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Strategies for the use of SPE

Reserch Article

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Introduction

In this paper we discuss four basic strategies used in solid phase extraction [1] filtration, [2] selective adsorption, [3] copolymeric extraction, and [4] immunoaffinity. Most extractions will 1fall into one of these categories.

Solid Phase Extraction (SPE) as a Filter

One of the first uses of SPE was as a filtration device in pharmaceutical laboratories where scientists were extracting active compounds from fermentation broths. SPE cartridges were used to remove as much of the matrix as possible, allowing active compounds to pass through the columns and to be collected. This technique has evolved into a more sophisticated large-scale type of chromatography, called flash chromatography, used by organic synthetic chemists. Flash chromatography has not found a place in forensic toxicology as yet. Figure 1 shows a graphic demonstration of this type of approach to SPE. The example shown is the isolation of γ-hydroxybutyric acid (GHB) from human urine. Urine samples are filtered by a special sorbent that holds interfering substances and allows the analytes of interest to pass through the column to be analyzed. In this case the biggest contaminant is urea, which is effectively removed by the SPE column.

The following describes a forensic application for GHB from human urine that uses SPE as a filtering step followed by additional liquid-liquid extraction techniques.

GHB has become widely known as the "sex drug for the 1990s. In the new "mi1lenium, GHB has become popular with college students and club-goers as a mood modulator. Cases of GHB use were rare, but have become numerous over the last two years. On ingestion, it reduces inhibitions and reportedly increases libido. Other popular street names include "scoop" and "liquid ecstasy." This drug has been classified as a date rape drug, along with lorazepam, ketamine, and flunitrazepam.

GHB is an endogenous human metabolite structurally similar to the neurotransmitter γ-amino butyric acid (GABA) [1,2]. It was first synthesized and used in Europe as an anesthetic, but was later discontinued because this drug was widely sold in health food stores as a weight control drug (functionally similar to L-tryptophan) and to induce the secretion of growth hormone for body building. In 1990 the sale and distribution of GHB was federally banned owing to its potentially harmful effects [3].

GHB is easily synthesized from γ-butyrolactone, a compound that is found in some commercial products. Figure 2 shows the chemical structures of GHB and butyrolactone. The Internet has popularized this drug by giving recipes on how to e manufacture it. One particular book claims the drug to be a "natural mood enhancer" [4]. In clandestine preparations the concentrations vary significantly, leading to problems with overdosage. Harmful contaminants from low-quality ingredients may also appear. In the 1990s more than 20 deaths in the United States were attributed to the of sort use of GHB, generally in conjunction with alcohol ingestion [5].

GHB is a small polar molecule that is very difficult to separate for a qualitative determination. In many methods it is extracted following conversion to butyrolactone and chemically derivatized by silylation [3,6,7]. This method allows for the chemical derivatization of the parent compound without the formation of the lactone. Instrumental analysis is performed using gas chromatography-mass spectrometry (GC-MS).

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Figure 1. The use of an SPE column as a filter

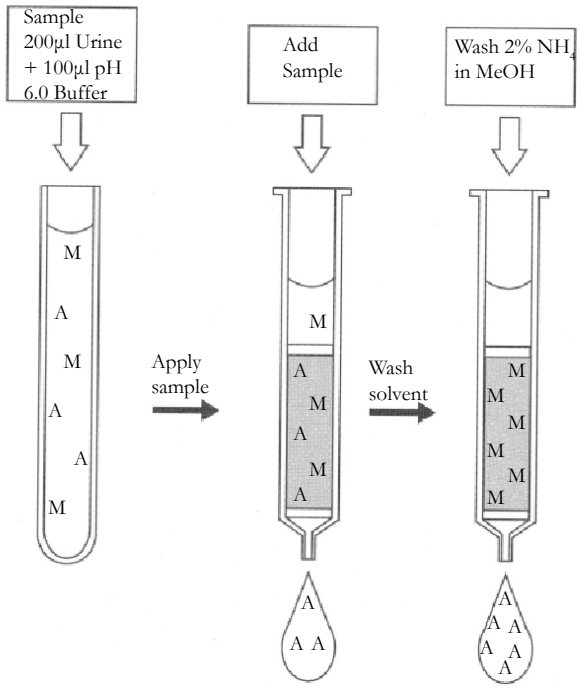
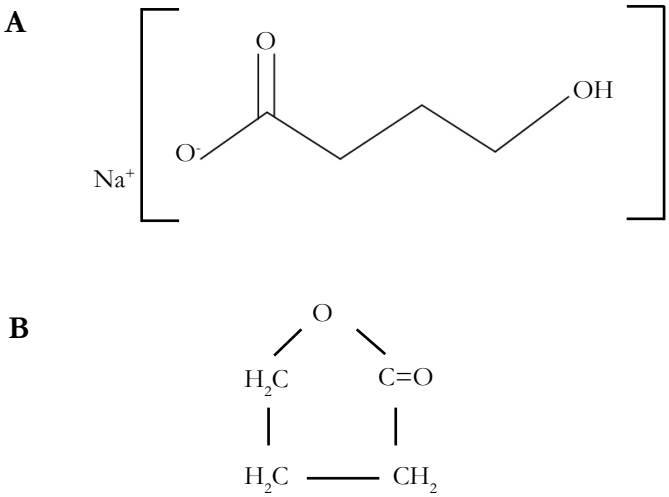


