Introduction to Chromatography

Laboratory Medicine Residency
Didactic Lecture Series

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Overview

There are *many* chromatographic assays (GC, GC/MS, HPLC, TLC, LC/MS/MS, etc.).

To understand them, it is best to break them down into their modular components/steps:

1) Sample preparation
2) Separation (the actual chromatography)
3) Detection (UV/Vis, Fluorescence, Mass spectrometry, etc.)

This talk will focus on the 2nd step: *separation*.

Chromatographic Components

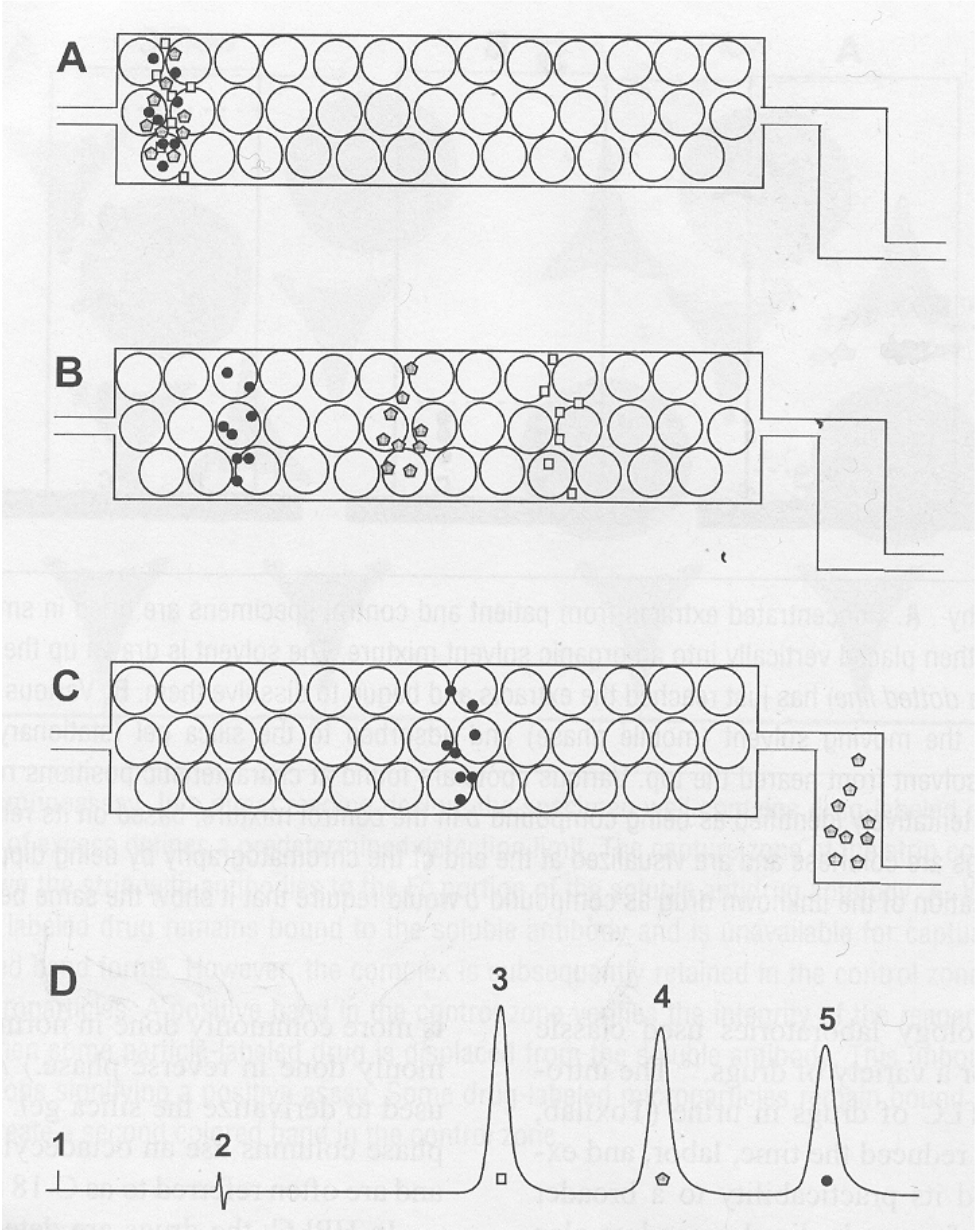
Each flavor of chromatography is best understood by breaking it down into modular components:

1) Sample “loading”
2) The “mobile phase” during separation.
3) The “stationary phase” during separation.

Separation of individual sample components is always based on their *relative affinity* for the *mobile* versus the *stationary* phases.

Because some molecules have higher affinity for the stationary phase, they will pass through the column *slower* than others and, therefore, will be separated from each other (we say they have been “retained” by the column).

Separation of Molecules by Chromatography



After injection, all molecules start out overlapping.

Due to varying relative affinity for the stationary versus the mobile phases, individual molecules begin to separate.

As the different molecules elute off of the column, they are detected as resolved “peaks”.

Type of Chromatography	Sample Loading	Mobile Phase	Stationary Phase
Gas Chromatography (GC)	Heating	Variety of Gases	Capillary column with a narrow hydrophobic coating.
Liquid Chromatography (LC)	Injection	Variety of Liquids (gradients also possible)	Column packed with small beads coated with a variety of chemical substituents.
Thin Layer Chromatography (TLC)	“Spotting”, followed by evaporation of the liquid.	Liquid driven by capillary action.	Paper or thin layer of silica plated on glass.

Relative Retention Times

- During separation, the *absolute* rates/times for movement of molecules are not always reproducible.
- For example, columns can get dirty, decreasing the amount of stationary phase available for interaction with molecules. This is equivalent to shortening the length of the column. There can also be variability in the timing of injection.
- However, such perturbations should affect the rates of ***all*** molecules in the same way. Therefore, their ***relative*** rates/times are usually highly reproducible (selection of an appropriate *internal standard* is critical for this reason).
- The "*relative retention time*" (RRT) is defined as the detection time for a individual peak divided by the detection time for a known *internal standard*. RRTs are characteristic and reproducible (but not always 100% specific) identifiers of individual molecules.
- Note that sometimes a "corrected retention time" is reported where the absolute retention time of an analyte is adjusted according to the variation in the retention time of the internal standard compared to a control sample:

$$\mathbf{CRT_{analyte} = RT_{analyte} * (RT_{std} / RT_{std,control})}$$

Quantification of Drug Concentrations

- Peak “area” generally correlates with the amount of drug loaded onto a column and, thus, the original drug concentration.
- However, there can be sample-to-sample variation due to extraction efficiency, loading volumes, detection efficiency, etc.
- Again, the *internal standard* is utilized to correct for variations.
  - Similar to the relative retention time, a *relative peak intensity* is defined and related to drug concentration.
  - Variations in the peak area are not always similar for all molecules (e.g. some molecules may have a lower extraction efficiency due to proteinuria and others may not; it is important that the internal standard shares the same *susceptibility* to interferences or variations in efficiency as the analyte).
  - Therefore, the internal standard is chosen to be chemically similar to the analyte of interest to best correct for variations.
  - However, adequate similarity is *not* easy to predict or establish.
  - Extensive validation is ultimately necessary to satisfy the rigor of your assay. Need to test all anticipated interferences and also un-expected variabilities using “real” patient samples, either with a large correlation study, with a gold standard method and/or with addition/recovery studies.

Protocol for Quantification of Analyte Concentration Based Upon a Calibration Curve

- A known amount of an internal standard is added to every sample (including controls and calibrators) before any other preparative step.
- All samples are brought through the identical preparative steps, separated by a chromatographic method and quantitatively detected.
- The relative peak intensities are measured for a series of calibrators with a fixed amount of internal standard and varying amounts of a known analyte. These relative peak intensities are fit to an equation, generally linear, to define a *calibration curve*.
- Similarly, the relative peak intensities of the unknown samples are calculated and related to the *calibration curve* to quantify the concentration of the analyte (drug) in the original clinical sample.

