Sample Preparation for Toxicological Analysis

Laboratory Medicine Residency
Didactic Lecture Series

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Potential Preparative Steps

- Cell lysis or tissue homogenation
- Removal of Soluble Protein
  - precipitation
  - filtration
- Extraction
  - single step liquid-liquid extraction
  - multiple step liquid-liquid extraction (“back-extraction”)
  - solid phase extraction
- Chemical Modification
  - derivatization for increased volatility
  - chemical or enzymatic hydrolysis of glucuronide
- Concentration
  - evaporation

Protein Removal

- In general, soluble protein will interfere with chromatography of small, hydrophobic molecules and must be separated from the drugs/analytes.
- Gas chromatography of alcohols and glycols does not require protein removal due to the high volatility of alcohols and glycols ("just heat and serve").
- Three effective methods:
  - Protein precipitation by chemical means
  - Protein filtration ("protein-free filtrate")
  - Organic phase extraction

Protein Precipitation

- Generally performed chemically:
  - Organic solvents (acetone, acetonitrile, methanol)
  - Zinc sulfate in methanol (pH 7)
  - 5-sulfosalicylic acid in methanol (pH 1-2)
  - Perchloric or trichloroacetic acid
  - Sodium tungstate in sulfuric acid
  - Ammonium sulfate in HCl, heated
- Many of these methods also lyse RBCs and can be used for whole blood analysis (e.g. zinc sulfate in methanol for immunosuppressants).
- **Major concern** with all methods involves potential "trapping" of protein-bound drug in the precipitate.
  - Needs to be tested for empirically, i.e. hard to predict.
  - Can sometimes be extracted by washing the precipitate, but not always reproducible. Use of an internal standard can help.

Protein Filtration

- Uses a nitrocellulose (or equivalent) filter that specifically retains proteins greater than a given size (generally 3000 daltons). Most commonly, the solution is “pushed” through the filter using centrifugation.
- Separates “protein-free” from “protein-bound” drug. Used to get “free” drug concentrations.
- Note that the ratio of free/bound drug is NOT changed during the procedure (as long as you don’t do a wash step); therefore, measured concentration of free drug is *accurate*.
- Generally requires about 1 ml of plasma/serum to get about 0.1 ml of protein-free filtrate.

Organic Extraction

- Most “drugs” are hydrophobic in nature and, therefore, have greater solubility in organic phases than in aqueous solution.
  - Note that a majority (but not all) of the physiologic components of plasma and urine (protein, carbohydrates, electrolytes) are highly water soluble and are not extracted into an organic phase.
- The “organic phase” can be another liquid (e.g. hexane) or a solid, generally in the form of a column packed with polymer-coated beads.
- Organic extraction can be *selective* because the aqueous solubility of many drugs are pH dependent.