Figure 2. Chemical structures of (A) γ-hydroxybutyric acid and (8) butyrolactone



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Materials and Methods for GHB Analysis

Reagents

Certified American Chemical Society (ACS)-grade hexane, sodium phosphate monobasic, sodium phosphate dibasic, dimethylformamide, ammonium hydroxide, and HPLC-grade methanol and ethyl acetate were all purchased from Mallinckrodt (Phillipsburg, PA). Distilled water was prepared using a millipore purification system, bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane (TMCS) from United Chemical Technologies, Inc. (Bristol, PA).

Standards and Solutions

a. GHB sodium salt, was purchased from Aldrich (Milwaukee, WI).
b. GHB-D6 was purchased from Cerrilliant (Austin, TX). The GHB and GHB-D6 were prepared to 0.1 mg/mL in methanol.
c. Prepare the 0.1 M phosphate buffer, pH 6.0, by dissolving 1.70 g of Na_2HPO_4 and 12.14g of $NaH_2PO_4 \cdot H_2O$ in 800 mL of DI H_2O. Dilute to 1000 mL using DI H_2O. Mix. The solution is stable for at least 1 month.
d. Adjust pH to 6.0 ±0.1 with 0.1 M monobasic or dibasic sodium phosphate.
e. The CH_3OH-NH_4OH (99:1) is prepared fresh daily.

Sorbent

The extraction columns were CLEAN SCREEN ZSGHB020 containing 200 mg of sorbent in a 10-mL column and were manufactured by United Chemical Technologies, Inc.

Instrumentation

A Hewlett Packard 5971A Mass Selective Detector, a 7673 Autosampler, and a 5890 Gas Chromatograph fitted with a 30-m, 0.25 mm i. d. , 0.25μm film thickness Rtx-5 (comparable to a DB-5 or HP-5) were from Restek (Bellefonte, PA).

GC conditions		**Oven temperature program**
Column head pressure	8 psi	Initial temperature 70°C, hold 1.00 min
Injection port temp.	2500C	Ramp to 100^0 C at 15^0 C/min
Transfer line temp.	2800 C	Ramp to 175^0 C at 25^0 C/min
Split vent flow	50 m L/min	Ramp to 280^0 C at 35^0 C/min
Septum purge flow	2.6 m L/min	
Equilibration time	0.5 min 0.1 min	Total run time = 9.00 min Purge on time

Injection Volume 1μL, Splitless injection
Run time is extended past the elution of the GHB to eliminate any residual BSTFA or urine byproducts.

GC-MS Analysis

The method uses selected ion monitoring (SIM) for three ions for each analyte. The dwell times were set to 30 ms per ion, resulting in 3.62 cycles per second. The most prevalent ions for GHB-diTMS are 147, 233, 148, 149, 204, 143, and 234 m/z. The most prevalent ions for GHB-D6-diTMS are 147, 239, 148, 149, 206, and 240 m/z. Urea is also derivatized by BSTFA to form a diTMS derivative. It elutes near GHB and has many of the same ions including 147, 148, and 149; therefore, some of the less abundant ions must be used for the SIM analysis. Table 1 lists these ions.

Urine GHB Extraction Procedure Using a ZS

1. Sample preparation
   a. a.To 200 μL of urine add internal standard (GHB·D6) and 100 μL of 0.1 M phosphate buffer, pH 6.0.
   b. Mix/vortex.

2. Condition CLEAN SCREEN@GHB extraction column.
   a. 1 x 3 mL of CH_3OH; aspirate.
   b. 1 x 3 mL of DI H_2O; aspirate.
   c. 1 x 0.5 mL 0.1 M phosphate buffer, pH 6.0; aspirate.
   Note: Aspirate at ≦3 in. Hg to prevent sorbent drying.

3. Apply sample.
   a. Place test tubes into vacuum manifold for collection.
   b. Collect both the sample loading and wash.
   c. Decant sample onto column. Aspirate at ~1 in. Hg.

4. Wash column
   a. Add 1 mL of CH3OH-NH4OH (99:1) to sample test tube; vortex.
   b. Decant wash onto column.
   Note: Aspirate at ~1 in. of Hg.