Amiodarone Report

Sample ID: TM6
Filename: C:\EZChrom Elite\DATA\012604A6
Method: C:\EZChrom Elite\METHODS\amiodarone.met
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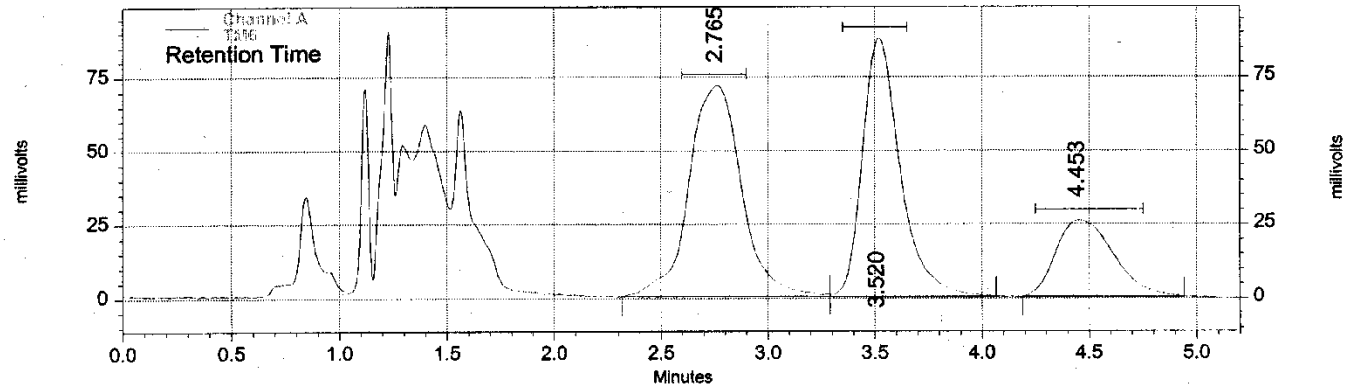
Printed: 1/26/04 11:16:24 AM



Name	Retention Time	Area	Concentration	Units
Amiodarone	2.77	122394	3.57	mg/l
Desethyl	3.52	107318	2.91	mg/l
IS	4.45	47721	1.00	mg/l
Totals		277433	7.49	

Amiodarone Report

Sample ID: S1
Filename: C:\EZChrom Elite\DATA\012604A02
Method: C:\EZChrom Elite\METHODS\amiodarone.met
Acquired: 1/26/04 11:45:45 AM
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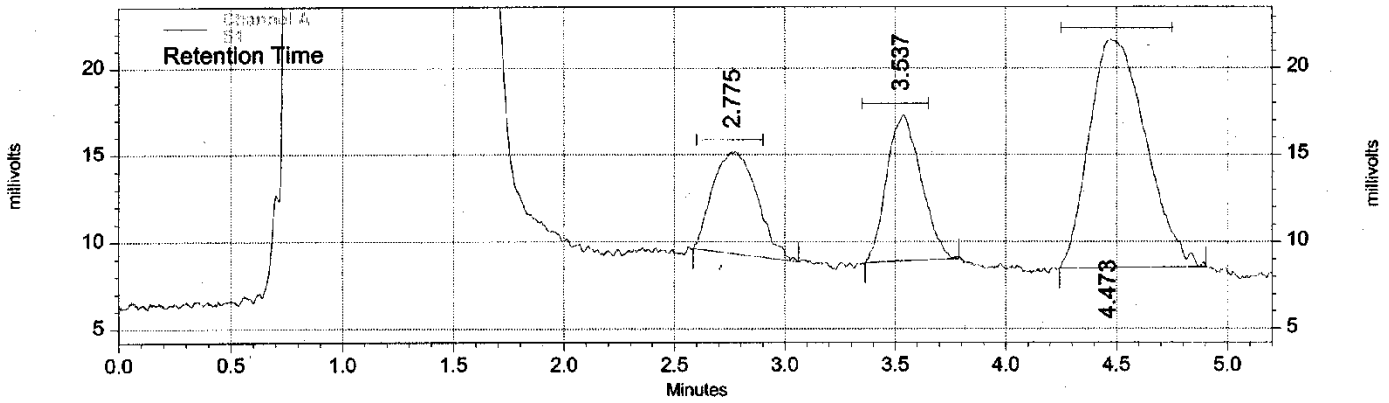

Name	Retention Time	Area	Concentration	Units
Amiodarone	2.77	8169	0.50 CAL	mg/l
Desethyl	3.54	9061	0.50 CAL	mg/l
IS	4.47	23261	1.00 CAL	mg/l
Totals		40491	2.00 CAL	

Amiodarone Report

Sample ID: S2
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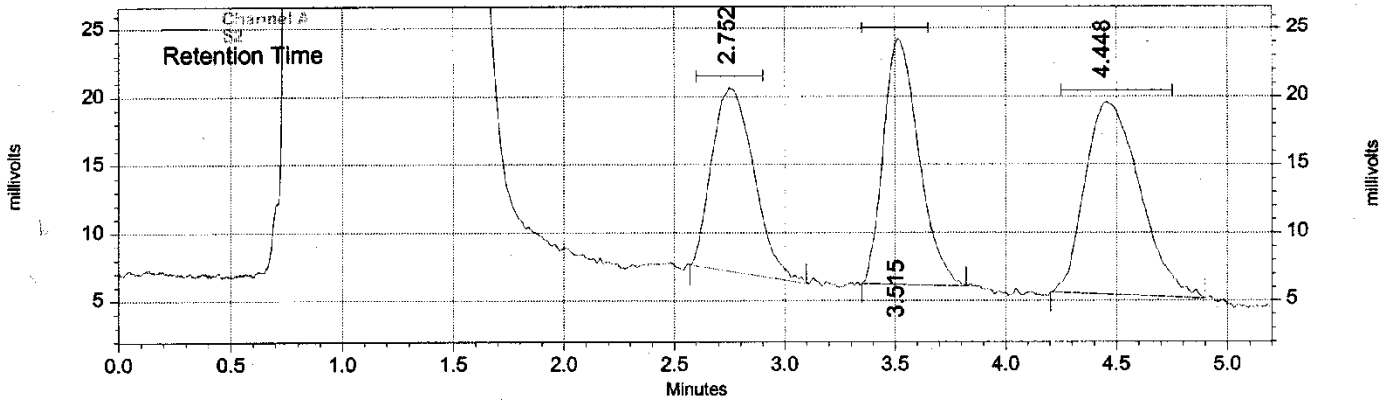

Name	Retention Time	Area	Concentration	Units
Amiodarone	2.75	17861	1.00 CAL	mg/l
Desethyl	3.52	20046	1.00 CAL	mg/l
IS	4.45	24280	1.00 CAL	mg/l
Totals		62187	3.00 CAL	

Amiodarone Report

Sample ID: S3
Filename: C:\EZChrom Elite\DATA\012604A04
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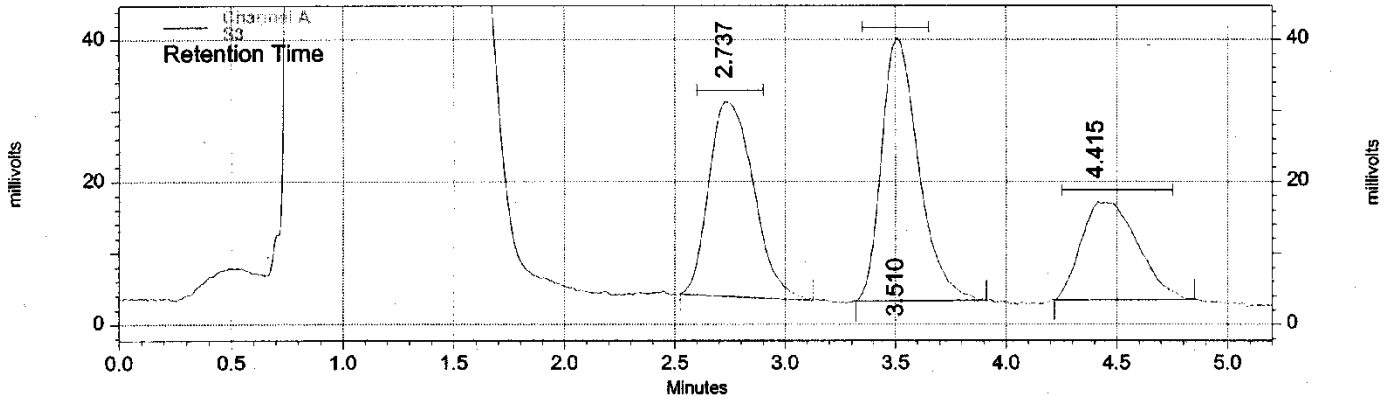
Acquired: 1/26/04 11:58:45 AM
Printed: 1/26/04 12:05:13 PM



Name	Retention Time	Area	Concentration	Units
Amiodarone	2.74	38281	2.00 CAL	mg/l
Desethyl	3.51	42913	2.00 CAL	mg/l
IS	4.41	24098	1.00 CAL	mg/l
Totals		105292	5.00 CAL	

