Comparison of Liquid/Liquid and Solid-Phase Extraction for Alkaline Drugs

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Abstract

The extraction of drugs from biological matrices is an essential specimen preparation step in current forensic postmortem laboratories. Traditionally, liquid/liquid extractions (LLE) were developed and employed to screen for the general unknown. However, solid-phase extractions (SPE) are becoming more popular as the availability of columns with suitable stationary phases increased. The purpose of this work was to determine if switching from an existing LLE to SPE was feasible. The limits of detection (LOD) for 122 drugs and metabolites were determined in blood following SPE and compared to previously determined LOD's by LLE, if available. There were 41 drugs that had LOD's in blood established by both methods; LLE had a lower LOD for 8 drugs (19.5%), SPE had a lower LOD for 16 (39%), and the LOD's were comparable in the remaining drugs. Although SPE cartridges were more expensive than LLE, SPE was determined to be a faster technique and doubled the number of specimens that could be extracted by one analyst within a specific timeframe. The SPE method utilized enabled the detection of several drugs not detectable after LLE (most notably, morphine and benzoylecgonine) and allowed the extraction of weakly acidic and neutral drugs with only one extra step.

Introduction

Biological matrices comprise multiple components and therefore, present challenges for drug analysis. Extraction techniques are utilized as preparatory procedures prior to instrumental analysis. They remove unwanted compounds such as lipids and allow the analyst to concentrate the target compounds into a small volume. This allows the identification and/or quantitation of drugs by gas chromatography (GC), GC–mass spectrometry (MS), high-pressure liquid chromatography (LC), and LC–MS. In clinical toxicology, specimens such as serum and urine are usually "clean" with little contamination, and so the requirement for a fast turn around time is often the determining factor in the choice of a sample preparation procedure. However, for the forensic toxicologist, specimen matrix and condition, such as hemolysis, decomposition, and contamination is a routine occurrence.

Traditionally, liquid/liquid extraction (LLE) is employed, based upon the manipulation of aqueous pH to extract drugs into an organic solvent. This method is used on a variety of specimens including blood, urine, bile, gastric contents, and tissue homogenates and acidic, neutral, and alkaline drugs may be extracted in one analytical scheme. Knowing the chemical properties of the drug(s) of interest allows the proper selection of organic solvents to perform a successful extraction from a biological specimen and a further purification of the extracts can be attained with a back extraction. The disadvantage of LLE is that relatively large volumes of organic solvents are required. This poses use and disposal problems. In addition, this technique requires a trained analyst with good hand-eye coordination to carefully pipet immiscible layers of similar appearance and each time a drug is extracted between an aqueous and organic layer, some loss will occur (1).

In the 1960's and 1970's, solid-phase extraction (SPE) was initially performed by toxicologists who made their own columns using sodium sulfate and cotton balls or materials containing silicon, such as diatomaceous earth (1). In 1974, three high-performance liquid chromatography (HPLC) researchers serendipitously discovered that the material used in HPLC columns had the ability to bind urinary steroids. After proper treatment of the column packing, the researchers were able to release the steroids (2). Chromatography companies such as Alltech, Supelco, and United Chemical Technologies, began to sell small cartridges comprised of hydrophobic material, such as long chains of carbon (C18), which would selectively bind non-polar drugs and allow extraction from a polar environment. Today, columns are based on different chemical principles, such as: hydrophobic, hydrophilic, cation-exchange, anion-exchange, and mixed mode (a combination of ion exchange and hydrophobic principles). For targeted analysis, the appropriate column's stationary phase is chosen based on the compound's chemical properties. Mixed mode columns, which allow the extraction of a wide range of drugs through the manipulation of polarity and pH, are the most appropriate for use when analyzing a general unknown.