5. Concentrate.
   a. Remove test tubes from vacuum manifold.
   b. Evaporate to dryness at 60^0 C using a stream of air or nitrogen gas.

6. Sample Cleanup.
   a. Add 200μL of dimethylformamide.
   b. Add 1 mL of hexane saturated with dimethylformamide.
   c. Mix by inversion for 5 min.
   d. Centrifuge at 1500g for 5 min.
   e. Transfer lower dimethylformamide layer to a clean test tube.

RT: retention time

Table 1: Ions Using SIMS Analysis and Quantitation Ion

RT (min)	Name	Target ion (% of target ion)	Second ion (% of target ion)	Third ion (% of target ion)
5.40	GHB-D6-diTMS	239.2 (100)	240.2 (20)	241.1 (9)
5.43	GHB-diTMS	233.1 (100)	234.1 (19)	235.0 (7)

GHB-D6-diTMS 239, 240, 241

7. Concentrate.
   a. Evaporate to dryness at 50^0 C using a stream of air or nitrogen gas.
8. Derivatize.
   a. Add 100μL of ethyl acetate and 100μL of BSTFA (with 1 % TMCS).
   b. Mix/vortex.
   c. No heating is required.
9. Quantitate.
   a. Inject a 1-to 2 μL sample onto GCMS.
   b. Monitor the following ions:
   GHB-diTMS 233, 234, 235

Results

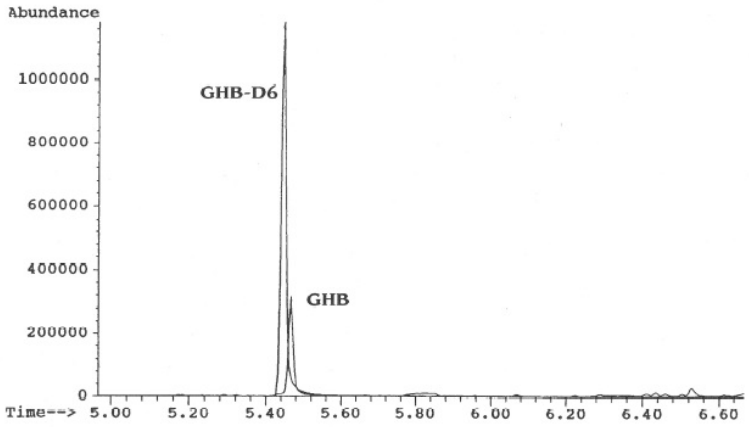
Chromatography

See Figure 3.

Recovery

Five 50 mg/L standards were prepared by adding 10μg of GHB to 200μL of drug-free urine. These standards were extracted using the previously described procedure. The internal standard

Figure 3: Chromatogram of the selected target ions showing GHB and GHB-D6 from a 5 m g/L extracted urine standard.



(4µg of GHB-D6) was added immediately prior to the evaporation of the DMF. Five 50 mg/L unextracted standards were prepared by adding 10µg of GHB and 4µg of GHB-D6 to a test tube that was dried at 50°C. All samples were derivatized using the procedure previously described. The recovery was calculated by comparing the area under the curve of the target ion for the extracted and the average of the unextracted standards. The average recovery was 67.2% (range =76%-58%).

Linearity

The assay is linear from 1 to 100 mg/L with the upper range limited by the saturation of the detector. The method could be run with a split injection if higher concentrations need to be quantified.

Stability

The diTMS derivatives are stable for more than 7 d at room temperature.

Sensitivity

The assay is sensitive to 1 mg/L.

Interferences

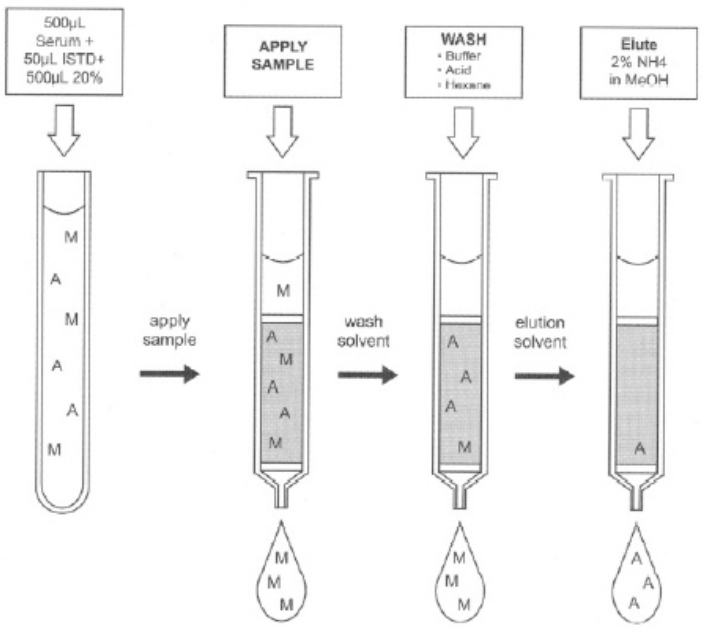
The only interference identified in this procedure was from urea. It is derivatized along with GHB to form urea-diTMS. Using the SIM ions listed above eliminates the interference.