Amiodarone Report

Sample ID: S4
Filename: C:\EZChrom Elite\DATA\012604A05
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Acquired: 1/26/04 12:05:15 PM
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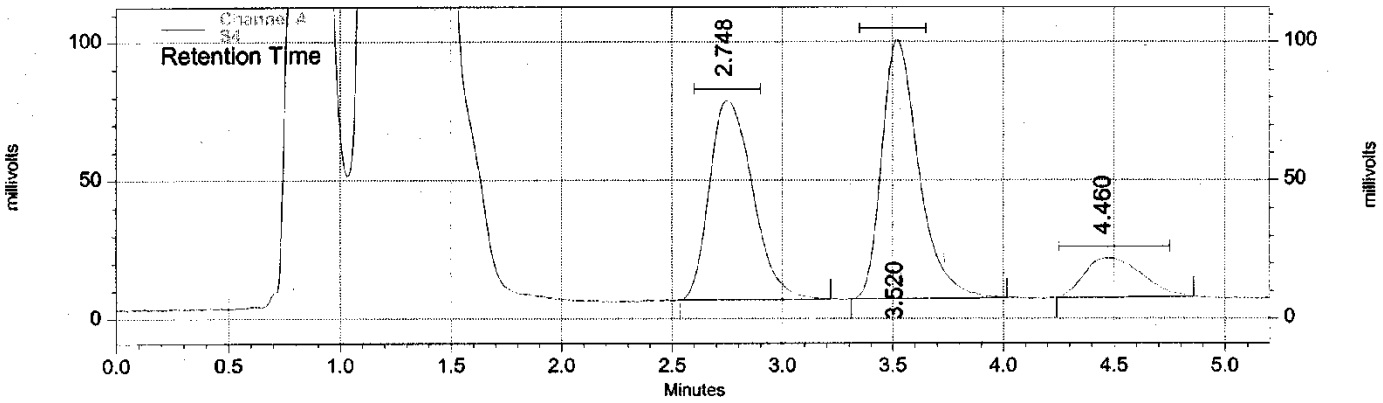


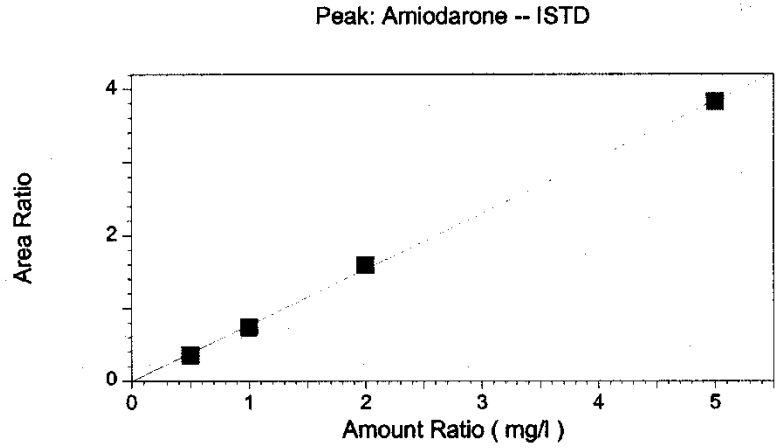
Name	Retention Time	Area	Concentration	Units
Amiodarone	2.75	98285	5.00 CAL	mg/l
Desethyl	3.52	108699	5.00 CAL	mg/l
IS	4.46	25698	1.00 CAL	mg/l
Totals		232682	11.00 CAL	

EZChrom *Elite*

Calibration Report

Method: C:\EZChrom Elite\METHODS\amiodarone.met
Print Time: 1/26/04 12:11:42 PM
User: System
Instrument: applied biosytem

Amiodarone (applied biosytem)
Average RF: 0.749301 RF StDev: 0.0393945 RF %RSD: 5.2575
Scaling: None LSQ Weighting: None Force Through Zero: On
Replicate Mode: Replace
Fit Type: Linear
y = 0.767319x + 0
Goodness of fit (r^2): 0.999296



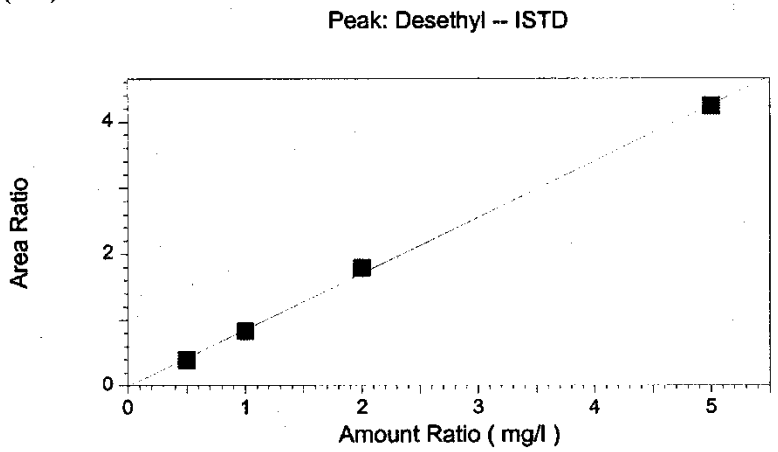
	Level 1	Level 2	Level 3	Level 4
Area Ratio	0.351189	0.735626	1.58856	3.82462
Amount Ratio	0.5	1	2	5
RF	0.702377	0.735626	0.794278	0.764923
Last Area Ratio				
Rep StDev				
Rep %RSD				
Rep Area Ratio 1	0.351189	0.735626	1.58856	3.82462

EZChrom *Elite*

Calibration Report

Method: C:\EZChrom Elite\METHODS\amiodarone.met
Print Time: 1/26/04 12:11:43 PM
User: System
Instrument: applied biosytem

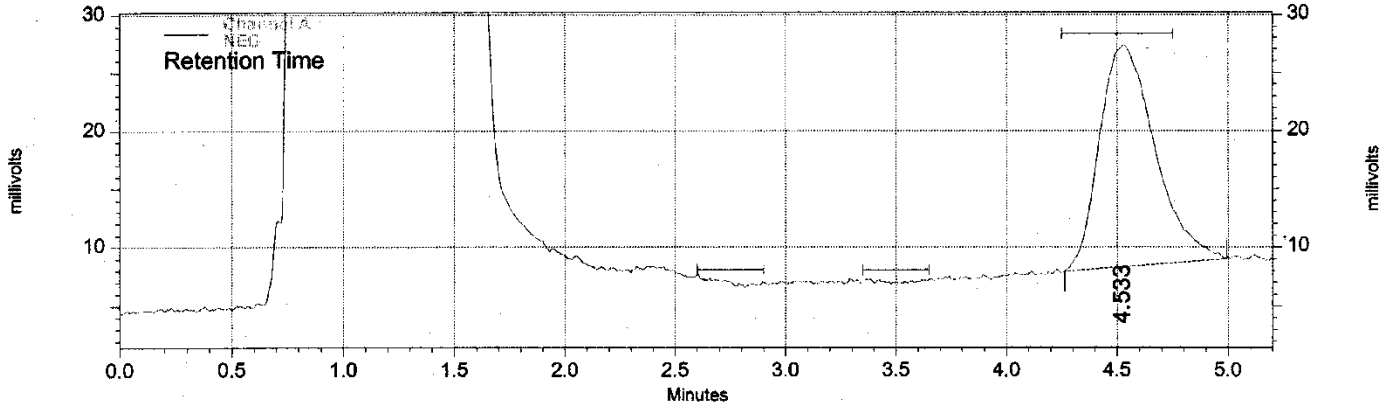
Desethyl (applied biosytem)
Average RF: 0.835262 RF StDev: 0.0462009 RF %RSD: 5.5313
Scaling: None LSQ Weighting: None Force Through Zero: On
Replicate Mode: Replace
Fit Type: Linear
y = 0.850619x + 0
Goodness of fit (r^2): 0.999008



	Level 1	Level 2	Level 3	Level 4
Area Ratio	0.389536	0.825618	1.78077	4.22986
Amount Ratio	0.5	1	2	5
RF	0.779072	0.825618	0.890385	0.845972
Last Area Ratio				
Rep StDev				
Rep %RSD				
Rep Area Ratio 1	0.389536	0.825618	1.78077	4.22986

Amiodarone Report

Sample ID: NEG
Filename: C:\EZChrom Elite\DATA\012604A23
Method: C:\EZChrom Elite\METHODS\amiodarone.met
Acquired: 1/26/04 2:30:41 PM
Printed: 1/26/04 2:37:25 PM