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Although in general, forensic toxicology laboratories were quick to use SPE technology for targeted drug analysis, few utilized the columns for a general unknown. The reasons included well validated and tested LLE methods, and the lack of time and resources to develop alternative procedures. In addition, LLE techniques for alkaline extractable drugs could detect > 100 compounds. Laboratories were unsure the SPE technology could fulfill this mandate. The purpose of this study was to evaluate the feasibility of switching an extraction method from LLE to SPE without loss of the ability to detect a similar range of drugs at comparable concentrations.

Experimental

Materials

All solvents used for both extraction techniques were purchased from Fisher Scientific (Pittsburgh, PA) and were analytical grade or better. Borosilicate test tubes were used for all extractions. Drug standards were purchased as methanolic standards from either Cerilliant Corporation (Round Rock, TX) or Alltech (State College, PA) or as powders from Sigma-Aldrich (St. Louis, MO). Solid-phase extraction columns were Clean Screen DAU purchased from United Chemical Technologies (Bristol, PA). The Clean Screen Columns are mixed mode columns, which have long chain alkyl groups, which act as a reverse phase, hydrophobically binding unionized compounds. The columns also have a benzenesulfonic acid group that is negatively charged and will bind positively charged compounds. Use of organic solvents and manipulation of the pH allows the elution of weakly acidic, neutral, and alkaline compounds as described later.

Liquid/liquid extraction

LLE was performed using 2 mL of blood following a traditional extraction scheme in place in the authors' laboratory for ~ 10 years. Briefly, to 2 mL blood, 2 mL saturated sodium borate (pH 9.3), 0.05 mL of internal standard (Methapyrilene 10,000 ng/mL), and 7 mL of toluene–hexane–isoamyl alcohol (78:20:2) were added. The test tubes were capped and mixed for 10 min on a rotary mixer. The samples were centrifuged for 10 min at 3000 rpm, and the top organic layer was transferred to new test tubes. A back extraction was performed with the addition of 2 mL 0.25M sulfuric acid. The tubes were capped, mixed for 5 min on a rotary mixer, and centrifuged for 5 min at 3000 rpm. The top organic layer was aspirated to waste and 2.5 mL 0.5 M sodium hydroxide and 2 mL n-butyl chloride added to each tube. The tubes were capped, rotated for 5 min, and centrifuged for 5 min at 3000 rpm. The top organic layer was transferred to another clean test tube, and the *n*-butyl chloride was evaporated under nitrogen at ~ 40°C. The extracts were reconstituted with 50 µL ethyl acetate and transferred to GC–MS autosampler vials for analysis.

Solid-phase extraction

The SPE scheme employed was a slight modification from the 2006 United Chemical Technologies (Bristol, PA) application manual. This method was chosen because of the purported ability by the manufacturer to extract multiple drugs. Briefly, to 2 mL blood, 0.05 mL of internal standard (methapyrilene 10,000 ng/mL and hexobarbital 40,000 ng/mL), 4 mL deionized water, and 2 mL 0.1 M potassium phosphate buffer (pH 6) were added. The test tubes were capped, vortexed vigorously, and centrifuged for 10 min at 3000 rpm. Samples were then ready for solid-phase extraction. The UCT CleanScreen columns were conditioned with 3 mL methanol, 3 mL deionized water, and 1 mL 0.1 M potassium phosphate buffer (pH 6) and care was taken not to allow the sorbent beds to dry. The specimens were added to the columns and allowed to flow through at 1–2 mL/min. The columns were washed with 3 mL deionized water, 1 mL 100 mM acetic acid, and dried under vacuum for 5 min at ~ 10 mm of Hg. In the next step, 2 mL of hexane was added and allowed to flow through. The weakly acidic and neutral drugs were eluted with 3 mL of hexane–ethyl acetate (50:50). The columns were washed with 3 mL of methanol and dried under vacuum for 5 min at ~ 10 mm of Hg. The alkaline drugs were eluted with 3 mL of freshly prepared methylene chloride–isopropanol–ammonium hydroxide (78:20:2). The eluate was evaporated to dryness under nitrogen at ~ 40°C. The extracts were reconstituted with 100 µL of ethyl acetate and transferred to GC–MS autosampler vials for analysis. The weakly acidic/neutral eluate and alkaline eluate were not combined for GC–MS analysis in this study. They were injected separately when determining limits of detection.