Summary

This procedure allows for the direct analysis of GHB and eliminates any possibility of forming or extracting γ-butyrolactone (GBL). Conversion to GBL is problematic in forensic analysis where litigation is involved because GHB is a scheduled drug in many US states and GBL is not. This method was designed to identify low levels of GHB in urine and requires only 200µL of sample. Expected concentrations in biological samples may be much higher and therefore a smaller sample size should be used [6]. The method utilizes a novel copolymeric sorbent employing SPE as a filter. A sample cleanup step and silylation followed by GCMS analysis is also incorporated into the method. The derivatization with BSTFA with 1% TMCS is accomplished without a heating step, owing to the high reactivity of the BSTFA to the GHB. The derivative is stable for more than a week at room-temperature conditions.

SPE-Selective Adsorption

A second approach to SPE is to selectively bond the analyte of interest while allowing the matrix to pass through. This is prob-



ably one of the more common approaches to SPE. Figure 4 is a graphic demonstration of this approach. The analysis of gabapentin in human serum is presented to illustrate how this sample preparation is performed.

Gabapentin [(l-aminomethyl-l-cyclohexyl) acetic acid] is an anticonvulsant agent used as an adjunctive therapeutic agent in the treatment of seizure disorders not adequately controlled by standard monoanticonvulsant therapy. Adequate drug levels are critical to controlling seizures.Chemically, gabapentin is an interesting drug to isolate because of three functional groups on its molecule an amine group (cationic), a carboxylic acid (anionic), and the ring structure (neutral, reversed phase extraction).

Figure 5 shows the chemical structures of gabapentin and the compound used as the internal standard in this analysis. Section 2.1 shows the extraction of gabapentin from serum or whole blood. Section 2.2 shows the data from the assay validation for this method.

Presented in this application is a specific and selective gas chromatographic method using a nitrogen-phosphorus detector (1). The selectivity of this method is illustrated in Table 2, which lists compounds found not to interfere with the gabapentin assay from more than 40 drugs screened for potential interference with this method. Figure 6 shows the chromatograms of the serum extracts.

Gabapentin in Serum, Plasma, or Whole Blood for GC or GC-MS Analysis Using a 200-mg CLEAN-UP C_{18} Extraction Column

1. Prepare sample
   a. Place 500µL of sample, calibrator, or control into a 10 x 25 mm disposable glass test tube and add 25µL of internal standard (5.0 mg/L).
   b. Vortex tube.

Figure 5: Gabapentin/Neurontin@ [(l-aminomethyl-l-cyclohexyl) acetic acid.

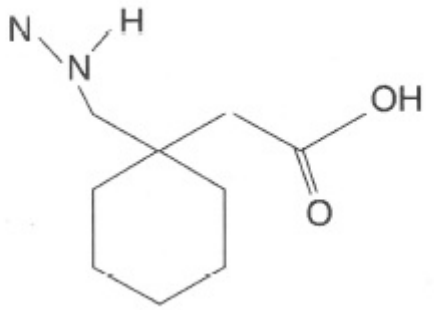
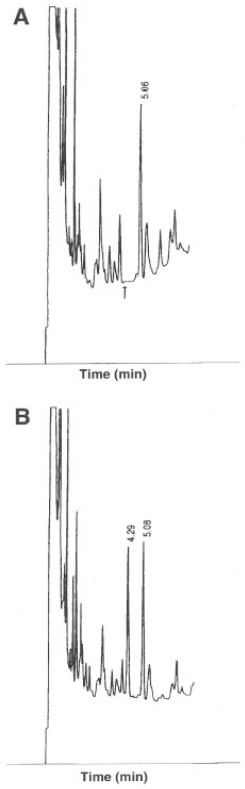


Table 2: Compounds Found Not to Interfere with Gabapentin Assay

Acetaminophen	Amikacin
Amylase	Amitriptyline
Benzoylecgonine	BUN*
Caffeine	Carbamazepine
Chloramphenicol	Cholesterol
Creatinine	Cyclosporine
Digoxin	Disopyramide
Estradiol	Ethosuximide
γ-Hydroxybutyrate	Gentamicin
Glucose	HCG*
LDH*	Lidocaine
Lithium	Methotrexate
m-Hydroxybenzoic acid	NAPA*
Netilmicin	Nortriptyline
p-Aminobenzoic acid (PABA)	Phenobarbital
Phenytoin	Primidone
Procainamide	Quinidine
Salicylate	Theophylline
Thyroxine	Tobramycin
Triiodothyronine	Triglycerides
Uric acid	Valproic acid
Vancomycin	