Name	Retention Time	Area	Concentration	Units
Amiodarone			0.00 BDL	mg/l
Desethyl			0.00 BDL	mg/l
IS	4.53	32979	1.00	mg/l
Totals				
		32979	1.00	

Amiodarone Report

Sample ID: LC
Filename: C:\EZChrom Elite\DATA\012604A06
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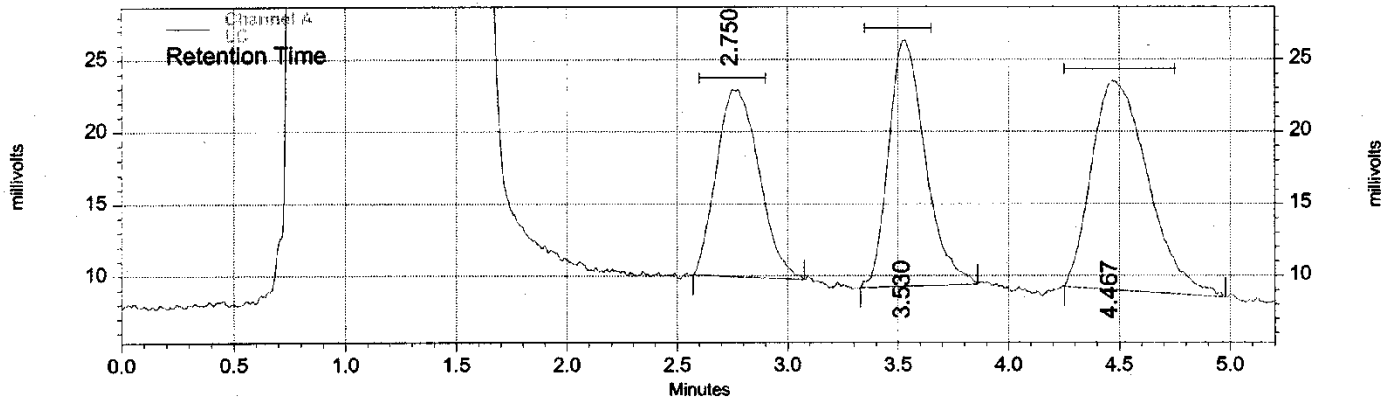
Acquired: 1/26/04 12:11:49 PM
Printed: 1/26/04 12:18:10 PM

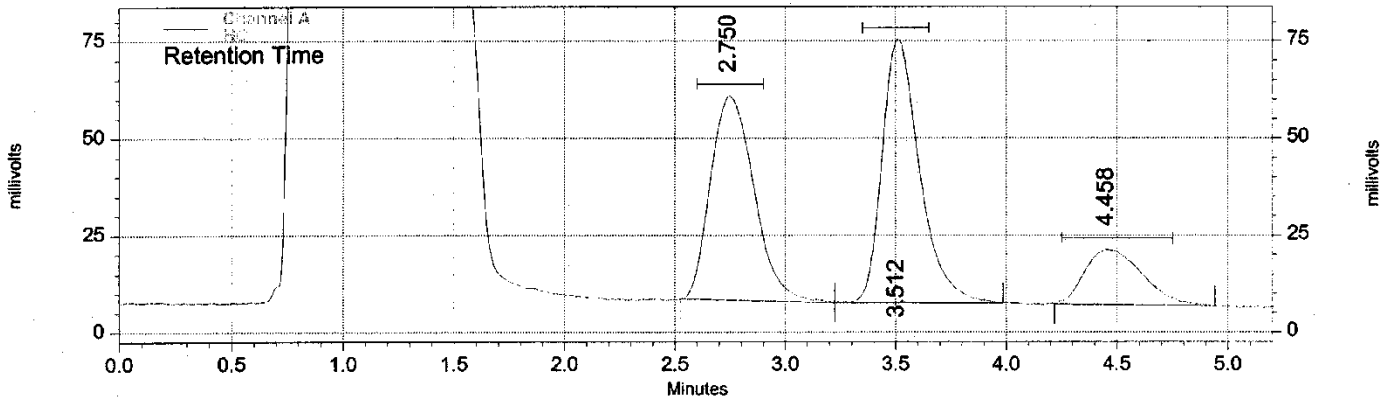


Name	Retention Time	Area	Concentration	Units
Amiodarone	2.75	17417	0.89	mg/l
Desethyl	3.53	19660	0.90	mg/l
IS	4.47	25642	1.00	mg/l
Totals		62719	2.79	

Amiodarone Report

Sample ID: HC
Filename: C:\EZChrom Elite\DATA\012604A07
Method: C:\EZChrom Elite\METHODS\amiodarone.met
Acquired: 1/26/04 12:18:13 PM
Printed: 1/26/04 12:24:40 PM

Rel. Ret. Time for Amiodarone = 2.75 / 4.46 = 0.62

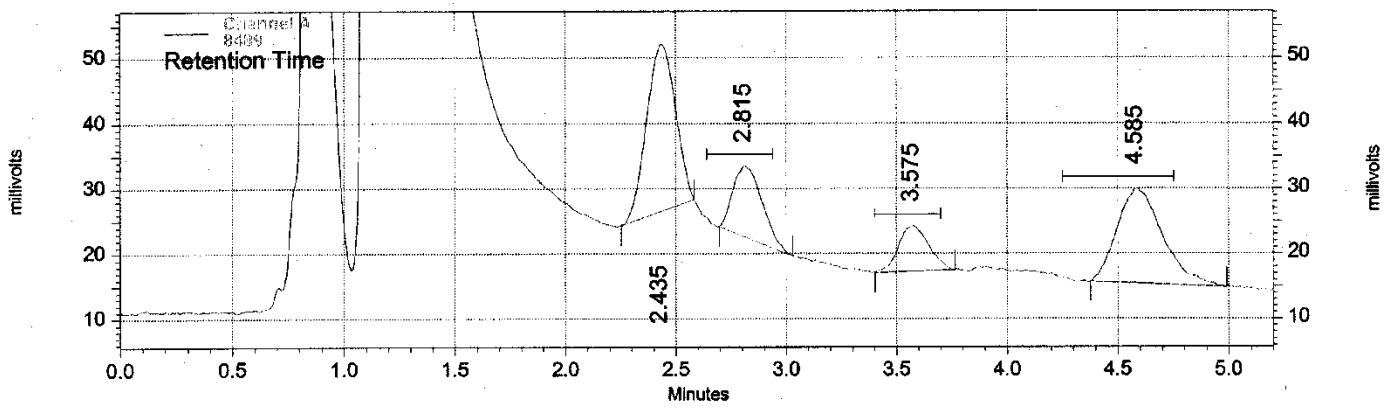


Name	Retention Time	Area	Concentration	Units
Amiodarone	2.75	72256	3.69	mg/l
Desethyl	3.51	80112	3.69	mg/l
IS	4.46	25530	1.00	mg/l
Totals		177898	8.38	

Amiodarone Report

Sample ID: 8489
Filename: C:\EZChrom Elite\DATA\012604A100
Method: C:\EZChrom Elite\METHODS\amiodarone.met
Acquired: 1/26/04 2:59:04 PM
Printed: 1/26/04 3:04:41 PM

