DEVELOPMENT OF A GENERAL SCREENING METHOD FOR ACIDIC, NEUTRAL, AND BASIC DRUGS FROM WHOLE BLOOD USING THE OASIS HLB® COLUMNS AND THE OASIS MCX® COLUMNS

BY

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A Thesis Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

Faculty of Pharmacy
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Abstract

Solid-phase extraction (SPE) is becoming a commonly used extraction technique. However, most of the existing solid-phase extraction methods extract a single drug from a relatively clean biological matrix (e.g. plasma or urine) using a silica based column. These methods are generally not satisfactory for forensic applications since the majority of biological samples are not clean (e.g. whole blood, bile, tissues) and the presence of drug is unknown. Silica based columns also have reproducibility and stability problems (i.e. irreproducible recoveries, sorbent instability at pH extremes). In this study, sequential extraction of acidic, neutral, and basic drugs from whole blood using the Oasis HLB® (Waters Corporation) and the Oasis MCX® (Waters Corporation) columns was attempted. Both columns are polymer based and are designed to overcome the limitations of silica based columns. Conditions for each step of the extraction sequence were optimized for a single drug or selected group of drugs then applied to a larger group of drugs consisting of the most commonly detected drugs in Manitoba forensic casework. A suitable extraction procedure for the Oasis HLB® columns was not found despite extensive testing; it was not possible to separate the acidic and basic drugs into two fractions. A switch to the Oasis MCX® column was found to be more promising. The final extraction procedure involved: a conditioning step with methanol then water, washing steps of water, then 0.1 M hydrochloric acid, then water/methanol (95:5), an elution step for the acidic and neutral drugs with acetone/chloroform (1:1), and an elution

step for the basic drugs with ethyl acetate/ammonium hydroxide (98:2). Acidic and neutral drugs eluted together into one fraction and the basic drugs eluted into a separate second fraction. Recoveries for the majority of drugs tested were greater than 70% with relative standard deviations less than 10%. The application of this procedure for the extraction of benzodiazepines was not as successful. Elution of this group of drugs produced dirty extracts which resulted in increased gas chromatograph maintenance costs making it unsuitable for routine screening. A suitable internal standard was not found although a number of compounds were tested. It is recommended that this SPE procedure using the Oasis MCX® column be used together with another technique to obtain the most comprehensive drug screen.

Acknowledgments

I would like to thank the RCMP Forensic Laboratory in Winnipeg, Manitoba for granting me the privilege of performing my Masters project at their facility. It was an experience I'll never forget. A special thank-you to Shirley Treacy for her patience, help and encouragement throughout the project, especially during the thesis writing. Also, thanks to Val Mason-Daniel, James Mitchell and Karen Zechel for all of their help and advice. I would also like to thank Frank Burczynski for accepting me as a graduate student from outside his field of research, and Wayne Hindmarsh for introducing me to the science of toxicology. Thank-you to the Waters Corporation for providing some Oasis® columns for us to use. To Ian and my family, thank you for your love and support throughout these years. Finally, I would like to thank Merck Frosst, the Canadian Society of Forensic Science, and the Buggey family for financial support from scholarships and awards.

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1. Introduction

1.1 Background

Systematic toxicological analysis (STA) is defined as the logical chemical-analytical search for potentially harmful substances whose presence is uncertain and whose identity is unknown (1). Extractions play a major role in STA. Extractions are able to simplify the isolation of compounds of interest from difficult to analyze matrices into an environment more suitable for analysis. Once isolated, the compounds of interest can be concentrated so that they are easier to detect, identify and quantify.

Traditionally, liquid-liquid extractions have been used. This method dates back to the mid-nineteenth century where it was used to extract drugs from poison victims (2). With the use of two immiscible solvents, compounds of interest partition into the solvent in which they are most soluble. Extraction efficiency can be influenced by solvent type, pH and volume. Although liquid-liquid extraction is still used today there are a number of drawbacks that limit its usefulness. Two major disadvantages include the decreased selectivity for compounds of interest and the formation of emulsions. All compounds with a similar solubility in the extraction solvent will be extracted, including undesired compounds (interferences). In addition, it can be difficult to extract relatively polar compounds from aqueous samples as they have limited solubility in organic solvents resulting in low recoveries (3). Emulsion formation is another potential problem. If an emulsion forms and is resistant to breakage, this causes a significant loss in the recovery of the compounds of interest, as the compounds of interest may become trapped within

the emulsion (2). Other disadvantages of liquid-liquid extractions include: increased solvent use and waste leading to environmental and safety issues, a procedure which is labor intensive and time consuming, automation difficulties, and reproducibility concerns (3). Other extraction methods such as solid-phase extraction have been developed because of these drawbacks.

Unlike liquid-liquid extraction where two liquids are used in the extraction process, solid-phase extraction involves the use of one liquid phase and one solid phase (sorbent). The solid-phase extraction mechanism is more specific than liquid-liquid extraction because it is based on interactions between sample components and the sorbent, as well as on solubility in the solvent. Compounds can be retained on the sorbent by non-polar, polar and ionic interactions as well as by covalent bonds, depending on the type of sorbent used. The more selective retention mechanism of solid-phase extraction has a number of advantages over liquid-liquid extraction including: cleaner extracts (4), increased selectivity for the compounds of interest, no emulsion formation, wider range of solvents available for use, and smaller volumes of solvent used for each extraction. In general, solid-phase extraction can extract smaller sample sizes. This is important when sample amount is limited (e.g. samples from infants). Chemicals from as little as 50-100 μL of plasma have been successfully extracted (5). Solid-phase extractions take less time. Therefore, volatile compounds can be analyzed without significant loss (5). Solid-phase extraction systems also can be automated which further increases the potential for time savings.

1.2 Project Hypothesis and Objective

Our hypothesis was that an extraction method using the Oasis® columns could be optimized for screening of common drugs and poisons from blood. The goal was to sequentially extract acidic, neutral and basic drugs from whole blood with an acceptable recovery and cleanliness *using only one column*. The acidic and neutral drugs would be eluted into one fraction. The basic drugs would be eluted into a second fraction.

Two columns were evaluated: the Oasis HLB® (150 mg) and the Oasis MCX® (100 mg), both manufactured by the Waters Corporation. Cleanliness of the extracts after extraction, visual appearance of the chromatogram and the percent recovery were used as evaluation parameters. A drug list, prepared by the RCMP Forensic Laboratory in Winnipeg, Manitoba, of the most commonly detected drugs in casework was used to evaluate the final procedure for the columns. A list of the drugs is presented in Appendix A. The extraction procedure was initially performed manually then transferred to an automated extraction system using the Zymark RapidTrace SPE Workstation.

2. Literature Review

2.1 Sorbents

The development of sorbents has increased dramatically since the use of permutite, diatomaceous earth, and silicic acid. Presently there are two main categories of sorbents: the silica based and the polymer based types.

2.1.1 Silica Based Sorbents

Initially silica (SiOH) was used as a sorbent but the development of HPLC column coatings in the 1970's revolutionized the solid-phase extraction sorbents (5). Bonded silicas created from the reaction of organosilanes with activated silica yield a product with the functional group of the organosilane attached to a silica backbone by a silyl ether linkage (6). Silicas having different properties are produced by altering the length and structure of the organosilane. Sorbents can be classified into four categories depending on their primary retention mechanism; reversed phase, normal phase, ion exchange, and adsorption (2). Reversed phases, such as C18, interact mainly via Van der Waals forces, thereby extracting relatively non-polar compounds. Normal phases, also called polar phases, can interact by dipole-dipole interactions extracting relatively polar compounds. Examples of polar phases are diol or amino sorbents. Ion exchange phases bind compounds through ionic interactions. These sorbents can be classified as either anionic or cationic; anionic sorbents retain negatively charged compounds and cationic resins retain positively charged compounds. Examples of ion exchange sorbents include

SCX (strong cation exchange) and WAX (weak anion exchange). Adsorption phases are not considered bonded phases as they do not have hydrocarbon chains attached to the backbone. These sorbents interact mainly via polar interactions and can therefore extract relatively polar analytes. An example of this type of sorbent is silica (SiOH).

Silica based sorbents have a number of drawbacks:

1. The reaction between the organosilane and the silica is often incomplete, leaving unreacted silanol groups which can act as cation exchange sites creating a secondary retention mechanism. End-capping reduces the amount of free silanols by converting the hydroxyl group to a methoxy group but this is not 100% effective (7).
2. The sorbent is not stable at pH extremes. At pH's lower than 2 there can be weakening of the silyl ether linkage. At high pH's there can be dissolution of the silica in aqueous solutions (6).
3. The sorbent must be wet before the sample is loaded. A dry sorbent will significantly decrease the recovery of the drug due to decreased interaction between the sample components and the sorbent (2).

The polymer based sorbents have overcome most of the limitations of silica based sorbents.