*Abbreviations: BUN, blood urea nitrogen; HCG, human chorionic gonadotropin; LDH, lactate dehydrogenase; NAPA, n-acetylprocainamide

Figure 6: Gabapentin analysis. Chromatograms of serum extracts: (A) Internal standard present (5.06 min). (B) Gabapentin 5.0 m g/L (4.3 min) and internal standard (5.08) present. Time (min)



c. Add 500µL of 20% acetic acid and vortex tube again.

2. Condition SPE column.
   a. 1 x 3 mL of CH_3OH; aspirate.
   b. 1 x 3 mL of DI H_2O; aspirate
   c. 1 x 3 mL of 1 N HC1; aspirate.

3. Apply sample.
   a. Load at 1 m L/min.

4. Wash column.
   a. 1 x 3 mL of DI H_2O; aspirate.
   b. 1 x 3 mL of ethyl acetate.
   c. 1 x 3 mL of hexane.
   d. Dry column (5 min at >10 in. Hg or until column is dry).

5. Elute Gabapentin.
   a. 1 x 1 mL of 2% NH_4OH in MeOH.
   b. Evaporate to dryness at 40^0C in a water bath.

6. Dervizatize.
   a. Add 50µL of MTBSTF A + 1 % BDMCS reagent to the residue.
   b. Cap tube and put into a water bath at 70^0 C for 30 min.
   c. Remove and allow to cool for 5-10 min.

7. Quantitate.
   a. Insert 1-2µL of the sample onto the chromatograph.

Assay Validation

a. Linearity was observed from 0.2 to 30 mg/L.
b. Within-run precision:
   7.0% for 1.1 mg/L (N= 10)
   3.4% for 4.5 mg/L (N= 10)
c. Between-run precision:
   12% for 1.1 mg/L (N= 16) over 3 wk
   5.0% for 4.5 mg/L (N=16) over 3 wk
d. Recovery of gabapentin: Internal standard
   1.0 mg/L: 46% 69% (N= 10)
   5.0 mg/L: 57%
   20.0 mg/L: 51 %

Summary

A simple, rapid, and selective method for gabapentin is presented that uses a reversed phase separation. Sample chromatograms can be seen in Figure 6. The pH of the sample was used to suppress the ionization of the carboxylic acid group. Ethyl acetate and hexane were used to wash the matrix away from the sorbent-isolated drug.

Gabapentin has a limited solubility in these solvents. Elution at an alkaline pH ionized the acid portion of the drug thus freeing the drug from the sorbent. Increasing the ammonium hydroxide concentration did not improve the recovery of the analyte and could produce ammonium salts, requiring further sample purification.

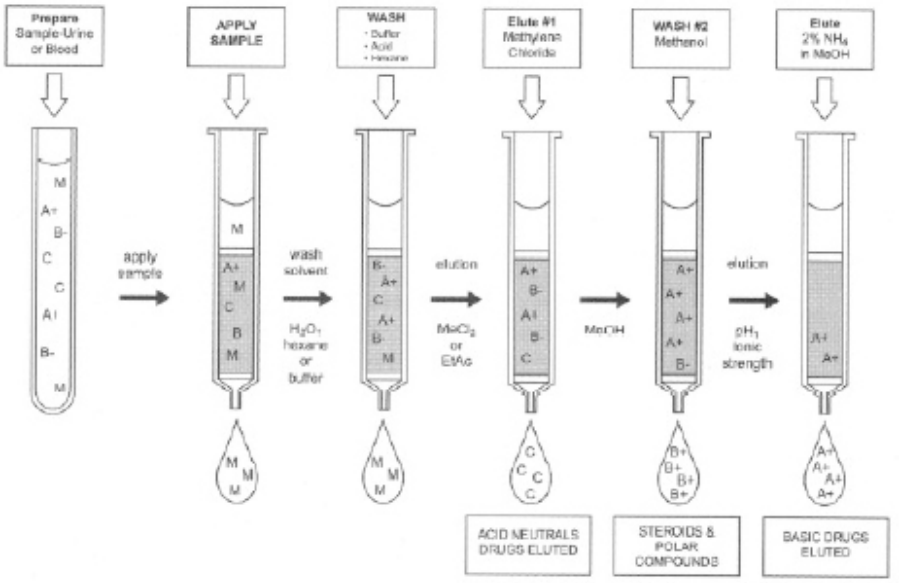
The derivatization of gabapentin with MTBSTFA and 1 % BDMCS improved the peak shape of the analytes and also increased the molecular weights, thus increasing their retention time on the chromatographic system.