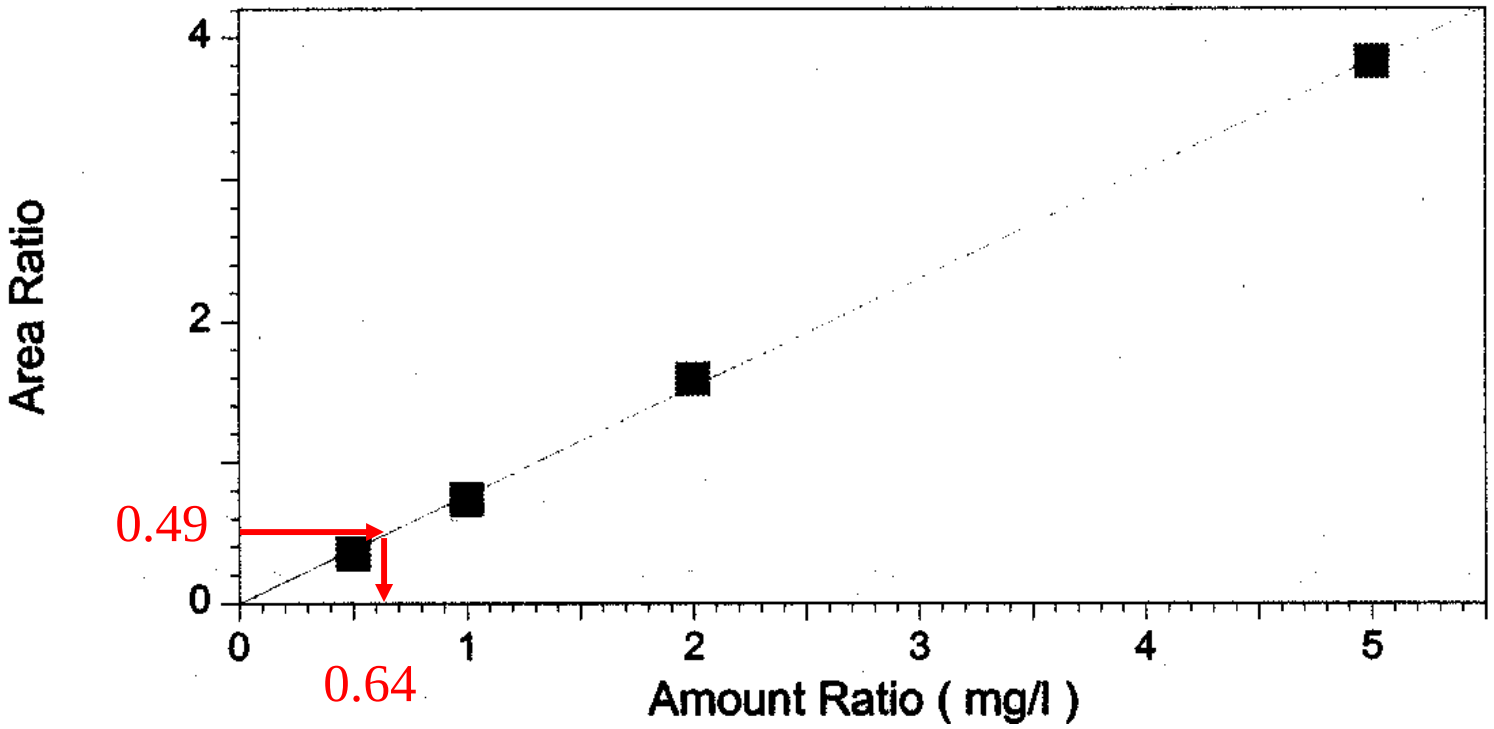
Rel. Ret. Time for Amiodarone = 2.82 / 4.59 = 0.61; agrees with standard and therefore confirms identity…



Name	Retention Time	Area	Concentration	Units
Amiodarone	2.82	10129	0.64	mg/l
Desethyl	3.58	6577	0.38	mg/l
IS	4.59	20579	1.00	mg/l
Totals		37285	2.02	

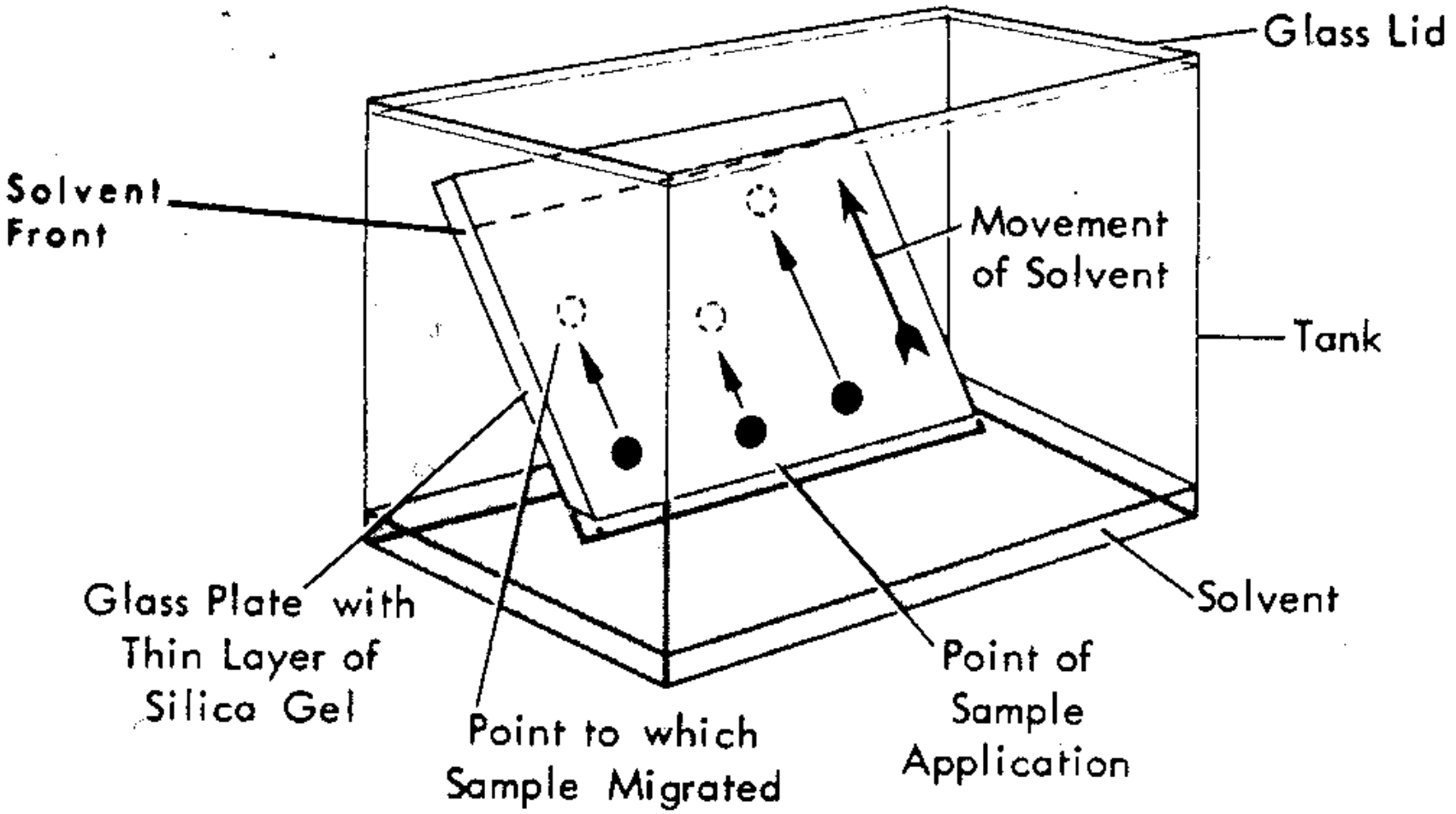
Rel. Peak Int. for Amiodarone = 10,129 / 20,579 = 0.49
Now look on curve…