Chromatographic conditions

The following GC–MS program was used for both the LLE and alkaline SPE. The samples (2 µL) were injected onto a Hewlett Packard 6890/5973 GC–MS equipped with a capillary column (HP1, 12 m, 0.2 mm i.d., 0.33 µm) and run in full-scan mode (scan range 40-400 *m/z*). The injector temperature was 250°C and the initial oven temperature was 70°C. The oven was initially ramped at 15°C/min to 150°C, followed by a 20°C/min ramp to 280°C. This temperature was held for eight min followed by a final ramp of 30°C/min to 320°C held for one min. Three mass spectral libraries were used: an in-house library with relative retention times determined using methapyrilene, the 2006 American Academy of Forensic Sciences (AAFS) library, and the National Institute of Standards and Technology (NIST) library. Enhanced ChemStation (MSD ChemStation D.03.00.611) was installed with the integration parameters listed in Table I.

Determination of limits of detection

Certified drug-free blood was spiked with various concentrations of each drug, normally beginning at 10 ng/mL and continuing incrementally until the drug was detected. The general scheme involved the following concentrations (ng/mL): 10, 25, 50, 100, 250, 500, and 1000. If a drug was not seen at 1000 ng/mL, the extractions were repeated at the following concentrations (ng/mL): 500, 1000, 1500, 2000, 3000, and 5000. Each spiked blood could contain multiple drugs (acidic, neutral, and alkaline), but drugs were chosen that had

appropriate separation on the GC column. The extracts were injected in increasing concentration to minimize the possibility of carryover. Each GC–MS chromatogram was examined for acceptability by determining the presence and appropriate responses of the internal standards. If an extract did not have the presence of the internal standard or its response was too low or high as compared to the controls, the extraction was repeated. Each chromatogram was then integrated using the previously mentioned parameters.

For acceptability, only integrated peaks were examined and the relative retention time on the GC (previously determined with purchased drug standards) had to be within ± 0.02 min. The mass spectra were examined for the presence of diagnostic ions and their appropriate abundance and the result from each mass spectral library had to have a quality match greater than 70%. The limit of detection was set as the extraction that provided an integrated peak, with a correct relative retention time and an appropriate mass spectrum.

For validation of the SPE method, 122 drugs/metabolites were analyzed.

Results and Discussion

Extraction Procedure

Each LLE extraction produced 7 mL of organic solvent waste (hexane–toluene–isoamyl alcohol) and 8 mL of aqueous buffer. The SPE method produced 20 mL of waste containing buffers and methanol and 2 mL of organic solvent waste (hexane). The SPE waste was contained within the extraction manifold, which allowed for easier disposal but required the purchase of the appropriate extraction apparatus. LLE required the ability to rotate or mix test tubes and both methods required a centrifuge and evaporation apparatus. The major financial difference between the two methods was the purchase of SPE columns (~ $2 per column) for each extraction.

Analyst time to perform LLE of 24 samples was ~ 3 h. The extraction was labor intensive and required excellent pipetting of the organic solvent from the aqueous solvent. Any aqueous solvent that was pipetted at the final step caused the extraction to fail and could ruin the GC column if injected. The SPE extraction could be performed in less than 3 h on twice the number of specimens, 48, using two SPE extraction manifolds. SPE demanded less hands-on activity and only required that the preparation and cleaning steps were performed in the appropriate order to insure proper extraction. The opportunity for failed extractions was also decreased as hand-eye coordination was not as important. The internal standard response for SPE was also more consistent than LLE due to the decreased manual pipetting.

Figure 1 shows a positive control extracted by LLE (A) and SPE (B). Note the absence of 6 "junk" peaks from the SPE chromatogram compared to the LLE. Most notably, the peak at 14.46 on the LLE is cholesterol, which is not present by SPE. The response for the internal standard is lower by SPE due to differences in reconstitution volume (50 µL LLE vs 100 µL SPE).