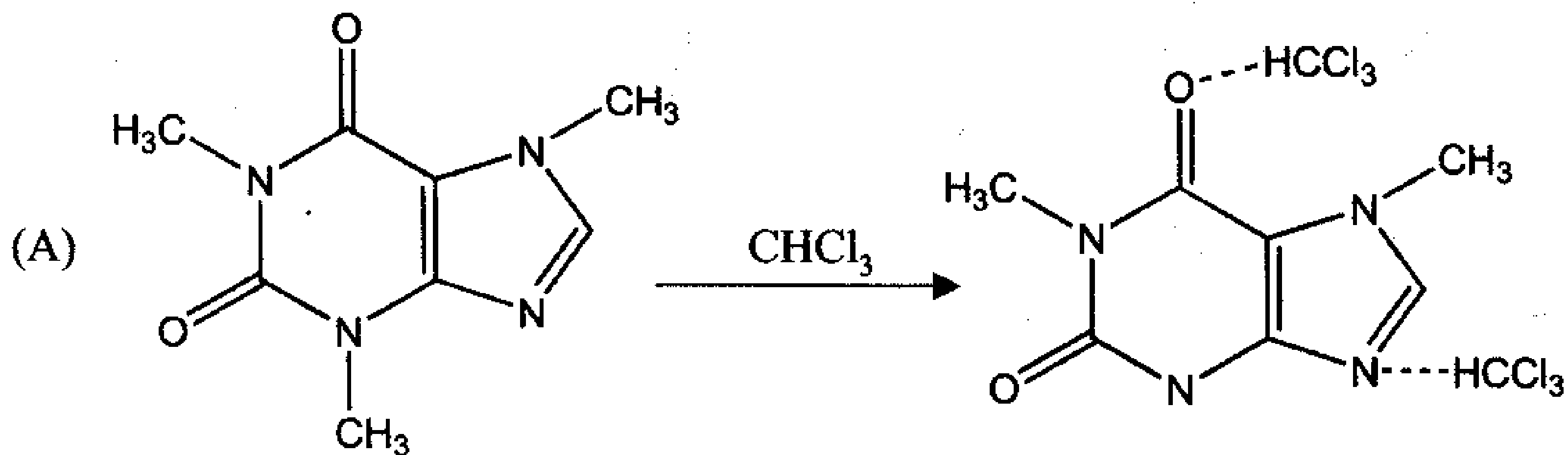
Organic Liquids Used for Extraction

Table 1. Key Properties of Organic Liquids Related to Their Utility as Extracting Solvents for Drug Substances

Solvent	Density (g/mL)	Boiling Point (°C)	g H_2O/L Saturation	Dielectric Constant	Hydrogen Bonds	
					H Donor	H Acceptor
n-Hexane	0.66	68	0.045	1.89	No	No
Toluene	0.87	111	0.46	2.38	No	No
1-Chloroform	0.89	78	0.90	7.4	No	No
Chloroform	1.49	61	1.24	4.8	Yes	No
Dichloromethane	1.34	40	11.9	9.08	No	No
Ethyl ether	0.71	35	17.0	4.33	No	Yes
Methyl t-butyl ether	0.74	55	20.3	na[a]	No	Yes
Ethyl acetate	0.90	77	29.4	6.0	No	Yes
1-Butanol	0.81	118	170	17.8	Yes	Yes
2-Propanol	0.79	82	Miscible	18.3	Yes	Yes
Acetone	0.79	56	Miscible	20.7	No	Yes
Acetonitrile	0.79	80	Miscible	37	Yes	Yes

[a]Value is not available.

Hydrogen Bonding with Solvent



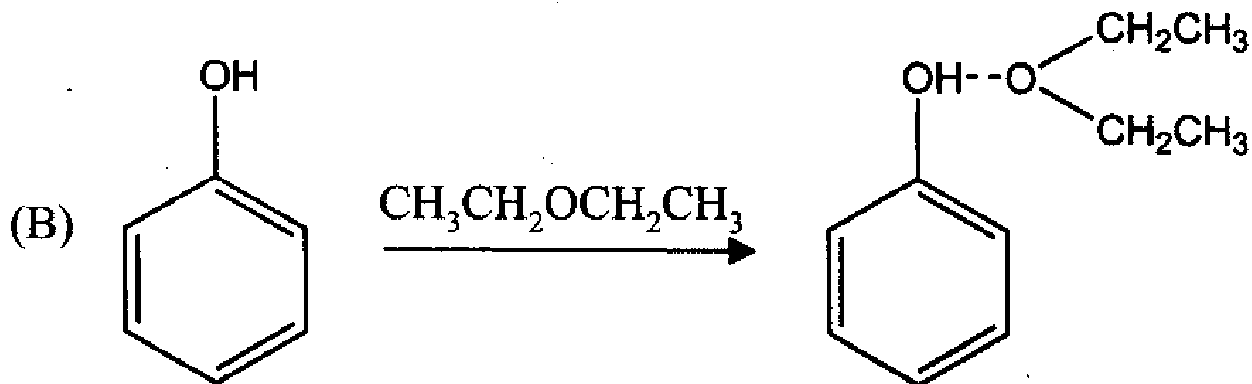


Table 2. Percent Recoveries of Fourteen Drugs by Extraction with Each of Eight Solvents (Extracting Solvent Partitioned Against An Equal Volume Aqueous Buffer)