2.1.2 Polymer Based

The first polymer based sorbent, XAD-2, was developed in the 1960's. This non-ionic sorbent is composed of styrene and divinylbenzene and is able to bind lipophilic but water-soluble organic molecules (2). Since the 1960's a number of other polymer based

sorbents have been developed using a similar polymer. Two of the most recent ones are the Oasis HLB® and Oasis MCX® columns developed by Waters Corporation. Both of these columns are made of poly(divinylbenzene-co-N-vinylpyrrolidone), a reversed phase sorbent. HLB refers to the hydrophilic-lipophilic balance which describes the sorbent's ability to bind both polar and nonpolar compounds through the use of Van der Waals forces and dipole-dipole interactions. The MCX® columns contain a mixed-mode cation exchange sorbent based on the polymer backbone with the addition of sulfonic acid groups to enable it to retain cations. It is considered a mixed-mode sorbent because it retains compounds by two different mechanisms; hydrophobic and ionic interactions. These sorbents are promoted for their unique ability to stay wet with water despite drying, retain a wide spectrum of both polar and non-polar compounds and remain stable from pH 1 to 14 (8). Better recoveries and increased reproducibility with the Oasis® columns compared to silica based columns have also been reported (8).

Sorbents are held in cartridges or columns which resemble empty syringe barrels. Within the column the sorbent is held in place between an upper and lower polyethylene frit. The empty space above the sorbent is called the reservoir. This reservoir holds the solvent or sample. Columns containing a minimum of 50 mg to a maximum of 1000 mg of sorbent are available. The choice of a sorbent volume depends on the application being considered; the more compounds of interest to be extracted from a sample, the greater the volume of sorbent required.

2.2 Extraction Procedures

2.2.1 General Extraction Procedure

The general extraction procedure consists of a number of steps: conditioning the sorbent, sample loading, washing the sorbent, drying the sorbent, and eluting the compounds of interest (6,9). The solvents used are either forced through the column by positive pressure using a stream of air or nitrogen, by negative pressure using vacuum, or by centrifugation.

Conditioning (or wetting) the sorbent is usually performed with a water miscible organic solvent, with the excess solvent being washed away using buffer or water. The purpose of this step is to open up the sorbent structure and increase the surface area available for interaction with the drugs. It also removes residues from the cartridge left from the manufacturing process. This step is very important because insufficiently wetted columns can result in decreased recovery of the compounds of interest and poor reproducibility due to inadequate interaction between the compounds and the sorbent.

Samples are then loaded onto a conditioned column at a relatively slow flow rate to allow the sorbent to bind sample compounds. Most biological samples require dilution, sonication, and/or centrifugation before loading onto the column to prevent clogging of the sorbent (3). Changing the pH of the sample may enhance retention by converting the analyte into either the ionized or nonionized form.

The wash step removes any undesirable compounds (interferences) loosely bound to the sorbent. An appropriate wash solvent is one that is strong enough to disrupt the

interference-sorbent bond but not the compound of interest-sorbent bond. More than one wash step is often used.

Drying involves the removal of the wash solvent from the column. This is done either by the application of air or the use of a solvent such as methanol. Since the goal is to have the cleanest elution extract possible, it is important to remove any undesirable compounds eluted by the wash step in this drying step.

Eluting the retained compounds requires the use of a sufficiently strong solvent to disrupt the bonds between the sorbent and the compounds of interest. The latter should have a high solubility in the selected solvent. Since one of the goals of extraction is to concentrate the compounds of interest, elution should be accomplished using the smallest volume of solvent possible. Elution may also require a change in pH to convert the analyte into the desired ionized or nonionized form.

2.2.2 Method Optimization

2.2.2.1 Drug Properties Influencing Solid-Phase Extraction

Knowledge of drug structure and pK_a is critical. Hydrophobic and ionic groups can predict how the drug will bind to the sorbent. Determining how soluble a drug is in a given solvent also is important. Solvents in which a drug has high solubility make appropriate elution solvents. Solvents in which a drug is insoluble may be appropriate wash solvents. In addition, some drugs are unstable in certain solvents. It is important to identify these solvents and avoid their use.

Protein binding is another factor that can influence extraction efficiency. Highly protein bound drugs may be unavailable for interaction with the sorbent, leading to a low extraction efficiency. There are a number of ways to break these bonds including denaturation or precipitation.

2.2.2.2 Matrix Concerns

The type of matrix or sample environment in which the compounds of interest are contained has a large impact on the type of pre-extraction preparation required. For example, whole blood requires a different preparation than urine because blood contains cells and proteins (6). The presence of protein in the sample, even if the drug is not highly protein bound, can potentially cause coating of the sorbent, preventing interaction between the sample and sorbent. Knowledge of the pH and ionic strength of the sample can enhance ion exchange extractions. An unsuitable pH and high ionic strength can hinder the success of the extraction.

2.2.2.3 Sorbent Considerations

Sorbent structure and its stability in certain solvents and at certain pH's are other factors to consider in the extraction procedure. Sorbent structure can determine the type of interactions being used to retain the compound. It is important to be aware of pH and solvent restrictions associated with the sorbent chosen for use. Performing an extraction at a pH or solvent not compatible with the sorbent may cause permanent sorbent changes leading to irreproducible results.

2.2.3 Screening Procedures

The majority of solid-phase extraction procedures have been optimized for the extraction of a single drug from a relatively clean biological sample such as plasma or urine. These methods are not satisfactory for forensic applications since the majority of samples submitted for analysis are whole blood and the presence of drugs is unknown, and depending on the state of decomposition, samples may contain large amounts of breakdown products creating a "dirty" sample.

General screening methods for the detection of acidic drugs (10) and basic drugs (11,12) have been developed for silica based mixed mode and reversed phase sorbents. Within each group there is no consensus as to the types of solvents used for each step.

One of the first screening methods was published in 1975 by Pranitis and Stolman using XAD-2, a polymer based sorbent (13). This procedure resembled column chromatography rather than solid-phase extraction because the columns were manually prepared by filling glass tubes with sorbent and large amounts of solvent (100 mL) were used for conditioning, washing and elution. Elution fractions were analyzed by thin-layer chromatography and UV spectrophotometry. A mixture of 22 acidic, neutral and basic drugs were extracted at a concentration of 50 μg%, which is in the therapeutic concentration range for many drugs. Percent recoveries were calculated for only eight drugs. Values ranged from 55 to 95%. Neither standard deviations nor the cleanliness of the extracts were reported. Since this first form of solid-phase extraction, columns have been made substantially smaller requiring less sorbent and less solvent.

Chen et al. developed a screening method for the isolation of acidic, neutral and basic drugs from serum, plasma, urine, whole blood and tissues using a silica based mixed-mode column (Bond Elut Certify®) (11,14,15). In this method the acidic and neutral drugs were eluted with acetone-chloroform (1:1). The basic drugs were eluted with 2% ammoniacal ethyl acetate. A total of 15 drugs were extracted from whole blood using this method. The percent recoveries were above 81% with a relative standard deviation of less than 9. The concentrations of drugs used in their study were above therapeutic levels. Application of this method to the extraction of therapeutic levels of drugs was not evaluated. This may limit its applicability in circumstances where it is important to detect low levels of drugs such as in forensic toxicology.

There have been a few articles published on the detection of single compounds or classes of drugs from clean samples (e.g. water, serum, plasma and urine) using the Oasis HLB® column (16-22). However, to date, there have been no known published reports of the use of the Oasis HLB® or MCX® columns for drug screening.

2.3 Automation

One of the advantages of solid-phase extraction is that it can be automated, decreasing the amount of manual labor required. There are a number of systems available. Some units perform the solid-phase extraction process on previously prepared (i.e. prediluted) samples. Others are capable of preparing the sample before extraction. Other automated systems have been developed which couple the solid-phase extraction process with on-line HPLC injection of the elution extract (2).

The RCMP Forensic Laboratory in Winnipeg, Manitoba uses the Zymark RapidTrace SPE Workstation shown in Figure 1. It is connected to a laptop computer running the RapidTrace software which can create and modify methods, and start extraction sequences. Each module of this automated system is capable of serially extracting up to ten samples at a time and can collect up to ten fractions per sample. Blood samples have to be prepared manually, i.e. diluted and centrifuged, before they can be extracted by the workstation. Samples are placed on racks, with the collection tubes and the extraction columns placed on turrets above them. Solvents are drawn from reservoirs by a syringe and forced through the column. Reagent flow rates can be controlled for each step of the procedure. Drying of the sorbent is done using a stream of nitrogen rather than an organic solvent.