Derivatized gabapentin samples were stable for up to 1 d with a<10% loss due to chemical degradation.

SPE in Copolymeric Interaction

Figure 7 shows a third approach to SPE, referred to as "copolymeric" or selective extraction. This is a most useful approach

Figure 7: Copolymeric interaction, forensic drug assay



in doing class extractions or drug-plus-metabolite extractions in groups of compounds. An example of this type of approach can be seen in the general method used for forensic samples.

The versatility of SPE can be best exhibited by its usage in the separation of a wide variety of drugs using a combination of separation strategies. The widest variety of separations have been developed using the combination of a C_8 with a cationic exchanger, usually benzene sulfonic acid. CLEAN SCREEN DAU, Bond Elute Certify@, SPECII@, Isolute@, and NARCII@ are a few of the products that currently use this combination and are termed "copolymeric sorbents".

This mechanism works by providing separation of the acid/neutral drugs using reversed phase C_8 functionality; the benzene sulfonic acid mechanism works on basic drugs by cation exchange of the amine functionalities. A variety of drugs that have been extracted by the CLEAN SCREEN DAU extraction column using the method found in sections 3.1 and 3.2. This method is an example of the kinds of methods that are developed in toxicology laboratories where a general method is used to identify drugs in biological samples for clinical management and toxicological significance. Section 3.2 shows the extraction procedure used for forensic drug analysis.

General GC-MS Method

GC-Column	Selectra@ CC-5 or DB-5, HP-5 or equivalent capillary column.
Specifications	30 m long x 0.25 mm internal diameter x 0.25µm film thickness
Temperature program	60^0 C hold 1 min; increase to 300^0 C at a rate of 25^0 C/min; hold for 3 min. The total run time is 13.6 min.
Conditions	The injector temperature is 250°C. Ultrapure helium is used as the carrier gas at 30 m L/min f1ow. The transfer line is run at 280°C.
MS Conditions	The mass spectrometer is run in the total ion mode. The photomultiplier voltage is set by the autotune program.

Selectra@ is a registered trademark of United Chemical Technologies, Inc. (Bristol, PA). HP 5@ is a registered trademark of Hewlett Packard Corporation (Palo Alto, CA). DB-5@ is a registered trademark of J & W Scientific Inc. (Folsom, CA). Bond Elute Certify@ is a registered trademark of Varian, Inc. (Harbor City, CA). SPECII@ is a registered trademark of Ansys (Harbor City, CA). Isolute@ is a registered trademark of International Sorbent Technologies, Inc. (Cardiff, UK). NARCII@ is a registered trademark of J. T. Baker (Phillipsburg, NJ).

Forensic Drug Analysis for GC or GC-MS Using a 200-mg CLEAN SCREEN Extraction Column (ZSDAU020 or ZC-DAU020)

1. Prepare sample.

1.1 Urine
- a To 5 mL of urine add 50-300µL of 1.0 M acetic acid to adjust sample pH to between 4.8 and 5.5.

1.2 Whole blood:
- a To 2 mL of blood add 8 mL of DI H2O. Mix/vortex and let stand 5 min.
- b Add 150-300µL of 1.0 M acetic acid to adjust sample pH to between 4.8 and 5.5.
- c Centrifuge for 10 min at 670g and discard pellet.

1.3 Tissue:
- a Homogenize 1 part tissue with 3 parts of DI H2O..
- b Centrifuge for 10 min at 670g and discard pellet.
- c Transfer 10 mL of supernatant to a clean tube.
- d Add 150-300µL of 1.0 M acetic acid to adjust sample pH to between 4.8 and 5.5.

2 Condition CLEAN SCREEN extraction column.
- a 1 x 3 mL of CH3OH; aspirate.
- b 1 x 3 mL of DI H2O; aspirate.
- c 1 x 1 mL of 0.1 M acetic acid; aspirate. Note: Aspirate at ≦3 in. Hg to prevent sorbent drying.

3 Apply sample.
   a Load at 1-2 mL/ min.

4 Wash column.
   a 1 x 3 mL of 0.1 M phosphate buffer, pH 6.0; aspirate.
   b 1 x 1 mL of 0.1M acetic acid; aspirate.
   c Dry column (5 min at ≦10 in. Hg).
   d 1 x 3 mL of hexane; aspirate.

5 Elute acidic and neutral drugs (faction A).
   a 2 x 2 mL of CH_2Cl_2; collect eluate at ≦5 mL/ min.
   b Evaporate to dryness at ≦40°C.