	Extracting Solvent							
Drug	Hexane	1-Chloro-butane	Dichloro-methane	Chloro-form	Isopropyl Ether	Ethyl Ether	Ethyl Acetate	1-Butanol
Chloramphenicol[a]	0	0	22	0	15	56	58	41
Hydrochlorothiazide[b]	0	4	8	0	12	30	92	84
Salicylic acid[c]	0	59	83	83	100	98	98	100
Morphine[d]	0	12	25	33	7	20	52	91
Caffeine[a]	0	10	81	90	6	9	37	54
Procainamide[e]	6	29	90	92	21	21	84	92
Pentobarbital[f]	4	66	97	96	98	95	98	99
Benzocaine[e]	29	82	100	100	88	88	94	94
Propoxyphene[e]	48	43	96	96	71	97	—	99
Imipramine[e]	35	79	88	98	84	70	99	100
Methaqualone[a]	78	90	93	93	89	91	95	97
2, 5-Dimethoxy-4-methylamphetamine[e]	87	98	100	100	95	95	93	100
Cocaine[e]	89	96	95	96	94	95	99	99
Haloperidol[e]	99	99	99	100	100	100	100	100

[a]pH is 7.
[b]Extracted at pH 4; poor recoveries were obtained at all pHs, with a pH of 2–6 giving the best recoveries.
[c]pH is 1.
[d]pH is 9.
[e]pH is 11.
[f]pH is 5.

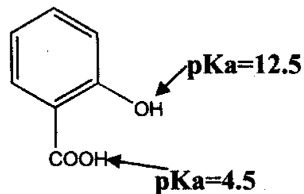
Acid-Base Equilibria

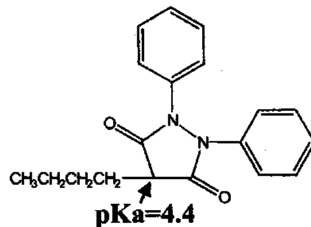
The two most common ionizable groups in organic molecules are carboxylates and amines, although others (such as hydroxyls) do exist.

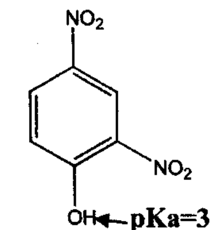
Acid-Base Equilibria: pKa

- The “pKa” quantifies the equilibrium between the “protonated” and “non-protonated” states:
  - “$pH = -\log[H^+]$” and “$pKa = -\log(Ka)$”
  - $pH = pKa + \log([\text{non-protonated}]/[\text{protonated}])$
- When the pH < pKa, there are more “protonated” molecules than “non-protonated”.
  - Carboxylate: $[COOH] > [COO^-]$, favors neutral charge and is more soluble in organic phase.
  - Amine: $[NH_3^+] > [NH_2]$, favors positive charge and is less soluble in organic phase.
  - For each pH unit, there is a ten-fold increase in ratio of protonated/non-protonated molecules.
- Of course, when the pH > pKa, the opposite is true.

(A) Strongly acidic drugs

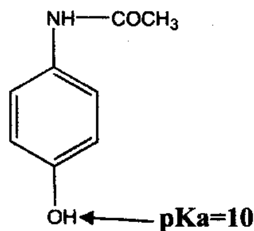


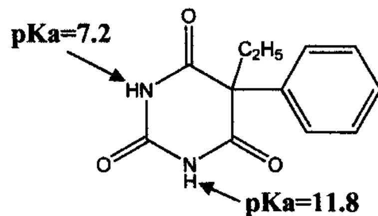


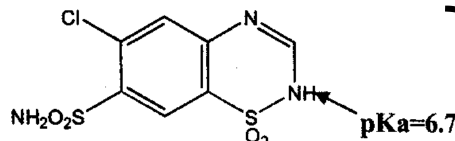


Generally negatively charged at neutral and mildly basic pH.