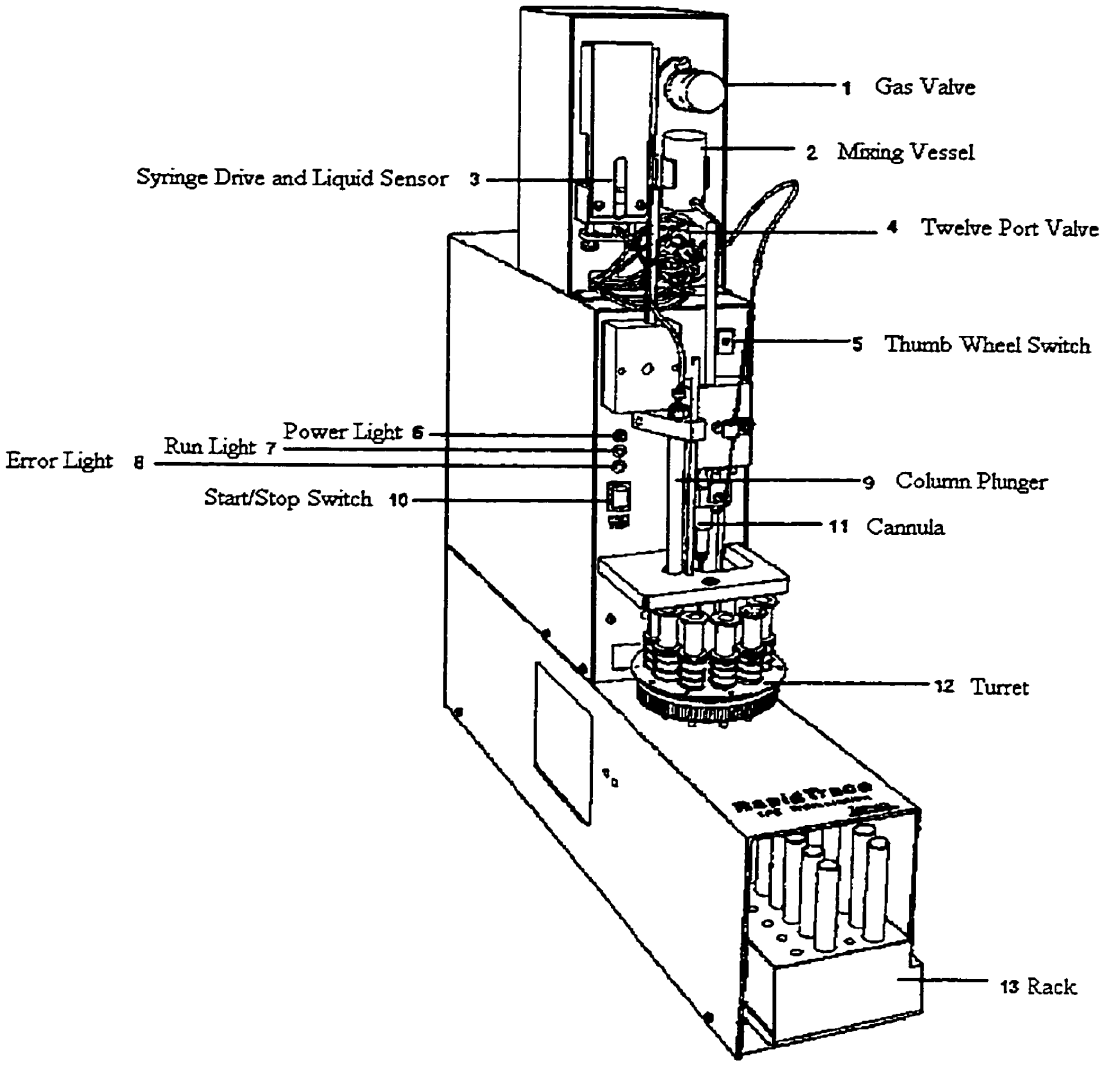

Figure 1 Zymark RapidTrace SPE Workstation Module

3. Materials

3.1 Reagents

Acetone, acetonitrile, chloroform, ethyl acetate, hexane, and methanol, all HPLC grade, were purchased from Caledon Laboratories, Georgetown, Ontario. Glacial acetic acid, hydrochloric acid, ammonium hydroxide and dichloromethane were of reagent grade and from Caledon Laboratories. Phosphate buffers pH 4 and 9 were also purchased from Caledon Laboratories. Isopropanol (HPLC grade) and potassium dihydrogen phosphate (KH_2PO_4) A.C.S. were purchased from Fisher Scientific, Fair Lawn, New Jersey. Anhydrous ethanol was purchased from Commercial Alcohol Inc., Toronto, Ontario. Water was obtained from a Milli-Q UF Plus system, Millipore Corporation, Bedford, Massachusetts.

Phosphate buffer (0.1 M) pH 6 was prepared by dissolving 6.81 g of potassium dihydrogen phosphate into 450 mL of deionized water, adjusting the pH to 6.0 with 1.0 M or 2.0 M potassium hydroxide, and making the total volume up to 500 mL with deionized water (11). Buffer of pH 7 was prepared the same way but adjusting to pH 7.0 with 2.0 M potassium hydroxide. Buffer of approximately pH 4.4 was prepared similarly but without pH adjustment.

3.2 Standards

All drugs and internal standards were obtained from different manufacturers. A list of the drug sources is summarized in Appendix B. Concentrated stock solutions were prepared from either existing solutions or the powdered drugs. Stock solutions were diluted with water or ethanol to the appropriate concentration before use.

3.3 Columns

Oasis HLB® and Oasis MCX® columns were purchased from Waters Corporation (Milford, Massachusetts).

4. Instrumentation

4.1 Gas Chromatography

A Varian Star 3600 CX Gas Chromatograph (GC) with a Varian 8200 CX autosampler was used for sample analysis for all drugs except benzodiazepines. The GC was equipped with a DB1 column (J&W Scientific, Brockville, Ontario) 15 m x 0.32 mm inner diameter (i.d.). The injector temperature was set at 250°C. The oven was programmed from 90°C to 320°C at 10 degrees per minute. The final temperature was held for 14 minutes. A nitrogen-phosphorus detector (NPD) set at 300°C was used to detect the compounds.

The benzodiazepine extracts were analyzed on a HP 5890 Gas Chromatograph with a HP 7673 Autosampler. A DB17 column (J&W Scientific, Brockville, Ontario)

15 m x 0.32 mm i.d. was used. The injector temperature was set at 250°C. The oven was programmed from 220°C for one minute to 290°C for 6.75 minutes at 8 degrees per minute. The electron capture detector was set at 300°C.

4.2 Automated Extraction System

The Zymark RapidTrace SPE Workstation was linked to a Dell Latitude LM laptop running the RapidTrace software. The workstation had two modules; each had the capability of extracting ten samples and collecting ten fractions. There were eight solvent reservoirs and a nitrogen gas line connected to the modules. Solvents were drawn from the reservoirs and forced through the column by a syringe providing negative and positive pressure. Columns were dried using a stream of nitrogen.

5. Methods

Method development began with the Oasis HLB® columns since the Oasis MCX® columns were not available at the beginning of this project. Methods used for the Oasis HLB® columns are described first followed by the method development for the Oasis MCX® columns. Method development is summarized in Tables 3 and 4 for the Oasis HLB® columns and the Oasis MCX® columns, respectively. Sample pre-treatment and method evaluation were the same for each column and are described below.

5.1 Drugs Used for Procedure Optimization

Codeine was used as the test drug for the Oasis HLB® procedure. Method development was performed with porcine blood spiked with 80 micrograms (μg) of codeine per 100 mL of blood (ug%). Nalorphine (2 μg/mL blood) was used as the internal standard.

A quality control standard for the basic drugs (NPQC, nitrogen-phosphate detector quality control standard) and a barbiturates mix were used as test drugs for the Oasis MCX® procedure. Porcine blood (1 mL) was spiked with 5 μg of each drug in the barbiturate mix and 100 μL of the NPQC. The drugs in each mix, as well as their respective concentrations are listed in Tables 1 and 2. Two internal standards were used in the MCX® procedure. Doxapram (250 ng) and tolylbarbital (25 ng) were added to each mL of blood. Nalorphine (2 μg to 1 mL of blood) was used instead of doxapram at the beginning of the MCX® method development.

Table 1 Drugs in NPQC

Drug	Concentration (ng/μL)
amphetamine	5
amitriptyline	5
benzocaine	10
caffeine	5
chlorpheniramine	5
codeine	5
diphenoxylate	5
ethosuximide	20
heroin	5
lidocaine	5
meperidine	5
methadone	5
methaqualone	5
methylphenidate	5
morphine	20
nefazodone	20
pentazocine	5
phenyltoloxamine	5
strychnine	10
trazodone	10

Table 2 Drugs in Barbiturate Mix

allobarbital
amobarbital
barbital
butabarbital
pentobarbital
phenobarbital
secobarbital
thiopental

5.2 Sample Pre-Treatment

Porcine blood samples (preserved with sodium fluoride and anticoagulated with potassium oxalate) were spiked with the drugs of interest, as well as the internal standards, and sonicated for 15 minutes. Phosphate buffer was added and the samples were vortexed for 10 seconds. The diluted blood samples were then centrifuged for 15 minutes at 1900 gravities (g). The supernatant was loaded onto the conditioned column. The remaining pellet (solid portion of the blood) was discarded.

5.3 Method Evalution

A goal of this project was to develop a procedure whereby drugs are eluted into the appropriate elution fraction but not during the wash or drying steps. To ensure that no drugs or internal standards were lost with any of the method modifications, the solvents used in the wash, pH adjustment and drying steps were collected and extracted with ethyl acetate. The prepared blood sample, after passing through the column, was also collected and extracted. An equal volume of ethyl acetate was added to the fraction, and the mixture was vortexed for 10 seconds then centrifuged for 5 minutes at 1900 g. Three-quarters of the extraction solvent were removed and the sample was evaporated to dryness. It was not possible to remove all of the solvent because some became trapped within an emulsion. These fractions were reconstituted with ethyl acetate (the GC injection solvent) and analyzed by GC for the presence of drugs. This procedure was used to obtain qualitative results since we were only interested in detecting the presence of drug and not the quantity. The presence of drug in either the wash or drying step fractions indicated that the modification was not acceptable.