Limit of detection of analytes

Table II is a listing of the LOD for drugs that were detected by both methods. Forty one drugs were tested, including benzodiazepines, antidepressants/antipsychotics, antihistamines, and drugs of abuse. LLE resulted in lower LOD's for eight drugs, representing a variety of drug classes including antihistamines (doxylamine), opioids (oxycodone), and antidepressants/antipsychotics (nortriptyline and haloperidol). The

Table I. ChemStation Integration Parameters

Integrator Event Name	Value	Time
Initial Area Reject	0	Initial
Initial Peak Width	0.1	Initial
Shoulder Detection	OFF	Initial
Initial Threshold	20.0	Initial
Area Reject	10	0.001
Baseline All Valleys	ON	1.0
Tangent Skim		1.0

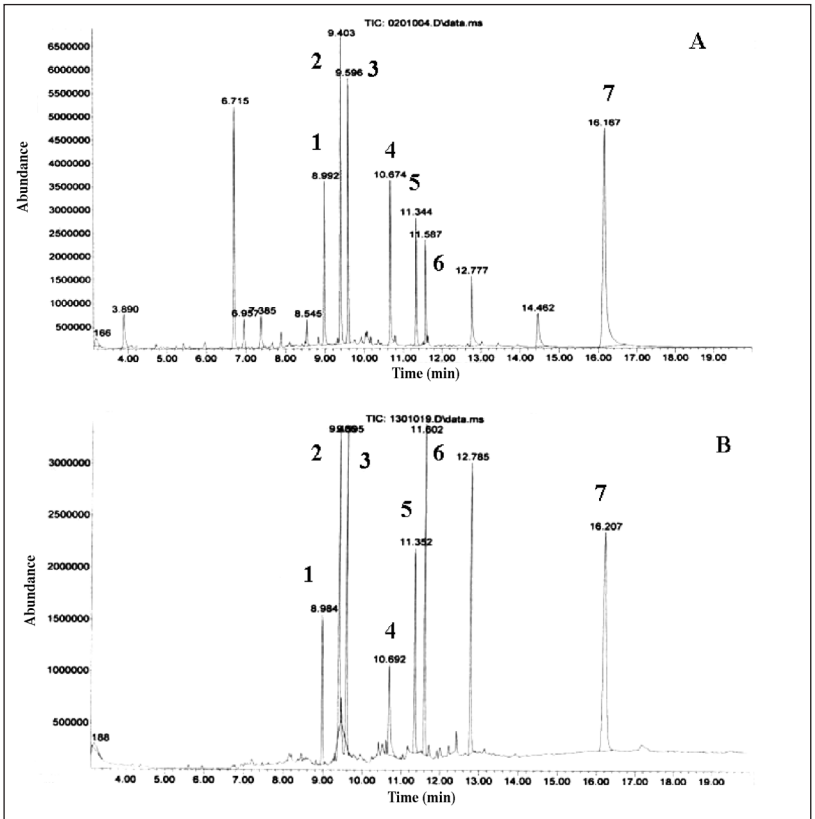

Figure I. Positive Control GC Chromatogram for Liquid/Liquid Extraction (A) and Solid-Phase Extraction (B). Peak numbers: 1, Diphenhydramine; 2, Tramadol; 3, Methapyrilene (IS); 4, Nortriptyline; 5, Sertraline; 6, Diazepam; 7, Trazodone.

differences in the LODs between techniques were significant for several drugs, such as haloperidol with a variation of five times. The result is that the SPE would be unable to detect non-toxic concentrations in blood. Similarly, there was a tenfold difference in the LOD for oxycodone between sample preparation procedures.

Sixteen drugs had limits of detection that were lower by SPE and these included the drug classes mentioned earlier. Notable findings were observed with benzodiazepines such as diazepam, which had almost a tenfold lower LOD by SPE. The LOD's were similar for 17 drugs, including cocaine, cyclobenzaprine, fentanyl, and zolpidem.