Calibration curve for Amiodarone

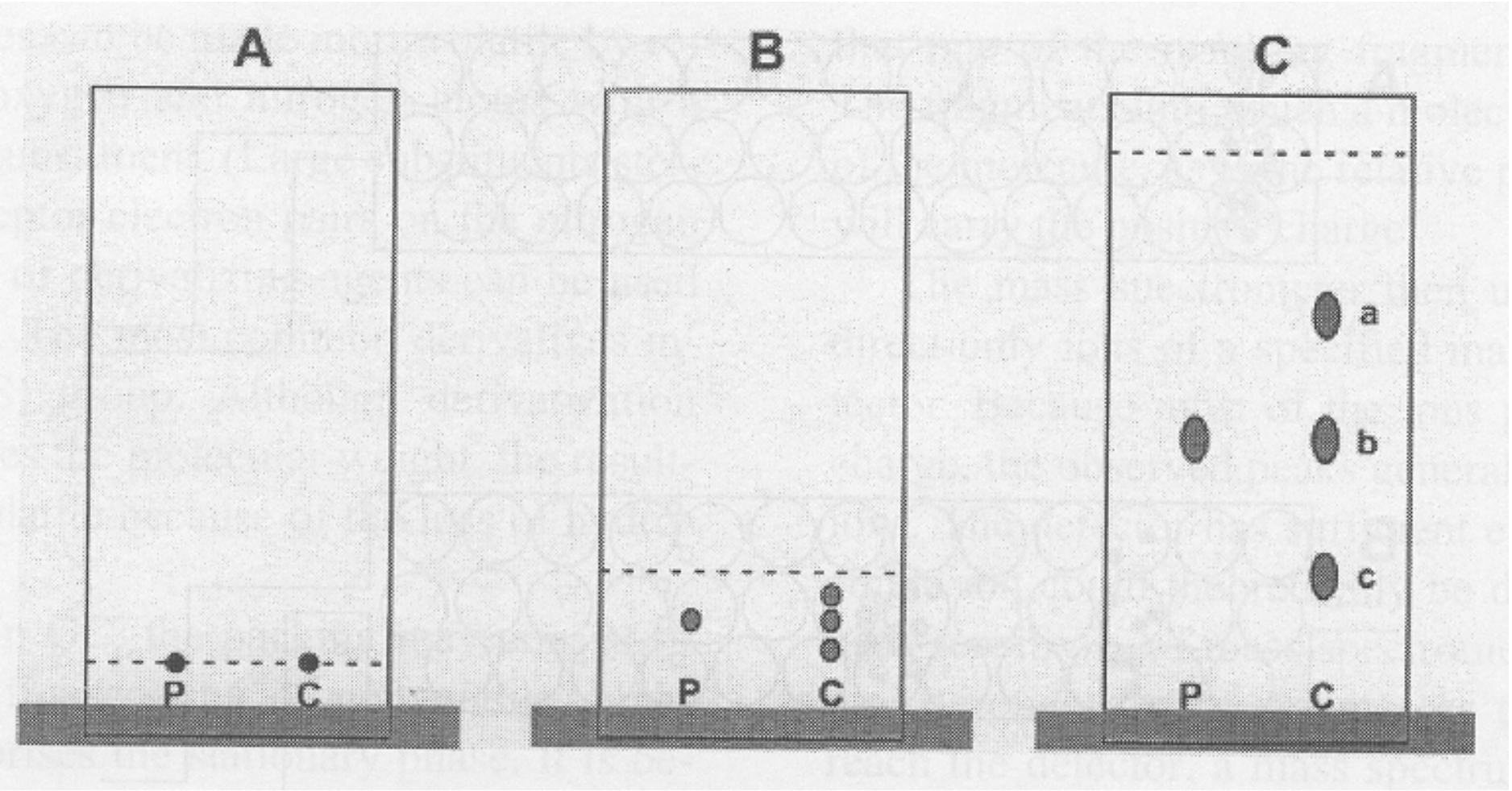


Although, the computer fits the measured relative peak intensity to this curve mathematically, visual inspection clearly identifies the correct concentration of amiodarone.