Percent recovery was calculated if no drug or internal standard had been lost prior to the elution step. A 100% extraction efficiency was simulated by extracting blood samples spiked with only the internal standards. The elution fractions of these samples were spiked with the drugs of interest. These samples were called unextracted samples. From their chromatograms, the peak area of the drug of interest was divided by the peak area of the internal standard. This provided a peak area ratio (PAR) for the unextracted samples. The same procedure was used for the spiked samples except the drugs of

interest were present in the initial blood sample and extracted through. The PAR of the spiked samples were calculated the same way as the unextracted samples. The percent recovery was calculated by dividing the PAR of the spiked samples by the PAR of the unextracted samples then multiplying the result by 100%. Standard deviation (SD) was calculated by the formula presented below (23). Percent relative standard deviation (%RSD) was calculated by dividing the standard deviation by the mean then multiplying the result by 100%. Recoveries above 70% with relative standard deviations below 10% were desirable.

$$\% \text{ recovery} = \frac{\text{PAR of spiked samples}}{\text{PAR of unextracted samples}} \times 100\%$$

$$\sigma = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n - 1}}$$

σ = standard deviation
x = percent recovery
$\bar{x}$ = average percent recovery (mean)
n = number of samples

Cleanliness of the elution fractions was also considered when choosing appropriate extraction conditions. Both the visual appearance of the elution fraction and the cleanliness of the chromatograms were noted. Fractions that contained precipitate, either before or after the evaporation step, or upon reconstitution might have caused problems during GC analysis and were considered unsuitable. Chromatograms of fractions that contained numerous peaks not representing drugs were considered "dirty" since they could potentially make identification of drugs more difficult. Method modifications that resulted in "dirty" chromatograms were also regarded as unsuitable.

A method that produced relatively clean extracts and acceptable percent recoveries of the test drugs was used to test the drugs from the prepared list. This list is

presented in Appendix A. The majority of drugs were of basic nature, consisting of a mix of primary, secondary and tertiary amines. These drugs were tested at therapeutic concentrations and were placed into groups based on their therapeutic concentration. A spiked water sample was extracted before the spiked blood samples to determine extractability under test conditions. Ethanolic drug standards were run on the GC-NPD to determine their retention times before extractions were performed. The student's t-test was used to compare drug recoveries between methods using two different elution solvents. Analysis of variance (ANOVA) was used to compare recoveries of drugs between methods using more than two different wash solvents or elution solvents.

5.4 Oasis HLB® Method Development

5.4.1 Initial Extraction Method

The method initially used was based upon a method developed by Chen et al. for Bond Elut Certify® column, a silica based mixed-mode cation exchange column (11). The solvents were forced through the sorbent by centrifugation.

Columns were conditioned with 2 mL of methanol and 2 mL of phosphate buffer each for 1 minute at 34 g. The conditioning solvents were discarded. Each solvent used in subsequent steps was collected into separate clean borosilicate test tubes, extracted and analyzed as described above under 5.3 Evaluation of Method. The supernate from the blood samples was loaded onto the conditioned columns and centrifuged for 5 minutes at 34 g. The sorbent was washed with 2 mL deionized water followed by 0.5 mL acetic acid

(0.01 M), each for 3 minutes at 400 g. Acidic and neutral drugs were eluted with 4 mL of acetone/chloroform (1:1) for 5 minutes at 8.5 g. Basic drugs were eluted with 4 mL of ethyl acetate/ammonium hydroxide (98:2) for 5 minutes at 8.5 g.

5.4.2 Initial Sample Preparation

5.4.2.1 Blood Volume

Two millilitres, 1.5 mL, and 1 mL volumes of undiluted porcine blood and 1 mL and 2 mL of 1.25x diluted porcine blood were tested. Samples were prepared as described above under 5.2 Sample Pre-Treatment.

5.4.2.2 Sample pH

Buffers at pH 4, 6, 7 and 9 were tested; 6 mL of each were added to the blood aliquot and vortexed for 10 seconds then centrifuged for 15 minutes at 19 g. The load fractions were collected, extracted with ethyl acetate, and analyzed by GC-NPD to determine if any drugs were lost during this step. The visual appearance of the sample and whether or not it clogged the column was noted.

5.4.3 Wash Solvents

Two millilitres of each wash solvent were tested. The wash fractions were collected, extracted with ethyl acetate and analyzed by GC-NPD. The solvents tried included: deionized water, water/acetonitrile (90:10), water/methanol (90:10),

water/methanol (95:5), water/methanol (96:4), water/methanol (97:3), water/ethyl acetate (95:5), and water/ethyl acetate (97:3).

5.4.4 pH Adjustment

Adjusting the pH with 0.5 mL of acetic acid (0.01 M) and hydrochloric acid (0.01M) was tried, as well as omitting this step altogether. The acid fractions were collected, extracted, and analyzed as described above.

5.4.5 Drying Solvent Volume

Methanol in volumes of 50, 210, 250 and 500 μL was used to dry the sorbent. This fraction was collected with the acid fraction, extracted and analyzed.

5.4.6 Elution Solvents

Each solvent mixture was freshly prepared before use. Solvent combinations with ammonium hydroxide were sonicated for 5 minutes before use to ensure homogeneity. Four millilitres of each solvent were tested: methanol, hexane/ethyl acetate (50:50), hexane/ethyl acetate (75:25), hexane/ethyl acetate/methanol (67.5:22.5:10), methanol/ammonium hydroxide (98:2), ethyl acetate/ammonium hydroxide (98:2), ethyl acetate/isopropanol/ammonium hydroxide (84:12:4), dichloromethane/isopropanol/ammonium hydroxide (78:20:2), hexane/ethyl acetate/ammonium hydroxide (74:24:2), hexane/ethyl acetate/methanol/ammonium hydroxide (67:22:10:1), and ethyl acetate/methanol/ammonium hydroxide (88:10:2).

A summary of the Oasis HLB® method development is presented in Table 3.

Table 3 Oasis HLB® Method Development Summary

Blood Volumes (mL)	2 1 (diluted)	1.5 2 (diluted)	1
Sample pH's	4 9	6	7
Wash Solvents	water water/MeOH 95:5 water/EtOA 95:5	water/ACN 90:10 water/MeOH 96:4 water/EtOA 97:3	water/MeOH 90:10 water/MeOH 97:3
pH Adjustment	0.01 M acetic acid	0.01 M HCl	no adjustment
Drying Volumes (µL)	50 500	210	250
Elution Solvents	MeOH hexane/EtOA 75:25 hexane/EtOA/MeOH/NH_4OH 67:22:10:1 EtOA/MeOH/NH_4OH 88:10:2	MeOH/NH_4OH 98:2 hexane/EtOA/MeOH 67.5:22.5:10 DCM/IPA/NH_4OH 78:20:2 EtOA/IPA/NH_4OH 84:12:4	hexane/EtOA 50:50 hexane/EtOA/NH_4OH 74:24:2 EtOA/NH_4OH 98:2

ACN = acetonitrile, DCM = dichloromethane, EtOA = ethyl acetate, HCl = hydrochloric acid, IPA = isopropanol, MeOH = methanol, NH_4OH = ammonium hydroxide

5.5 Oasis MCX® Method Development

5.5.1 Initial Extraction Method

The methodology was provided by the Waters Corporation for the extraction of whole blood using the Oasis MCX® columns (Waters Corporation personal communication). The solvents were pulled through the sorbent using a vacuum. The procedure began with centrifugation but was transferred to the Zymark RapidTrace SPE Workstation once the method was working satisfactorily. The workstation used positive pressure applied by a syringe to force the solvents through the column. The flow rates used in the Zymark procedure are listed in brackets after the centrifuge parameters.

Column conditioning involved the use of 2 mL of methanol and 2 mL of phosphate buffer each for 1 minute at 34 g (12 mL/min). The blood sample supernate was loaded onto the column and centrifuged for 5 minutes at 34 g (1.2 mL/min). The sorbent was washed with 2 mL of deionized water, 2 mL 0.1 M hydrochloric acid and 2 mL of 5% methanol in water, each for 1 minute at 400 g (9 mL/min). Two different elution solvents were recommended for the elution of acidic and neutral drugs: 2 mL ethanol or acetonitrile/methanol (70:30) for 5 minutes at 8.5 g (1.2 mL/min). Basic drugs were eluted with 2 mL 5% ammonium hydroxide in either ethyl acetate or ethanol for 5 minutes at 8.5 g (1.2 mL/min).

5.5.2 Initial Sample Preparation

5.5.2.1 Blood Volume

Different blood volumes were not tested on the Oasis MCX® columns due to the extensive testing with the Oasis HLB® columns. The blood volume used (1 mL) was the same as that chosen for the Oasis HLB® columns.

5.5.2.2 Sample pH

Potassium dihydrogen phosphate buffer pH ~4.4 (9 mL), as recommended by Waters Corporation, was added to the blood sample.

5.5.3 Wash Solvents

There were three wash steps recommended for the Oasis MCX® columns: 2 mL water, 2 mL 0.1 M HCl and 2 mL 5% methanol in water (Waters Corporation personal communication). The first modification of the original wash method was to use 3 mL of each solvent. Subsequent modifications focused on optimizing the solvent for the last wash step (originally 2 mL of 5% methanol in water) and included: 10% methanol in water, 0.1 M HCl with 5% MeOH, and 5% acetone in water. Two millilitre volumes of each solvent were tested. Again, the solvents were collected and treated as described above.

