with either colloidal gold or a colored latex bead.

3) The solution of primary antibody conjugate and free drug travel as a mixture until they reach the capture zone.

4) The capture zone has an immobilized drug that recognizes the primary antibody conjugate.

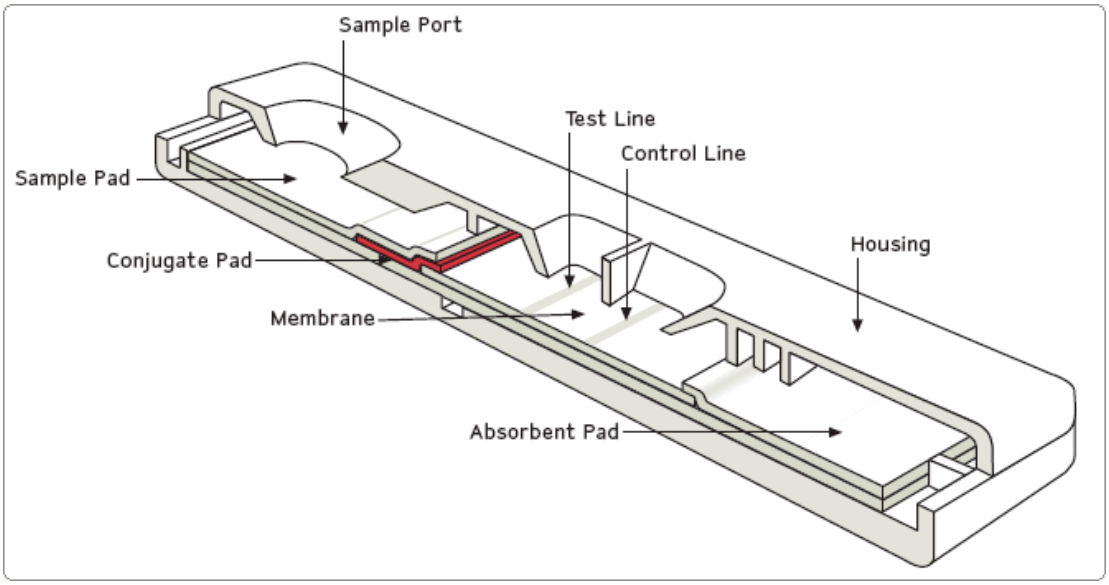
    a) Negative Test: When no drug is present the primary antibody conjugate binds the to the immobilized drug conjugate and forms a colored Test Line in the capture zone.

    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 recognize 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.



A little more detail: The principle behind the test is straightforward; Basically, Lateral Flow Immunochromatographic Assays are a simple device intended to detect the presence (or absence) of a target analyte in sample (matrix). Most commonly these tests are used for medical diagnostics either for home testing, point of care testing, or laboratory use. Often produced in a dipstick format, Lateral flow tests are a form of immunoassay in which the test sample flows along a solid substrate via capillary action. After the sample is applied to the test it encounters a coloured reagent which mixes with the sample and transits the substrate encountering lines or zones which have been pretreated with an antibody or antigen. Depending upon the analytes present in the sample the coloured reagent can become bound at the test line or zone. Lateral Flow Tests can operate as either competitive or sandwich assays.

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, 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

	TesTcard 9®		Syva RapidTest d.a.u. 10®		Triage TOX Drug Screen®	
Compound	FP	FN	FP	FN	FP	FN
Amphetamines/methamphetamines	4	0	2	0	0	0
Opiates	3	0	2	0	0	0
Methadone	-	-	0	1	0	1
Cocaine	0	0	0	0	0	1
Cannabis	0	3	4	0	0	1
Benzodiazepines	3	5	1	3	2	0
Barbiturates	2	0	1	0	0	0
Tricyclic antidepressants	2	0	0	0	1	0

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. http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/DrugsofAbuseTests/default.htm

The major drug chains carry home testing kits. They are also available on line. 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.