6 Extract and analyze faction A.
   a Add 1 mL of hexane and 1 mL of CH_3OH/H_2O (80:20). Mix/vortex.
   b Centrifuge to separate layers. Aspirate and discard hexane (upper) layer.
   c Evaporate again to dryness at ≦40°C.
   d Reconstitute with 100µL of ethyl acetate and inject 1 to 2µL onto chromatograph.

7 Wash column.
   a 1 x 2 mL of methanol; aspirate.
   b Dry column (5 min ≦10 in. Hg).

8 Elute basic drugs (fraction B).
   a 1 x 2.0 mL of methanol-NH_4OH (98:2); collect eluate at 1-2 m L/ min. Note: Prepare elution solvent daily.

9 Extract and analyze fraction B.
   a Add 3.0 mL of DI H_2O and 250 µL chloroform to eluate. Mix/vortex 30 s.
   b Centrifuge to separate phases. Aspirate and discard aqueous (upper) layer.
   c Inject 1-2µL of the chloroform layer onto chromatograph. Note: Fractions A and B can be combined before analysis and evaporated together.

The Future of SPE

The future of SPE is illustrated in Fig. 8, which shows that immunoaffinity can be used to isolate the analytes of interest. This type of SPE is dependent on the attachment of a biologically active molecule to a matrix surface by the use of an aldehydic silane. This forms a covalent bond with the amine groups of the substrate, allowing for the immobilization of the biomolecule with the surface of a variety of materials.

SPE is a very robust technique; however, what if we could improve on the selectivity of our separations by the use of antibodies, enzymes, peptides, proteins, or the attachment of any biological substrate to pick up our analytes?

This type of technology was introduced many years ago by the gluteraldehyde procedure [1-3] and was used for the immobilization of antibodies for benzodiazepines [4]. This immunoaffinity column could pick up most benzodiazepines including their glucuronides. This process could eliminate the acid-base or enzymatic hydrolysis step in conventional SPE procedures.

The only problems with this procedure were in the formation of two Schiff bases in the covalent linkage, making it sterically strained and also susceptible to hydrolysis by various mechanisms. These two limitations represented challenges that limited the effectiveness of this procedure. The recent introduction of a new line of aldehydic silanes (Bio Conext) offers many advantages above the present technologies in ligand immobilization. Aldehydic silanes attach easily to many matrices that contain hydroxyl functions. This includes glass, ELISA plates, silica, plastic beads, metal, agarose, and many polymeric resins. The bonding to primary amines of the substrate creates only one Schiff base attachment. This provides for a more stable linkage that is not sterically hindered. The use of various carbon lengths in the chains from the matrix surface to the biomolecule allow access to active sites and also allow the binding to larger size molecules.

Figure 8 illustrates the chemistry involved in the attachment of the aldehydic silane to a biologically active substrate. Figure 9 explains, in a step-by-step fashion, an actual synthesis of bonding the aldehydic silane first to the matrix (silica) then to Protein A.