5.5.4 Sorbent Drying

Samples extracted on the Zymark RapidTrace SPE Workstation were dried with a stream of nitrogen applied to the sorbent for 5 minutes.

5.5.5 Elution Solvents

A number of elution solvents were tried for the acidic and basic elution fraction to achieve the cleanest extract with an acceptable recovery. The following were used for acidic drug elution with the Oasis MCX® columns: 2 mL acetonitrile/methanol (70:30), 4 mL acetonitrile/methanol (70:30), 4 mL acetone/chloroform (1:1), 4 mL hexane/ethyl acetate (75:25), and 4 mL hexane/ethyl acetate/methanol (68:22:10).

The following were tested for the elution of basic drugs from the MCX® columns: 5% ammonium hydroxide in ethyl acetate, 2% ammonium hydroxide in ethyl acetate, 1% ammonium hydroxide in ethyl acetate, and 2% ammonium hydroxide in ethanol. A volume of 4 mL was used for each.

Solvent combinations with ammonium hydroxide were sonicated for 5 minutes before use to ensure homogeneity. Fractions were visually evaluated for the presence of particulate matter and color. After evaporation they were reconstituted and analyzed by GC-NPD. Percent recoveries were calculated.

A summary of the method development for the Oasis MCX® columns is presented in Table 4.

Table 4 Oasis MCX® Method Development Summary

Blood Volume (mL)	Sample pH	Wash Solvents	Elution Solvents
1 mL (diluted 1.25x)	4.4	<u>Step 1</u> 2 mL water 3 mL water <u>Step 2</u> 2 mL 0.1 M HCl 3 mL 0.1 M HCl 2 mL 5% MeOH in 0.1 M HCl <u>Step 3</u> 2 mL 5% MeOH in water 3 mL 5% MeOH in water 2 mL 10% MeOH in water 2 mL 5% acetone in water	<u>Acidic Elution</u> 2 mL ACN/MeOH 70:30 4 mL ACN/MeOH 70:30 4 mL A/C 1:1 4 mL hexane/EtOA 75:25 4 mL hexane/EtOA/MeOH 68:22:10 <u>Basic Elution (4 mL)</u> 5% NH_4OH in EtOA 2% NH_4OH in EtOA 1% NH_4OH in EtOA 2% NH_4OH in EtOH

A/C = acetone/chloroform, ACN = acetonitrile, EtOA = ethyl acetate, EtOH = ethanol, HCl = hydrochloric acid, MeOH = methanol, NH_4OH = ammonium hydroxide

6. Results

6.1 Oasis HLB® Method

6.1.1 Sample Preparation

6.1.1.1 Blood Volume

Use of undiluted porcine blood, regardless of volume (1, 1.5, or 2 mL), clogged columns during the loading step. Use of 2 mL diluted porcine blood also caused some clogging but not with every sample. One millilitre of diluted porcine blood produced no clogging so this was the aliquot volume used in subsequent studies.

6.1.1.2 Sample pH

Initially, 6 mL of 0.1 M phosphate buffer pH 6 was used to dilute the blood aliquot. However, analysis of the collected fractions showed that some codeine and nalorphine were eluted in the pH adjustment step, the drying step, and acidic/neutral elution fractions. When the buffer was changed to pH 9, the drugs were eluted into the basic elution fraction but the extracts were very dirty and unsuitable for routine analysis. A pH 4 phosphate buffer caused precipitate formation which resulted in column clogging and therefore, was also unsuitable. Use of pH 7 phosphate buffer produced elution of codeine and nalorphine into the acidic/neutral elution fraction only.

6.1.2 Wash Solvents

Washing the sorbent with 2 mL of water/methanol (90:10), water/methanol (95:5), water/acetonitrile (90:10), water/ethyl acetate (95:5), or water/ethyl acetate (97:3) caused elution of the codeine and/or nalorphine in the wash and pH/dry fractions. These drugs were not eluted in the wash step when 2 mL water, water/methanol (96:4) or water/methanol (97:3) were used. Percent recoveries of codeine were calculated for these wash solvents and are presented in Table 5. The average percent recovery (mean ± SD) using water/methanol (96:4) was 88.4 ± 16.7% and using water/methanol (97:3) was 31.1 ± 1.3%. The average percent recovery (mean ± SD) using water was 83.0 ± 13.2%. ANOVA and Tukey's Test were used to detect statistical differences between groups. Washing with water/methanol (96:4) resulted in higher recoveries of codeine compared to washing with water/methanol (97:3) which were statistically significant ($p < 0.05$). There was no statistically significant difference ($p > 0.05$) in percent recovery between water and water/methanol (96:4).

Table 5 Percent Recovery of Codeine with Different Wash Solvents

Wash Solvent	Spiked Sample Size	Unextracted Sample Size	% Recovery ± SD
Water	10	6	83.0 ± 13.2
Water/MeOH 96:4	15	15	88.4 ± 16.7
Water/MeOH 97:3	3	2	31.1 ± 1.3

MeOH = methanol, SD = standard deviation

6.1.3 pH Adjustment

Use of acetic acid, hydrochloric acid or the elimination of the pH adjustment step did not affect which fraction eluted the codeine and nalorphine; both drugs eluted into the acidic elution fraction. Omitting the pH adjustment step produced very dirty fractions. At this point it was decided to eliminate the acidic drug elution step since none of the modifications performed eluted the codeine and nalorphine in the basic elution fraction.

6.1.4 Drying Solvent Volume

In the original method, 50 μL of methanol was recommended to dry the sorbent (11). This volume was not sufficient to thoroughly dry the Oasis HLB® sorbent (i.e. the sorbent still looked wet near the bottom frit). Using 210 μL was also an insufficient amount of methanol. Five hundred microlitres of methanol caused elution of codeine. Two hundred and fifty microlitres of methanol was found to sufficiently dry the sorbent without causing elution of drugs.

6.1.5 Elution Solvents

Once the acidic drug elution step was eliminated from the procedure, the elution solvents were tested only for basic/neutral drug elution. Results of successful attempts are summarized in Table 6.

Elution with methanol produced fractions that left a dark red precipitate following evaporation. When reconsituted, the fractions were clear in appearance. The recovery (mean ± SD) of codeine was 91.6 ± 9.7%.

The average percent recovery (mean ± SD) of codeine in samples eluted with hexane/ethyl acetate (50:50) mixture was 76.8 ± 7.1% and hexane/ethyl acetate (75:25) was 83.2 ± 8.3%.

Hexane/ethyl acetate/methanol (67.5:22.5:10) produced a very high recovery for codeine (95.1 ± 26.0%).

Use of hexane/ethyl acetate/methanol/ammonium hydroxide (67:22:10:1) produced very low recoveries. The average recovery (mean ± SD) of codeine was 32.1 ± 14.7%.

Average recovery (mean ± SD) of codeine from samples eluted with ethyl acetate/ammonium hydroxide (98:2) was 79.0 ± 18.9%.

Ethyl acetate/methanol/ammonium hydroxide (88:10:2) produced an average percent recovery of 86.1 ± 7.5%.

Elution with methanol/ammonium hydroxide (98:2) produced dark brown fractions with very dark precipitate. These fractions were not analyzed because of the precipitate.

Dichloromethane/isopropanol/ammonium hydroxide (78:20:2) did not extract nalorphine (ISTD) in any of the samples extracted, although codeine was detected in all samples. The chromatograms contained many extraneous peaks suggesting that the extracts were dirty.

Hexane/ethyl acetate/ammonium hydroxide (74:24:2) did not elute nalorphine in any sample and codeine in only one sample. Percent recoveries were not calculated because the internal standard was not extracted.

Ethyl acetate/isopropanol/ammonium hydroxide (84:12:4) did not elute nalorphine in any of the samples extracted. Codeine was detected in all of the samples. Percent recoveries were not calculated because the internal standard was not extracted.

Table 6 Percent Recovery of Codeine from Different Elution Solvents

Elution Solvent	Spiked Sample Size	Unextracted Sample Size	% Recovery ± SD
MeOH	6	6	91.6 ± 9.7
Hexane/EtOA (50:50)	9	9	76.8 ± 7.1
Hexane/EtOA (75:25)	8	8	83.2 ± 8.3
Hexane/EtOA/MeOH (67.5:22.5:10)	9	9	95.1 ± 26.0
Hexane/EtOA/MeOH/NH_4OH (67:22:10:1)	6	6	32.1 ± 14.7
EtOA/NH_4OH (98:2)	24	24	79.0 ± 18.9
EtOA/MeOH/NH_4OH (88:10:2)	9	9	86.1 ± 7.5

EtOA = ethyl acetate, NH_4OH = ammonium hydroxide, MeOH = methanol, SD = standard deviation

ANOVA and Tukey's Test determined that all solvent mixtures resulted in higher recoveries than hexane/ethyl acetate/methanol/ammonium hydroxide (67:22:10:1). These results were statistically significant ($p < 0.05$). No significant differences between the other solvent mixes were found ($p > 0.05$).

6.2 Oasis MCX® Method

6.2.1 Sample Preparation

6.2.1.1 Blood Volume

Extraction of 1 mL of diluted porcine blood through the Oasis MCX® columns was successful. Other volumes were not tested.