To further validate the SPE method, the LOD for an additional 65 drugs was determined solely by SPE to insure that they were detected and at concentrations normally seen in casework (Table III).

The detection of hydroxyzine was difficult by LLE, and an LOD by SPE was unable to be established. Our laboratory has detected it in routine casework since switching to SPE; however, the LOD must be greater than 5000 ng/mL. The LLE was designed for alkaline drugs; however, the following weakly acidic and neutral drugs were detected following LLE (with determined LOD in parentheses, if available): carbamazepine (2500 ng/mL), carisoprodol (5000 ng/mL), meprobamate (not determined), caffeine (not determined), and oxcarbazepine breakdown product (not determined). These drugs were not detected in the alkaline fraction of the SPE, but when the weak acid/neutral fraction was collected and analyzed, the drugs were detected generally with a lower LOD. Table IV contains the LOD's determined for weakly acidic and neutral drugs by SPE.

Following the LOD studies, our laboratory decided to switch methodologies to SPE. To date over 2000 case specimens have been assayed by this methodology (including blood, urine,

Table II. LOD Comparison between LLE and SPE

	LLE LOD (ng/mL)	SPE LOD (ng/mL)	Methodology with lower LOD
Alprazolam	350	250	SPE
Amitriptyline	25	25	Equal
Bupropion	100	50	SPE
Chlordiazepoxide	50	10	SPE
Chlorpheniramine	50	25	SPE
Chlorpromazine	50	25	SPE
Citalopram	25	25	Equal
Cocaine	25	25	Equal
Codeine	100	50	SPE
Cyclobenzaprine	50	25	Equal
Desipramine	50	100	LLE
Desmethyldoxepin	150	50	SPE
Dextromethorphan	50	25	SPE
Diazepam	200	25	SPE
Diphenhydramine	50	25	SPE
Doxepin	50	25	SPE
Doxylamine	100	250	LLE
Fentanyl	50	50	Equal
Fluoxetine	100	100	Equal
Haloperidol	50	250	LLE
Hydrocodone	50	50	Equal
Imipramine	25	25	Equal
Ketamine	25	25	Equal
Meperidine	100	25	SPE
Methadone	30	25	SPE
Methylphenidate	50	50	Equal
Midazolam	50	50	Equal
Nordiazepam	50	50	Equal
Norfluoxetine	200	250	LLE
Normeperidine	100	100	Equal
Norpropoxyphene	100	100	Equal
Nortriptyline	50	250	LLE
Oxycodone	25	250	LLE
Paroxetine	150	100	SPE
Phencyclidine	25	10	SPE
Propoxyphene	50	100	LLE
Sertraline	50	25	SPE
Tramadol	25	25	Equal
Trazodone	100	250	LLE
Verapamil	50	50	Equal
Zolpidem	50	50	Equal

Table III. SPE LODs for Drugs Also Seen by LLE

Drug	SPE LOD (ng/mL)	Drug	SPE LOD (ng/mL)
Alfentanil	50	Mesoridazine	100
Amantadine	500	Methamphetamine	100
Amoxapine	50	Metoclopramide	250
Amphetamine	100	Metoprolol	500
Atomoxetine	100	Mirtazapine	25
Atropine	50	Nicotine	50
Benzphetamine	10	Norcocaine	25
Brompheniramine	25	Norcodeine	100
Bupivacaine	25	Norverapamil	250
Buspirone	100	Olanzapine	50
Butorphanol	100	Orphenadrine	50
Clomipramine	10	Papaverine	25
Clonidine	25	Pentazocine	50
Clozapine	50	Phentermine	100
Cocaethylene	25	Procainamide	500
Dicyclomine	25	Prochlorperazine	500
Dihydrocodeine	50	Promethazine	50
Diltiazem	50	Propranolol	250
Ephedrine	100	Protriptyline	100
Etomidate	25	Pyrilamine	50
Fenfluramine	50	Quetiapine	1000
Fluconazole	50	Quinine/quinidine	500
Lamotrigine	50	Scopolamine	250
Levamisole	100	Strychnine	250
Levorphanol	100	Thioridazine	100
Lidocaine	25	Tocainide	500
Loperamide	< 500	Trifluoperazine	50
Loxapine	25	Trihexyphenidyl	25
Maprotiline	50	Trimipramine	10
MDA	50	Venlafaxine	50
MDMA	50	Xylazine	25
Meclizine	50	Yohimbine	1000
Mepivacaine	50		