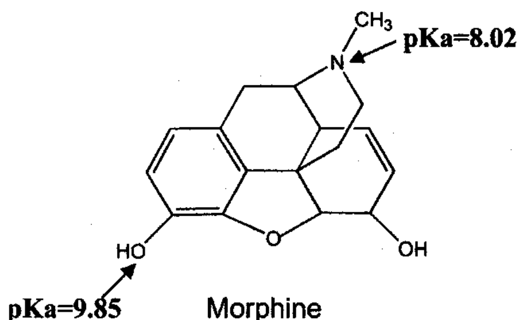
(B) Weakly acidic drugs

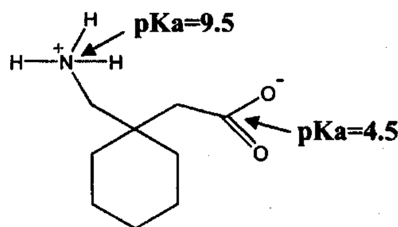


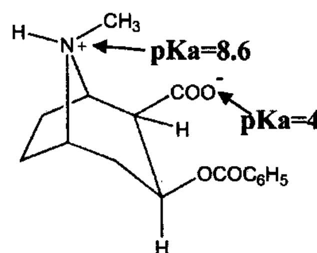




(C) Amphoteric basic drugs

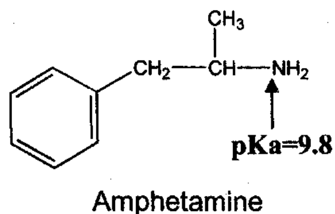


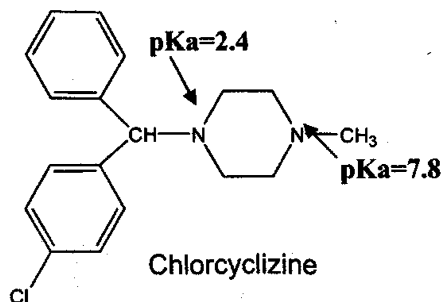




Variably charged across a range of pH values.

(D) Strongly basic drugs





Generally positively charged at neutral and mildly acidic pH.

Example Separation of Three Drugs

Salicylic Acid: -COOH (pKa 4.5) & -OH (pKa 12.5)

BZE: -COOH (pKa 4) & $-R_3NH^+$ (pKa 8.6)

Amphetamine: $-RNH_2^+$ (pKa 9.8)

Step 1: Acidify to pH = 2 and extract with organic phase

- Salicylic Acid: -COOH and -OH, net neutral charge (**soluble in organic phase**)
- BZE: -COOH and $-R_3NH^+$, net positive charge (water soluble)
- Amphetamine: $-RNH_2^+$, net positive charge (water soluble)

Step 2: Add strong base (NaOH) to bring pH > 11 and extract with organic phase

- BZE: $-COO^-$ and $-R_3N$, net negative charge (water soluble)
- Amphetamine: -RNH, net neutral charge (**soluble in organic phase**)

Step 3: BZE remains in the aqueous phase and can be further purified.

“pKa-based” Selection of Drug Class

Amphetamine: $-RNH_2^+$ (pKa 9.8)

Step 1: Add strong base to bring pH = 11

Amphetamine: -RNH, net neutral charge (**soluble in organic phase**)

Step 2: Extract with organic phase to bring any neutral drug or other base with pKa < 11.