6.2.1.2 Sample pH

Nine millilitres of 0.1 M potassium dihydrogen phosphate buffer pH~4.4 was used to dilute the blood. No clogging or precipitate formation occurred. Other pH's were not tested.

6.2.2 Wash Solvents

All wash solvents were centrifuged through the column for 1 minute at 400 g instead of 3 minutes. One minute was sufficient to force the solvent through the sorbent thereby saving 2 minutes per wash step.

The original method from Waters Corporation suggested using 2 mL each of three wash solvents: water, 0.1 M hydrochloric acid, and 5% methanol in water (Waters Corporation, personal communication). Analysis of the wash fractions indicated that no drugs were eluted when this wash sequence was used. The chromatograms of the elution fractions were relatively clean except for a large artifact in the basic elution fraction. This artifact was large enough to obscure any drugs eluted in this area of the

chromatogram (see Figure 2). Increasing the volume of water and 0.1 M hydrochloric acid to 3 mL did not result in loss of drugs but 3 mL of 5% methanol in water did cause elution of some barbiturates. The artifact peak size was not reduced. Use of 2 mL of 10% methanol in water also caused a loss of barbiturates without reducing the artifact peak size. Acetone in water (5%) and methanol in 0.1 M hydrochloric acid (5%) did not cause elution of drugs but did not help to clean the samples as well as the other solvents.

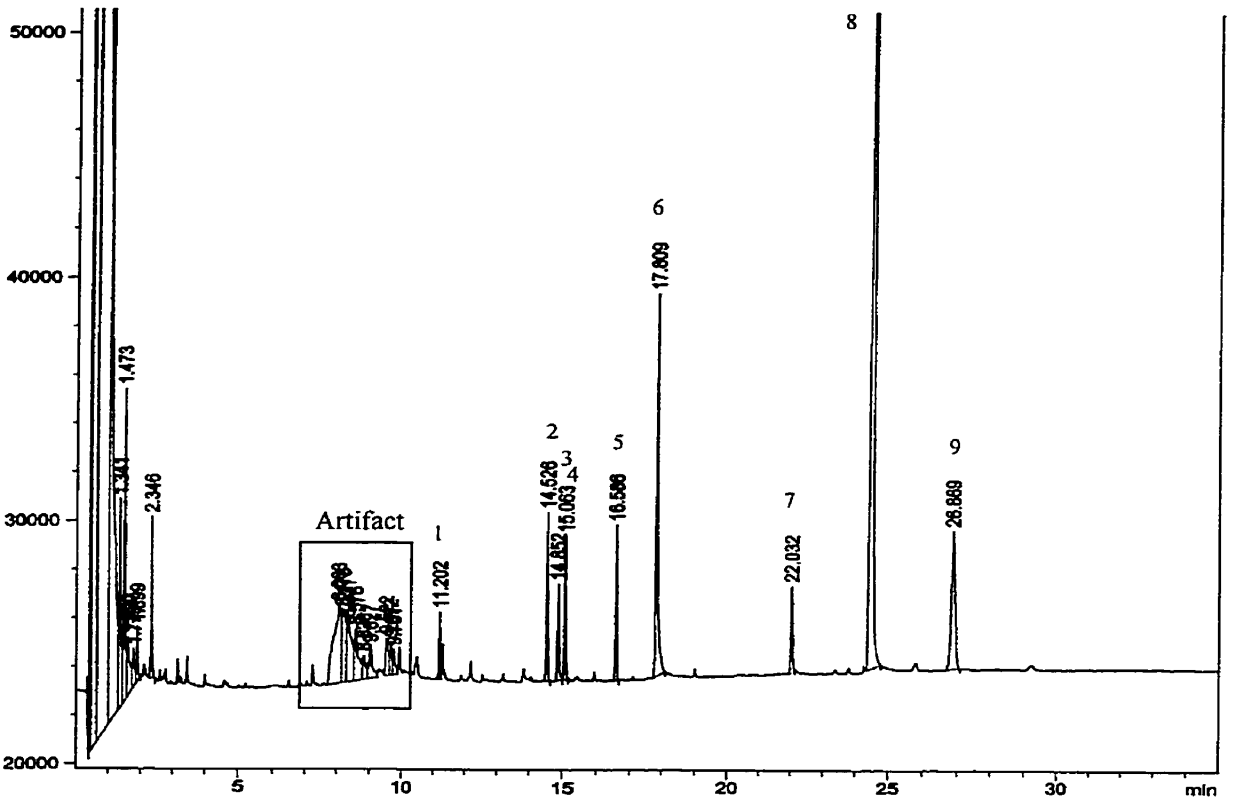

Figure 2 Artifact in basic elution fraction. Peak Identification: (1) fluoxetine, (2) orphenadrine, (3) propranolol, (4) imipramine, (5) maprotiline, (6) nalorphine (ISTD), (7) quinidine, (8) and (9) ethyl acetate artifacts

6.2.3 Drying

The sorbent was thoroughly dried using a stream of nitrogen for 5 minutes instead of methanol.

6.2.4 Elution Solvents

6.2.4.1 Acidic Elution Solvents

The use of 2 mL of acetonitrile/methanol (70:30) was insufficient to remove all barbiturates. Some were eluted in the basic elution fraction. When the volume of elution

solvent was increased to 4 mL, the barbiturates were completely eluted into the acidic fraction. Four millilitres of all other elution solvents were also used.

Elution with acetone/chloroform (1:1) resulted in higher recoveries of all barbiturates (except barbital) as compared to acetonitrile/methanol (70:30). Statistical analysis using the student's t-test (unpaired) found significantly higher recoveries for amobarbital ($p < 0.05$), pentobarbital ($p < 0.05$), phenobarbital ($p < 0.05$), and secobarbital ($p < 0.05$). Recoveries for allobarbital and thiopental were not significant ($p > 0.05$ and $p > 0.5$, respectively). Results are summarized in Table 7 for acetonitrile/methanol (70:30) and acetone/chloroform (1:1).

Table 7 Percent Recoveries of Barbiturates using Different Acidic Elution Solvents

Drug	% Recovery ± SD	
	acetonitrile/methanol (70:30) ss = 2, us = 2	acetone/chloroform (1:1) ss = 10, us = 10
allobarbital	77.1 ± 3.2	81.2 ± 5.1
amobarbital	75.6 ± 2.7	90.0 ± 3.7
barbital	52.9 ± 2.3	28.0 ± 3.2
butabarbital*	-	90.4 ± 5.9
pentobarbital	76.5 ± 2.2	89.5 ± 4.0
phenobarbital	77.9 ± 0.5	92.7 ± 5.1
secobarbital	75.8 ± 1.3	86.6 ± 4.5
thiopental	72.8 ± 1.3	78.8 ± 12.5

SD = standard deviation, ss = spiked sample size, us = unextracted sample size
* samples extracted with acetonitrile/methanol (70:30) were not spiked with butabarbital

Hexane/ethyl acetate (75:25) and hexane/ethyl acetate/methanol (68:22:10) as solvent mixtures did not elute tolylbarbital (acidic ISTD). Chromatograms from the elution fractions of these solvents were dirtier than the acetonitrile/methanol fractions. Percent recoveries were not calculated.

6.2.4.2 Basic Elution Solvents

The average percent recoveries (mean ± SD) of the basic drugs eluted with the different basic elution solvents are summarized in Table 8.

Initially, 5% ammonium hydroxide in ethyl acetate was used. All of the basic drugs were eluted and the chromatograms were relatively free from extraneous peaks.

The use of 2% ammonium hydroxide in ethyl acetate produced fractions that were visually similar to the fractions eluted with 5% ammonium hydroxide in ethyl acetate. There was no significant difference in the recovery of drugs when using 2% versus 5% ammonium hydroxide in ethyl acetate ($p > 0.05$, ANOVA).

Eluting the basic drugs with 1% ammonium hydroxide in ethyl acetate produced fractions that were visually similar in appearance compared to the fractions eluted with 5% and 2% ammonium hydroxide in ethyl acetate. However, significantly lower recoveries were obtained with this solvent mix compared to the other elution solvents ($p < 0.05$, ANOVA).

Elution with 2% ammonium hydroxide in ethanol produced very dirty fractions. The elution fractions were dark green with a precipitate. These fractions were not reconstituted or analyzed.