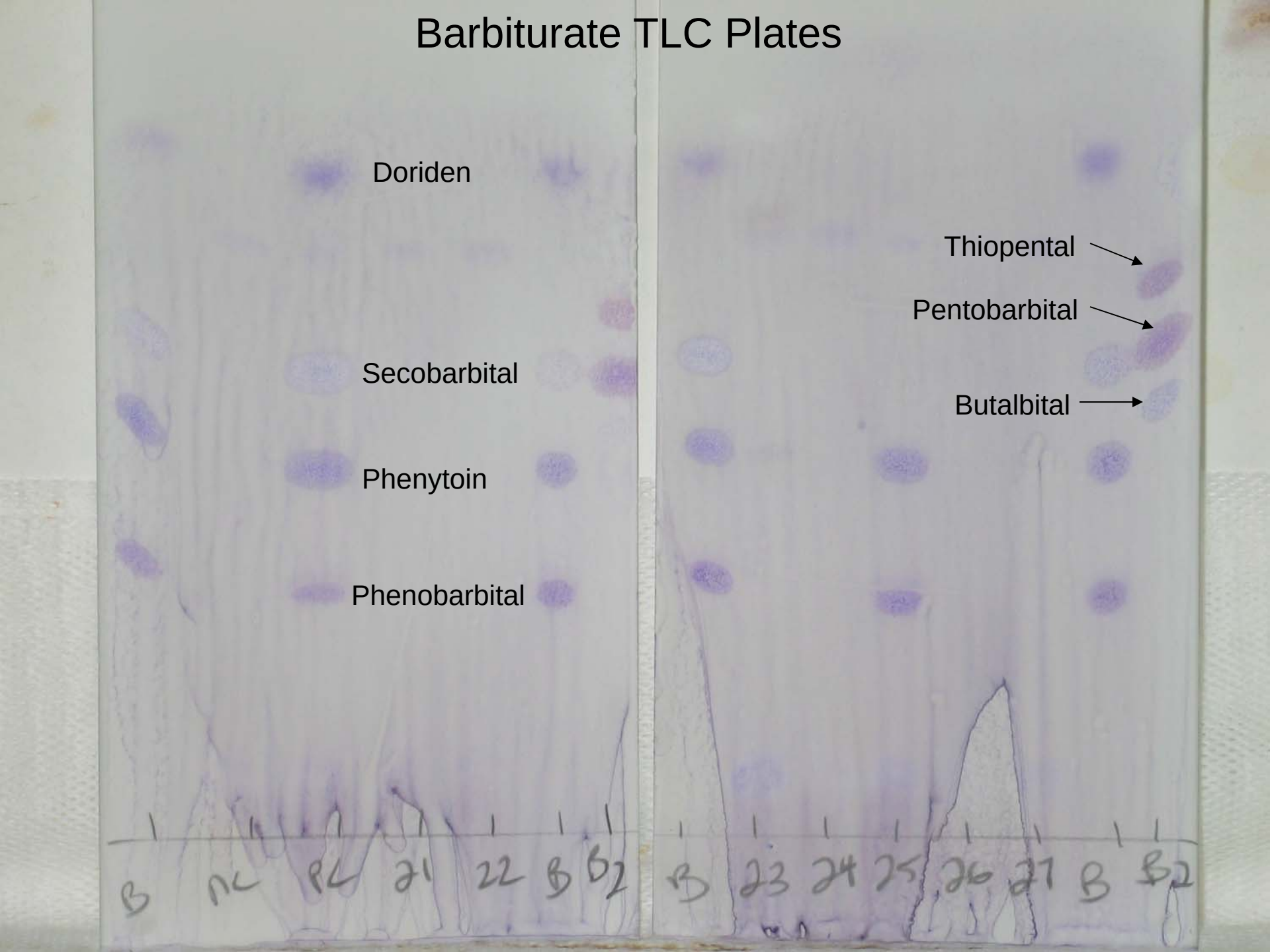
Thin Layer Chromatography



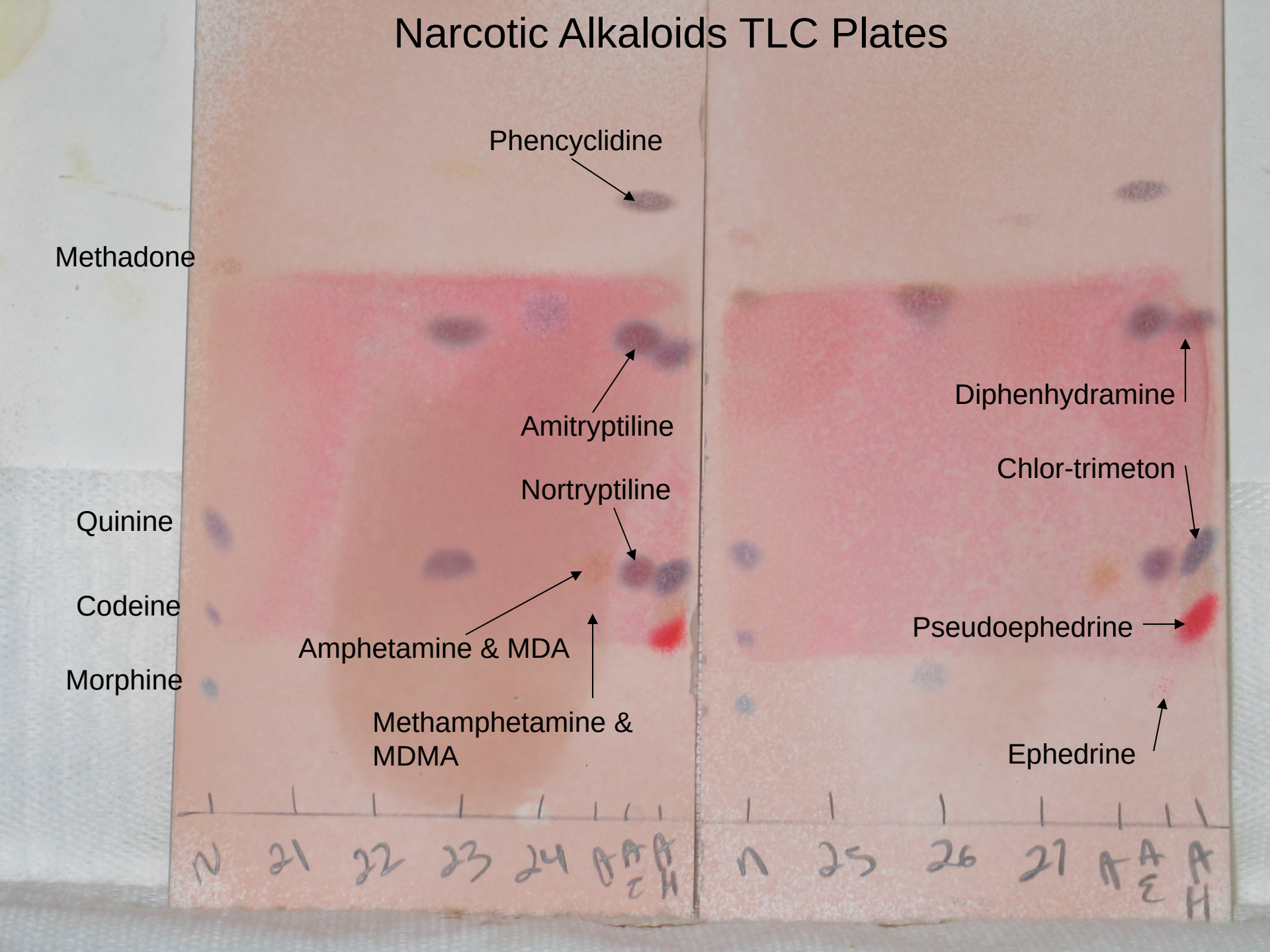
Thin Layer Chromatography



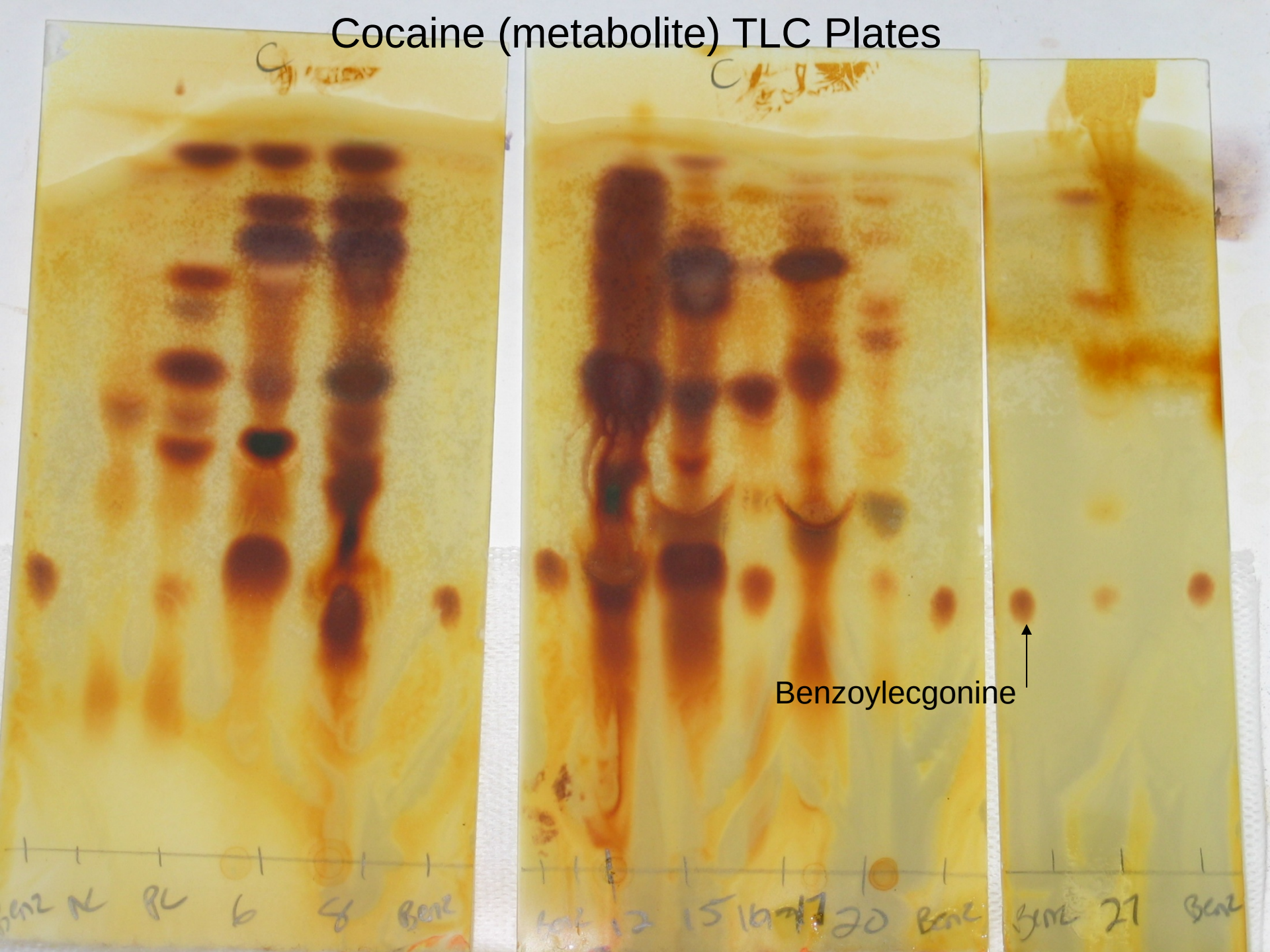
Barbiturate TLC Plates



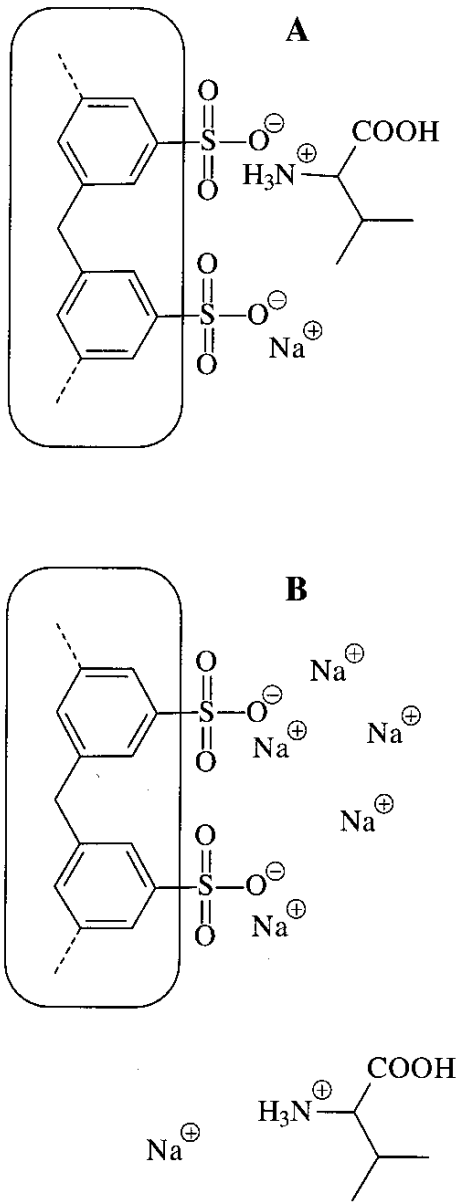
Narcotic Alkaloids TLC Plates

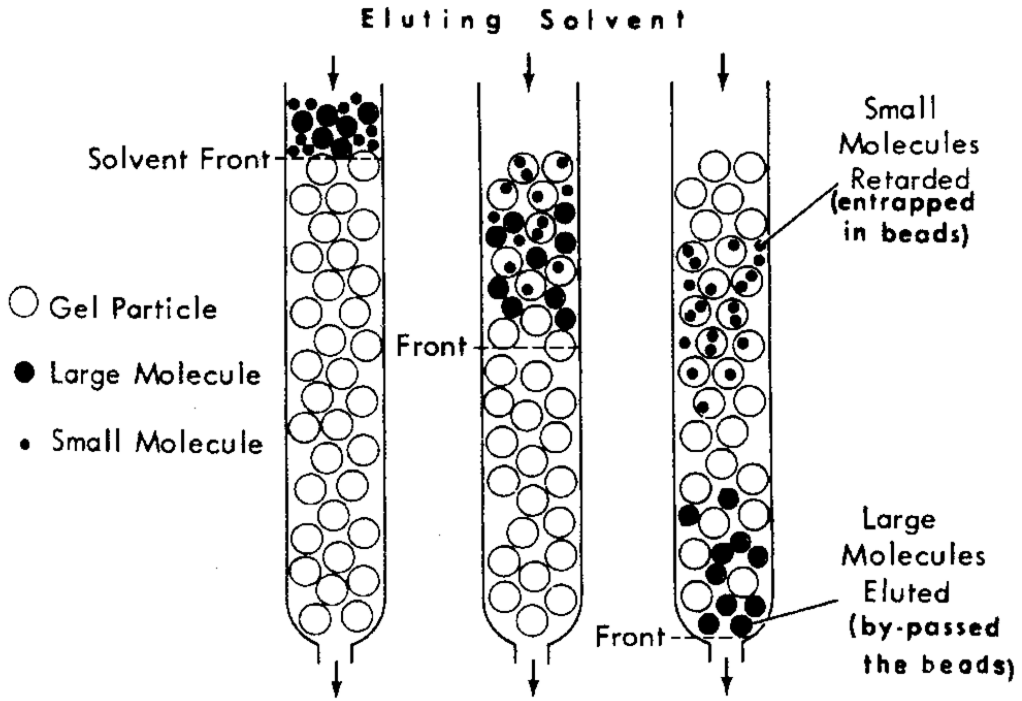


Cocaine (metabolite) TLC Plates



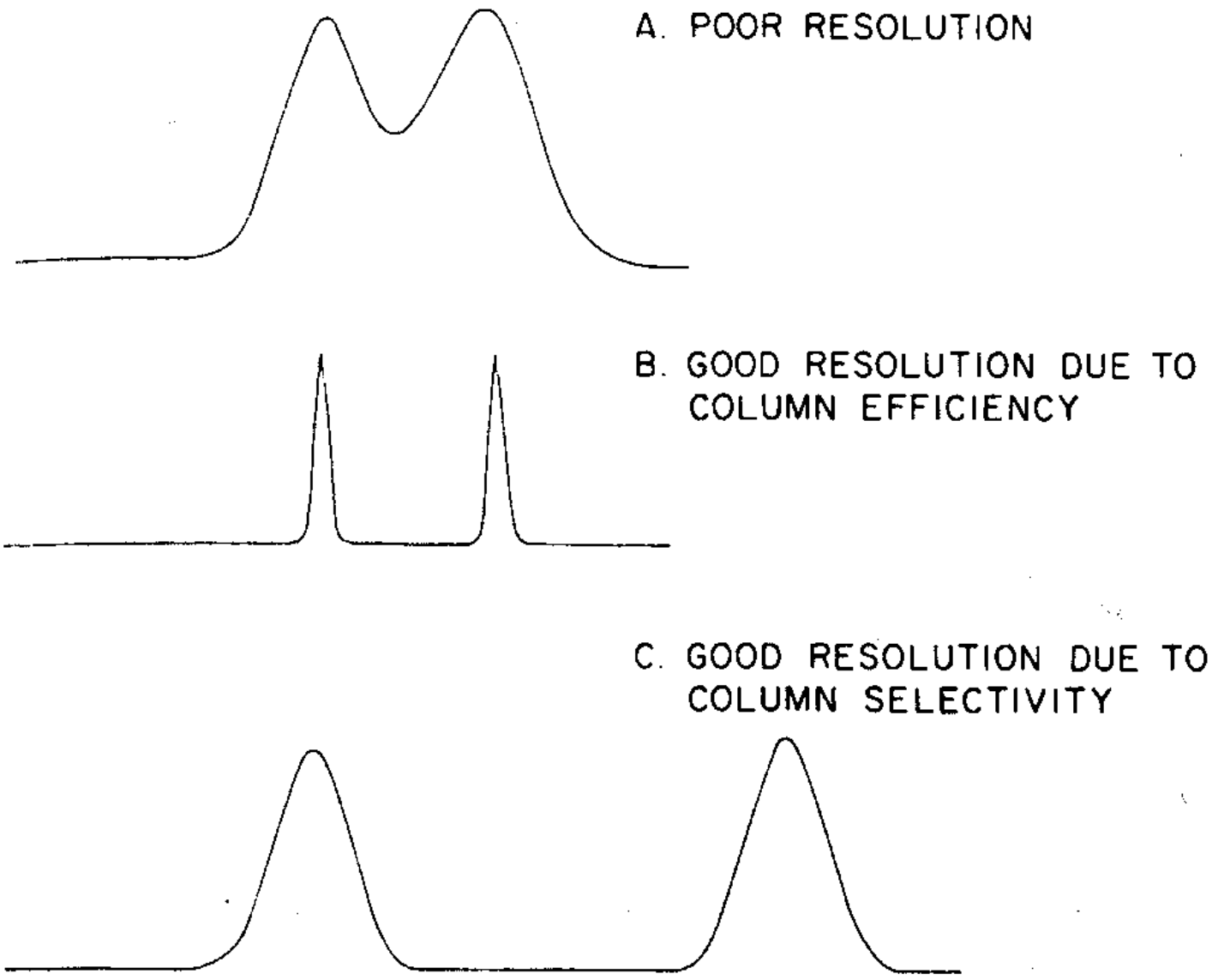
Affinity Chromatography





"Size Exclusion" or "Gel Filtration" Chromatography

Peak Resolution



Examples of LC Detection Methods

TABLE **8-4** | **Examples of Detectors Used in High-Performance Liquid Chromatographs**

Type of Detector	Principle of Operation	Range of Application	Detection Limit	Comments
UV photometer (fixed wavelength)	Measures absorbance of UV light	Selective	<1 ng	Analyte must absorb UV light or must be derivatized.
UV photometer (variable wavelength)	Measures absorbance of UV light	Selective	<1 ng	Can be "tuned" to a specific wavelength.
Diode array	Measures absorbance of light	Selective	<1 ng	Provides complete spectra.
Fluorometer	Measures fluorescence	Very selective	pg–ng	Analyte must fluoresce or must be derivatized.
Refractometer	Measures change in refractive index	Universal	1 μg	
Electrochemical	Electrochemically measures oxidized/reduced analyte	Selective	pg–ng	Useful for catecholamines.

TABLE 8-2 | Examples of Detectors Used in Gas Chromatographs

Type of Detector	Principle of Operation	Selectivity	Limit of Detection	Comments
Thermal conductance (TCD)	Measures thermal conductivity change in carrier gas on elution of compounds	Universal	<400 pg propane/mL He	
Flame ionization (FID)	$CHNO + heat \rightarrow CHNO^+ + e^-$; electrons collected for detection	Hydrocarbon	10–100 pg CHO	
Thermionic selective (TSD; NPD)	Alkali bead selectively ionizes N- or P-containing compounds	N, P	0.4–10 pg N 0.1–1.0 pg P	