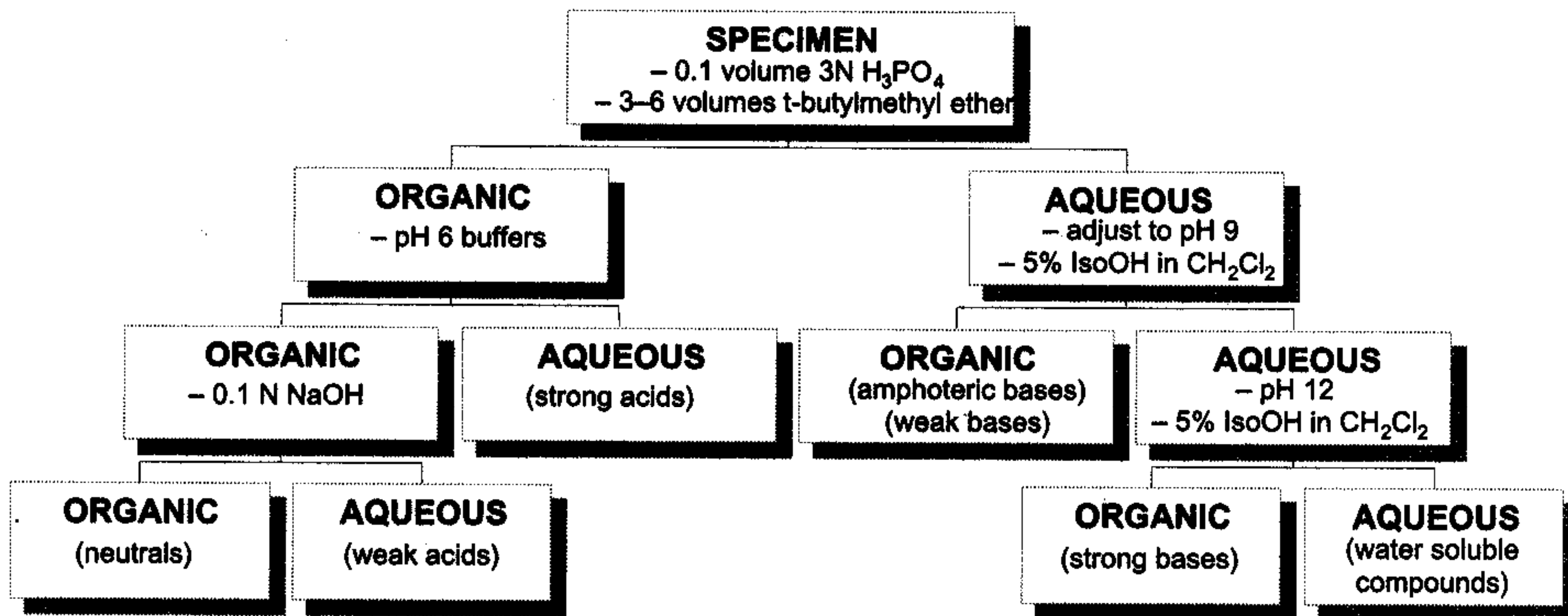
Step 3: Prepare an aqueous buffer for “back-extraction” with pH = 8.5.

Amphetamine: $-RNH_2^+$, net positive charge (**water soluble**)

Step 4: Back-extraction of organic phase from “step 2” will now bring back any drugs that have become charged at pH = 8.5 and will leave behind all neutral compounds.

Hence, this protocol has selectively purified drugs that are neutral at pH 11 and charged at pH 8.5, thus eliminating a LOT of other drugs (one of the “three sources of specificity”).

Generic Scheme for Fractionation of a Mixture of Drugs with Varying Acidity/Basicity



H_3PO_4 = phosphoric acid
IsoOH = isopropanol
NaOH = sodium hydroxide
CH_2CL_2 = dichloromethane

Three Sources of Specifity/Drug Identification

1. Sample Preparation
   - selective extraction or purification
2. Chromatography
   - separation of individual molecules
   - identification by relative retention times
3. Detection
   - detection methods can be selective for drug class
   - of course, mass spectrometry is most selective

Solid Phase Extraction

- Works essentially the same as liquid extraction, but substitutes a column packed with polymer-coated beads for the organic phase.
- Basic protocol is to adjust the pH of the solution, run it over the column, wash the column with a buffer of the same pH, and then elute the drugs off of the column using either an organic solvent or an aqueous solution of a different pH.
- Note that the columns may be “multi-functional” combining a charged group with hydrophobic surface area, as used for our GC/MS procedure for drug confirmation.
- Some columns are clogged by lipid or protein, so may need to clean up sample initially.

Hydrolysis

- Many drugs are excreted after hepatic conjugation (most often to glucuronide),
  - rendering them less reactive in immunoassays
  - and preventing them from being extracted into organic phases for chromatographic analysis.
- Glucuronide can be hydrolyzed either chemically (e.g. strong acid) or enzymatically to give the free drug.
- We use a glucuronidase before GC/MS confirmation of opiates. We might consider doing this as well for the benzodiazepine and opiate immunoassays (EMITs).