Table 8 Percent Recovery of Drugs in NPQC using Different Basic Elution Solvents

Drug	% Recovery ± SD		
	5% NH_4OH in EtOA ss = 2, us = 2	2% NH_4OH in EtOA ss = 2, us = 2	1% NH_4OH in EtOA ss = 2, us = 2
amitriptyline	53.7 ± 7.7	73.8 ± 11.9	35.5 ± 0.4
amphetamine	ND	ND	ND
benzocaine	ND	ND	ND
caffeine*	-	-	-
chlorpheniramine	77.7 ± 3.5	92.3 ± 20.9	51.2 ± 1.5
codeine	73.9 ± 3.0	93.5 ±7.3	67.0 ± 1.8
diphenoxylate	20.9 ± 12.0	27.8 ± 3.1	11.8 ± 1.1
heroin	63.7 ± 0.9	85.4 ± 14.5	55.6 ± 0.7
lidocaine	90.3 ± 0.3	105.2 ± 22.9	71.9 ± 2.3
meperidine	140.0 ± 12.6	124.0 ± 16.8	42.3 ± 1.5
methadone	73.1 ± 2.2	93.9 ± 16.8	40.3 ± 1.5
methaqualone	63.7 ± 3.7	90.1 ± 7.2	65.2 ± 1.9
methylphenidate	118.1 ± 4.9	103.3 ± 45.8	47.7 ± 0
morphine	72.4 ± 7.1	85.1 ± 3.3	38.2 ± 0.8
nefazodone	44.5 ± 12.4	55.9 ± 5.9	38.2 ●2.9
pentazocine	62.8 ± 1.9	90.0 ± 8.9	43.0 ± 1.1
phenyltoloxamine	80.7 ± 7.3	97.5 ± 29.4	52.4 ± 2.8
strychnine	86.5 ± 0.4	93.2 ± 2.7	58.1 ± 0.9
trazodone	77.8 ± 2.1	85.0 ± 3.2	65.0 ± 4.7

* caffeine was detected at sub-therapeutic levels, percent recovery was not calculated
EtOA = ethyl acetate, ND = not detected, NH_4OH = ammonium hydroxide, SD = standard deviation, ss = spiked sample size, us = unextracted sample size

6.2.5 Artifact Removal

An attempt was made to determine the source of the artifact. Different approaches were attempted with the results summarized in Table 9.

Table 9 Approaches in Determining the Source of the Artifact

Approach	Result
injection of extract on other GC columns (DB1 and RTX)	artifact present
liquid-liquid extraction of unspiked porcine blood using ethyl acetate	dirty extract, possible presence of artifact
GC analysis of pure solvents and drug mixes	artifact absent
extraction of water blank	artifact absent

Once it was determined that the artifact did not arise from the GC column, solvents, drugs mixes, or the Oasis® column itself, the source of blood and its storage container were examined. Samples of porcine blood stored in plastic, human blood stored in plastic, and human blood stored in glass were extracted. All chromatograms of the basic elution fractions showed the artifact. The artifact size was the smallest from the human blood stored in glass. This suggested that the artifact might be due to the leaching of a plasticizer from the plastic storage bottle. Due to the limited supply of human blood stored in glass, fresh porcine blood was collected and stored in glass bottles. This blood supply was free of the artifact (see Figure 3) and was used for the remainder of the project.

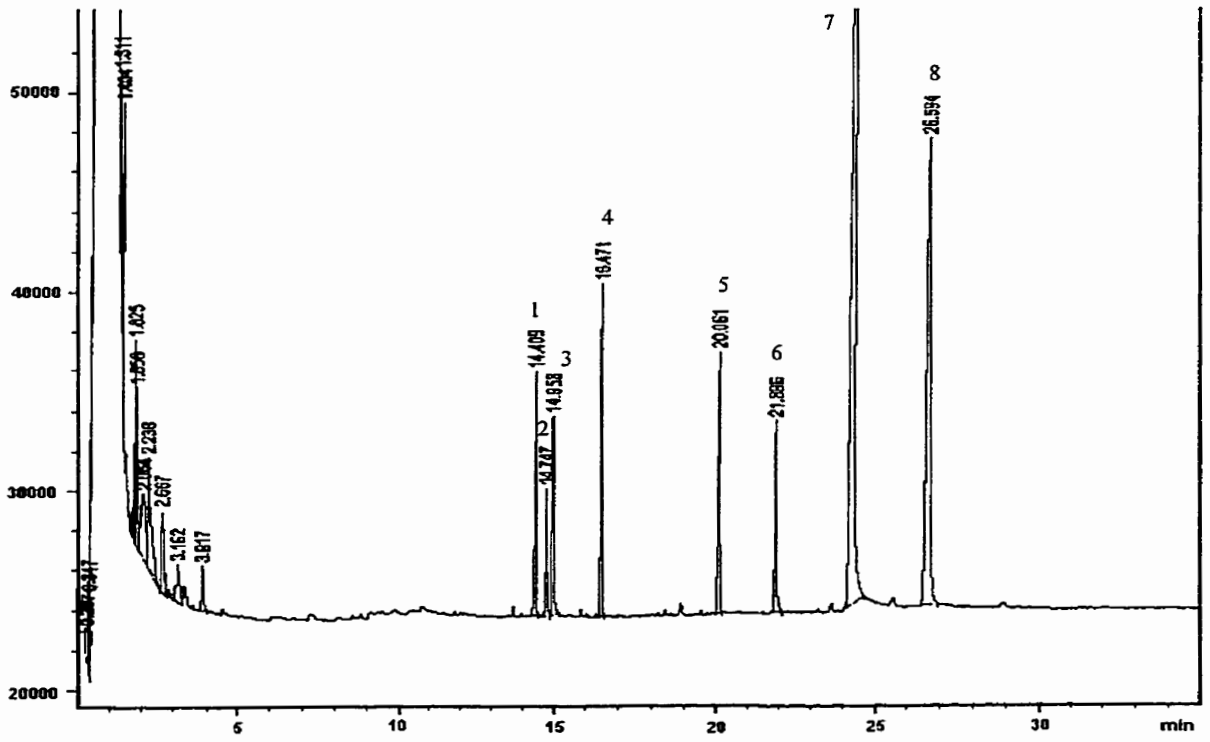

Figure 3 Basic elution fraction from the extraction of fresh porcine blood.
Peak Identification: (1) orphenadrine, (2) propranolol, (3) imipramine, (4) maprotiline, (5) doxapram (ISTD), (6) quinidine, (7) and (8) ethyl acetate artifacts.

The final method chosen is presented in Figure 4. It was used to extract the benzodiazepines and the drugs listed in Appendix A. Results for the drugs listed in Appendix A are summarized in Table 10. Chromatograms of the extracted drugs are presented in Appendix C. Results for the benzodiazepines are described in the next section.