Electron capture (ECD)	$e^- + R + N_2 \rightarrow Re^- + N_2 + e^-$; excess electrons collected; concentration inversely related	Electronegative groups	0.05–1.0 pg Cl-containing compounds	Relative response: I_2, Br_3, Cl_n, F_n – 10^6; I, Br_2, Cl_n, F_n – 10^5; Br, Cl_2, F_2 – 10^3; Cl, F, ketones, amines—10^2, ethers, esters—10; C_nH_{n+2} – 1
Mass spectrometer (MSD)	$e^- + ABC \rightarrow A^+ + BC$; monitor mass-to-charge ratio by either scanning or single-ion monitoring (SIM)	Universal (tunable)	1-ng scan 10-pg SIM	Can provide structural confirmation; ion ratios constant in SIM
Photoionization (PID)	$CHNO + photon \rightarrow CHNO^+ + e^-$; detect electron	Hydrocarbon	1–10 pg CHO	May be improvement on FID
Electrolytic conductivity (Hall)	Postcolumn reaction detector for selective detection of halogen-, S-, or N-containing compounds	Halogen-, S-, and N-containing compounds	0.1–1.0 pg Cl 2.0 pg S 4.0 pg N	
Flame photometric (FPD)	P- and S-containing hydrocarbons emit light when burned in an FID-type flame; emitted light detected	P- and S-containing compounds	0.9 pg CHP 20 pg CHS	
Fourier transform infrared (FTIR)	Infrared wavelength light absorbed by the compound of interest	Universal (tunable)	1 ng of strong infrared absorber	Can be scanned for structural information or absorbance-measured for quantitation

Summary of Major Learning Points

- Modular nature of chromatograpy.
  - Assays are divided into *sample preparation, separation* and *detection*.
  - The separation step involves *sample loading,* a *mobile phase* and a *stationary phase*.
- Importance of an internal standard for
  - Calculating "*relative retention times*",
  - Calculation of *relative peak areas* and generation of a calibration curve for quantification of drug concentrations in the original clinical sample.
- Analytical *specificity* provided by
  - Sample preparation techniques
  - Separation during chromatography (RRT)
  - Method chosen for detection