bile, and tissue homogenates) and have detected the following drugs that had previously not been observed by LLE: benzoylecgonine, 6-acetylmorphine, morphine, cotinine, naloxone, 7-aminoclonazepam, 7-aminoflunitrazepam, ticlopidine, and clopidogrel. In LLE extractions, the nortramadol (*N*-desmethyltramadol) metabolite was most often seen when tramadol was present in casework. In SPE extractions, the nortramadol peak is either absent or very small and instead, *O*-desmethyltramadol is seen. A peak identified as nicotinamide appears early on the SPE chromatograms and was not seen by LLE. We are continuing to monitor extractions for new mass spectra that match either the AAFS or NIST spectral libraries. Due to the difficulty with tissue homogenates passing through the columns, we only extract 1 mL and reconstitute with 50 µL of ethyl acetate before injection. Severely decomposed specimens may contain too many interferences for GC–MS analysis.

Comparison with previous work

The majority of published SPE methods have been targeted drug analysis (3–5). The current study was undertaken to evaluate whether SPE could be utilized for a general unknown without loss of the ability to detect a similar number of drugs at the same or lower concentration compared with LLE extraction. A study by Decaestecker et al. found that SPE had the possibility to be used for screening by LC–MS, but they lacked an optimized SPE method (6). Our results are similar to a study using Bond-Elut Certify LRC and Isolute Confirm HCX mixed-mode columns to extract urine that showed SPE to be better than LLE (7). The authors presented data on the recovery of 16 drugs at toxic concentrations ≥ 2000 ng/mL. In forensic toxicology, it is important to be able to extract drugs in overdose cases, but it is also important to be able to detect drugs at therapeutic concentrations to determine multiple drug intoxications or to evaluate compliance. The inclusion of LOD's in our study enables a forensic toxicologist to know whether an SPE method would extract drugs at both therapeutic and toxic concentrations. The LOD's listed here demonstrate SPE is capable of general unknown screening for both therapeutic and toxic concentrations and does not have to remain as a selective drug extraction method in the forensic toxicology field.

Table IV. Weak Acid/Neutral Drugs Detected by SPE

Drug	LOD (ng/mL)
Amobarbital	500
Butabarbital	500
Butalbital	250
Carbamazepine	500
Carisoprodol	500
Hexobarbital (IS)	N/A
Ibuprofen	2000
Mephobarbital	500
Meprobamate	2500
Oxcarbazepine	250
Phenobarbital	2500
Phenytoin	500
Secobarbital	250
Theophylline	1000
Thiopental	Coelutes with IS

In another study (8), automated SPE was performed using an Oasis MCX mixed-mode column on a variety of drugs (acidic/neutral/alkaline). The authors determined extraction efficiency for many drugs, but did not examine LODs. They did note that their procedure was unable to extract brompheniramine, paroxetine, haloperidol, and ephedrine, all of which were detected in our laboratory with LOD's below 250 ng/mL.

The SPE method allows our analysts to extract double the number of specimens within the same time period. This method also allows for the collection of the weakly acidic/neutral drug fraction when indicated by case history without the use of additional specimen. The detection of 6-acetylmorphine and benzoylecgonine allows the qualitative identification of cocaine and heroin use following a positive immunoassay result without the need for selective GC–MS analysis. The SPE method described in this study has proven to be reliable for the analysis of forensic postmortem specimens such as blood, and also urine, bile and tissue homogenates, and a suitable substitute for the more time consuming LLE.

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