Figure 8: Aldehydic silane attachment

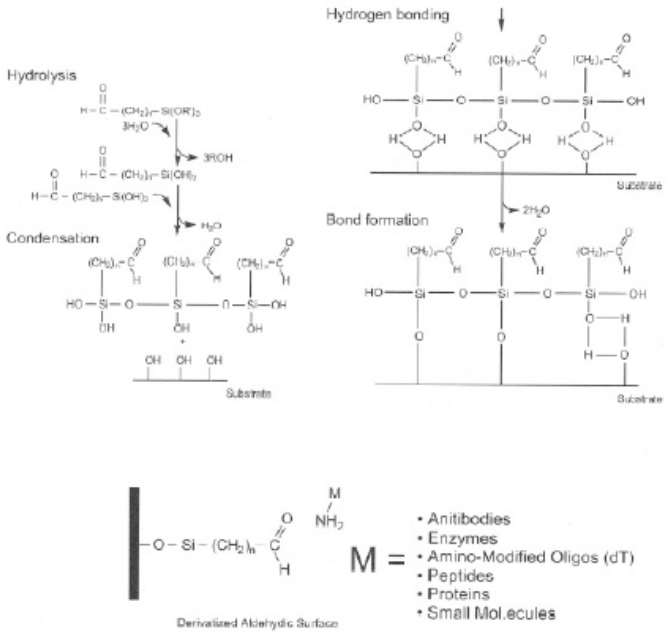


Figure 9: Aldehydic silane bonding

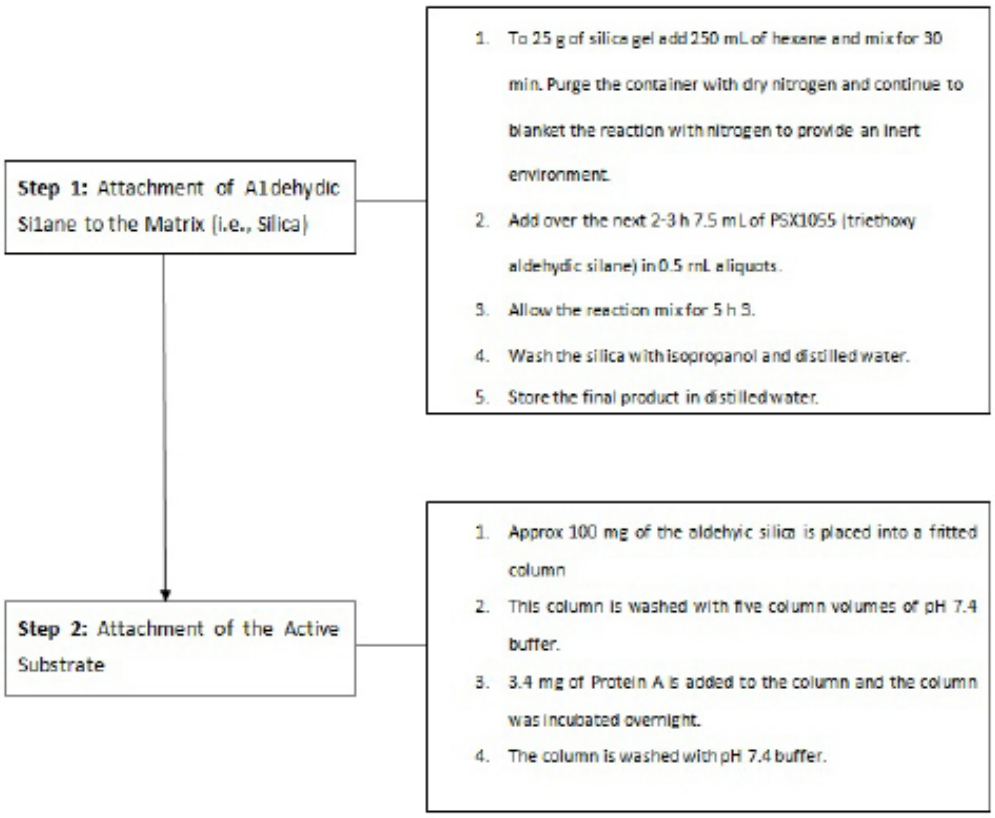


Figure 10: Immunoaffinity SPE Procedure

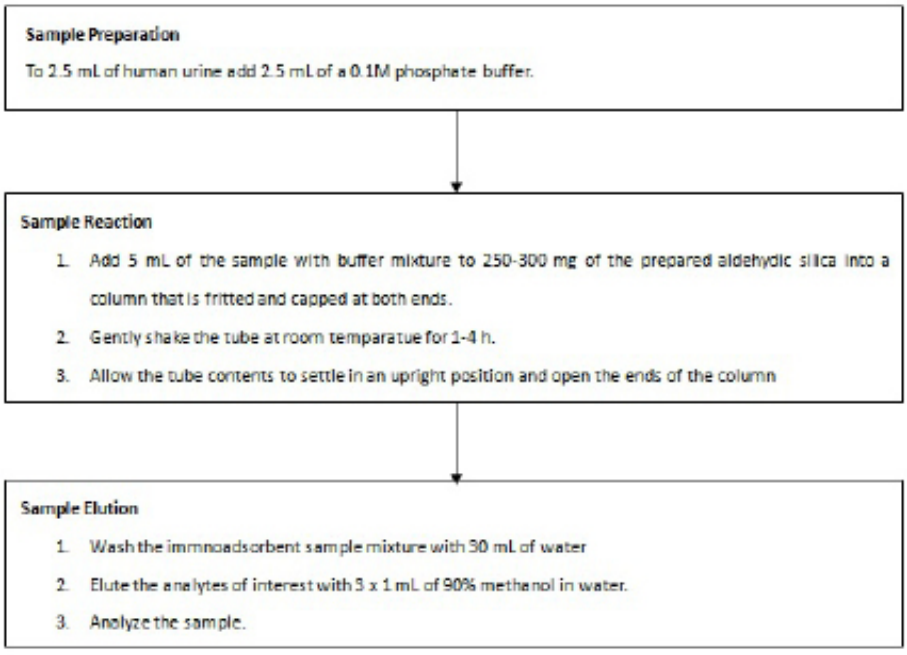


Figure 10 illustrates how an immunoaffinity procedure would be performed. The simplicity of the method, along with selectivity and the limitless possibilities for attachment and analysis, illustrate the direction that SPE in the future may take.

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