Derivatization

- Gas Chromatography requires a volatile compound for analysis.
- Alcohols and glycols are highly volatile; hence, with gentle heating, they selectively evaporate from serum/plasma and can be analyzed.
- On the other hand, a majority of drugs are not sufficiently volatile for effective detection by GC/MS.
- A number of chemicals (MSTFA, BSTFA, etc.) can be added to urine that react with charged or polar atoms in small molecules to give "trimethylsilyl" (TMS) derivatives that are more volatile than the parent compounds. Hence, during GC/MS we often detect "TMS-BZE" or "TMS-morphine" instead of the original compounds.
- Note that derivatization also changes the fragmentation pattern during MS; this can be utilized to advantage if the original compound won't fragment or if the product ions are overlapped.

TABLE 8-3 | Common Derivatization Reactions for Gas Chromatography

Functional Group	Reaction	Typical Reaction	Comments
$R-OH$	Acylation	$R-OH \xrightarrow{CH_3C(=O)-O-C(=O)CH_3} R-OC(=O)CH_3$	Acetylation is a stable derivative for GC and GC/MS.
	Silylation	$R-OH \xrightarrow{F_3C-C(=O)-N(CH_3)-Si(CH_3)_3} R-O-Si(CH_3)_2-CH_3$	MSTFA is useful for acid-sensitive compounds.
$R-COOH$	Esterification	$R-COOH \xrightarrow{CH_3OH \cdot HCl} R-C(=O)O-CH_3$	
	Silylation	$R-COOH \xrightarrow{F_3C-C(=O)-N(CH_3)-Si(CH_3)_2-C(CH_3)_2-CH_3} R-C(=O)-O-Si(CH_3)_2-C(CH_3)_2-CH_3$	MTBSTFA derivative is particularly useful for CG/MS; loss of m/z 57 (*t*-butyl group) is prominent ion.
$R-C(=O)-R'$	Oximation	$R-C(=O)-R' \xrightarrow{H_2NOH} R-C(=N-OH)-R'$	Oximation usually results in formation of two isomers that may be separable by GC.
$R-NH_2$	Acylation	$R-NH_2 \xrightarrow{F_3C-F_2C-C(=O)-O-C(=O)-CF_2-CF_3} R-N(H)-C(=O)-CF_2-CF_3$	Acylation with fluorocarbons provides a more volatile derivative.
	Silylation	$R-NH_2 \xrightarrow{F_3C-C(O-Si(CH_3)_3)=N-Si(CH_3)_3} R-N(H)-Si(CH_3)_2-CH_3$	BSTFA is a relatively reactive silylation reagent; reaction with secondary amines may require forcing conditions.

Sample Preparation for GC/MS

Step 1: “Hydrolysis”

- add β-glucuronidase to 1.0 ml of patient urine
- incubate at 65 °C for 3 hours
- centrifuge to remove debris after cooling to room temperature

Step 2: “Extraction”

- add 10 μl of internal standard solution to each sample.
- add 0.5 of 100 mM phosphate buffer at pH 6.0 (check pH and adjust)
- run each sample over a pre-washed solid-phase extraction column
- wash column extensively with aqueous phosphate buffer
- acidify with 0.1 N HCl and elute with methanol

Step 3: “Concentration”

- evaporate methanol by blowing dry nitrogen over sample

Step 4: “Derivatization”

- add 75 μl of MSTFA and heat at 70 °C for 20 minutes
- volume remaining after derivatization is transferred to autosampler for injection

Review of Preparative Steps

- Cell lysis or tissue homogenation
- Removal of Soluble Protein
  - precipitation
  - filtration
- Extraction
  - single step liquid-liquid extraction
  - multiple step liquid-liquid extraction (“back-extraction”)
  - solid phase extraction
- Chemical Modification
  - derivatization for increased volatility
  - chemical or enzymatic hydrolysis of glucuronide
- Concentration
  - evaporation