Figure 4 Final Oasis MCX® Procedure using Zymark RapidTrace SPE Workstation

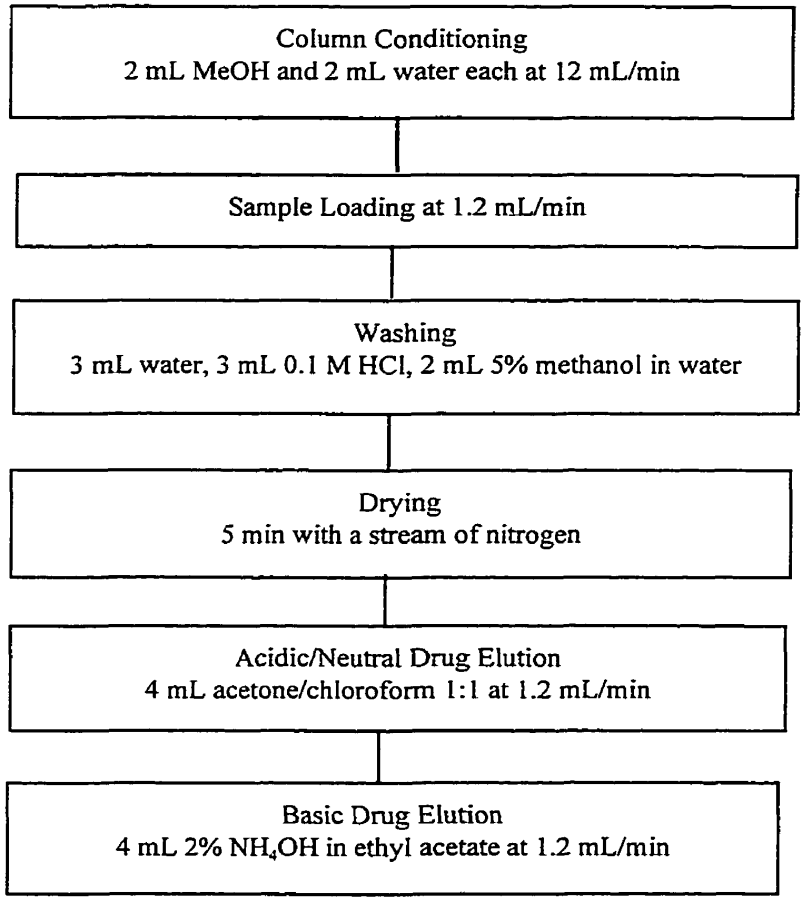

Table 10 Recoveries of Drugs from Appendix A using Oasis MCX® Columns

Drug	Concentration (µg%)	Number of Samples ss;us	% Recovery ± SD	%RSD	Comments
amitriptyline	25	9;9	97.4 ± 6.5	6.6	
amoxapine	10	10;10	81.8 ± 14.3	17.5	
amphetamine	≤ 50	37;37	ND	-	
azacyclonal	10*	†	ND	-	below peak area reject[§]
azatadine	1.4	†	ND	-	
benzocaine	100	8;8	74.0 ± 5.4	7.3	
benztropine	12	3;10	109.1 ± 8.3	7.6	
brompheniramine	2.2	†	ND	-	
chlordiazepoxide	300	†	ND	-	multiple peaks; parent drug not detected
chlorpheniramine	5*	10;10	121.8 ± 7.4	6.1	
chlorpromazine	25	10;10	78.1 ± 12.4	15.9	
chlorprothixene	25	15;15	71.4 ± 8.5	11.8	
clemastine	10*	3;5	68.1 ± 3.9	5.8	
clomipramine	25	10;10	69.8 ± 2.3	3.2	
cocaine	25	10;10	113.8 ± 14.5	12.7	
codeine	10	10;10	86.7 ± 7.4	8.6	
desipramine	25	10;10	81.0 ± 6.3	7.8	
dextromethorphan	5	10;10	85.2 ± 3.6	4.2	
dextrorphan	25	10;10	86.9 ± 4.6	5.3	
dicyclomine	8	15;15	92.8 ± 11.4	12.3	
diethylpropion	10	10;10	ND	-	
diltiazem	25	10;10	88.2 ± 3.3	3.8	
diphenhydramine	5	10;10	87.7 ± 4.8	5.5	
diphenoxylate	5*	9;10	73.6 ± 10.4	14.1	
ephedrine	5	5;5	ND	-	
ethosuximide	200	37;37	ND	-	
flunarizine	10	10;10	55.9 ± 6.6	11.8	
fluoxetine	25	10;10	69.2 ± 4.2	6.1	
fluvoxamine		EtOH standard	ND	-	
haloperidol	1.5	†	ND	-	
heroin	5	8;8	92.9 ± 8.8	9.4	
hydrocodone	2	†	ND	-	
hydroxyzine	5	†	ND	-	coeluted with ISTD
imipramine	25	10;10	79.3 ± 4.2	5.3	
ketamine	10	10;10	83.1 ± 6.4	7.7	
levorphan		EtOH standard	ND	-	
lidocaine	50	8;8	95.5 ± 4.9	5.2	
loxapine	5	15;15	88.2 ± 5.8	6.5	

* tested above therapeutic concentration, † single spiked water sample, § peaks with area counts below 2500 were not considered due to insufficient signal to noise ratio, EtOH = ethanol, ISTD = internal standard, ND = not detected, RSD = relative standard deviation, SD = standard deviation, ss = sample size, us = unextracted sample size

Table 10 Recoveries of Drugs from Appendix A using Oasis MCX® Columns (continued)

Drug	Concentration (µg%)	Number of Samples ss;us	% Recovery ± SD	%RSD	Comments
maprotiline	25	10;10	69.9 ± 4.8	6.9	
meperidine	50	8;8	91.6 ± 6.9	7.5	
methadone	50	8;8	90.2 ± 4.6	5.1	
methamphetamine	10	10;10	ND	-	coeluted with other peak
methaqualone	50	8;8	86.6 ± 3.2	3.7	
methotrimeprazine	5	10;5	114.1 ± 9.5	8.3	
methylecgonine	10*	5;10	75.3 ± 8.1	10.7	
MDA	50	9;10	58.2 ± 14.8	25.4	
methylphenidate	5	10;10	133.6 ± 25.2	18.8	
methyprylon	500	10;10	85.7 ± 4.4	5.1	
metoprolol	25	15;15	87.2 ± 17.5	20.1	
midazolam	25	10;10	56.3 ± 11.7	20.8	
morphine	20	10;10	86.2 ± 41.1	47.7	
nefazodone	100	9;9	78.4 ± 7.7	9.8	
nordoxepin	10	†	ND	-	
nortriptyline	5	5;5	ND	-	
orphenadrine	25	10;10	85.2 ± 3.8	4.5	
paroxetine	5	10;10	ND	-	
pentazocine	25	9;9	96.1 ± 5.0	5.2	
perphenazine	500	†	ND	-	coeluted with EtOA artifact
phencyclidine	7	10;10	100.4 ± 7.6	7.6	
pheniramine	5	7;2	89.3 ± 3.9	4.4	
PPA	100	EtOH standard	ND	-	
phenyltoloxamine	5*	10;10	122.6 ± 16.2	13.3	
prochlorperazine	1	†	ND	-	
procyclidine	25	10;10	83.9 ± 5.3	6.3	
propoxyphene	25	10;10	84.8 ± 7.2	8.5	
propranolol	25	10;10	76.4 ± 9.9	13.0	
pseudoephedrine	75	10;10	136.4 ± 28.0	20.6	
quinidine	25	10;10	81.2 ± 26.7	33.2	
sertraline	5	10;10	58.2 ± 5.6	9.6	
strychnine	50	8;8	83.1 ± 20.7	24.5	
thioridazine	25	10;10	83.6 ± 8.2	9.8	
trazodone	100	8;8	87.0 ± 8.1	9.0	
trifluoperazine	5	10;10	ND	-	below peak area reject[§]
trimipramine	5	15;15	90.9 ± 6.5	7.2	
triprolidine	4	†	ND	-	
verapamil	15	10;10	121.9 ± 9.8	8.0	
zopiclone	5	3;5	107.3 ± 24.0	22.4	

* tested above therapeutic concentration, † single spiked water sample, § peaks with area counts below 2500 were not considered due to insufficient signal to noise ratio, EtOA = ethyl acetate, EtOH = ethanol, ISTD = internal standard, MDA = methylenedioxyamphetamine, ND = not detected, PPA = phenylpropanolamine, RSD = relative standard deviation, SD = standard deviation, ss = sample size, us = unextracted sample size

6.2.6 Benzodiazepines

The benzodiazepine mixture consisted of the drugs listed in Table 11. The internal standard used for the benzodiazepines was methylnitrazepam (50 ng per millilitre of blood). When extracted it was found that the benzodiazepines eluted into both the acidic and basic elution fractions. Clonazepam, lorazepam, oxazepam, and temazepam eluted in the acidic fraction while all others eluted into the basic fraction. A chromatogram of each fraction is shown in Figures 5 and 6. Methylnitrazepam (ISTD) split unevenly between the two fractions. The search for another internal standard was undertaken. The possiblities included: CBP (1-(4-chlorobenzhydryl)-piperazine), carbamazepine, demoxepam, halazepam, lormetazepam, and prazepam (see Table 12).

Table 11 Benzodiazepine Mix

Drug	Concentration (µg%)
alprazolam	8
clonazepam	4
desalklyflurazepam	8
diazepam	20
lorazepam	8
nitrazepam	8
nordiazepam	20
oxazepam	20
temazepam	40
triazolam	4

Table 12 Summary of Internal Standard (ISTD) Search

ISTD Possibility	Result
CBP	not extracted into any elution fraction
carbamazepine	not extracted into any elution fraction
demoxepam	split into multiple peaks on GC
halazepam	split between acidic and basic elution fractions
lormetazepam	split into multiple peaks on GC
prazepam	eluted in basic fraction only

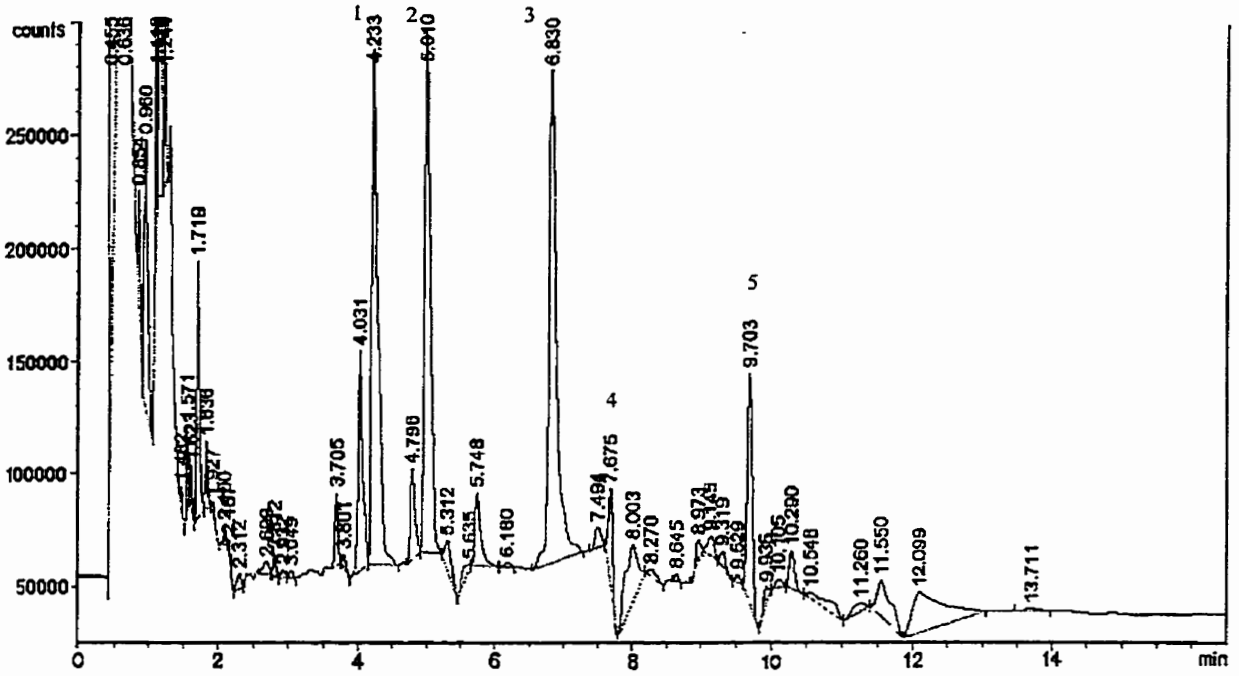

Figure 5 Benzodiazepine Acidic Elution Fraction. Peak Identification: (1) oxazepam, (2) lorazepam, (3) temazepam, (4) methylnitrazepam (ISTD), (5) clonazepam

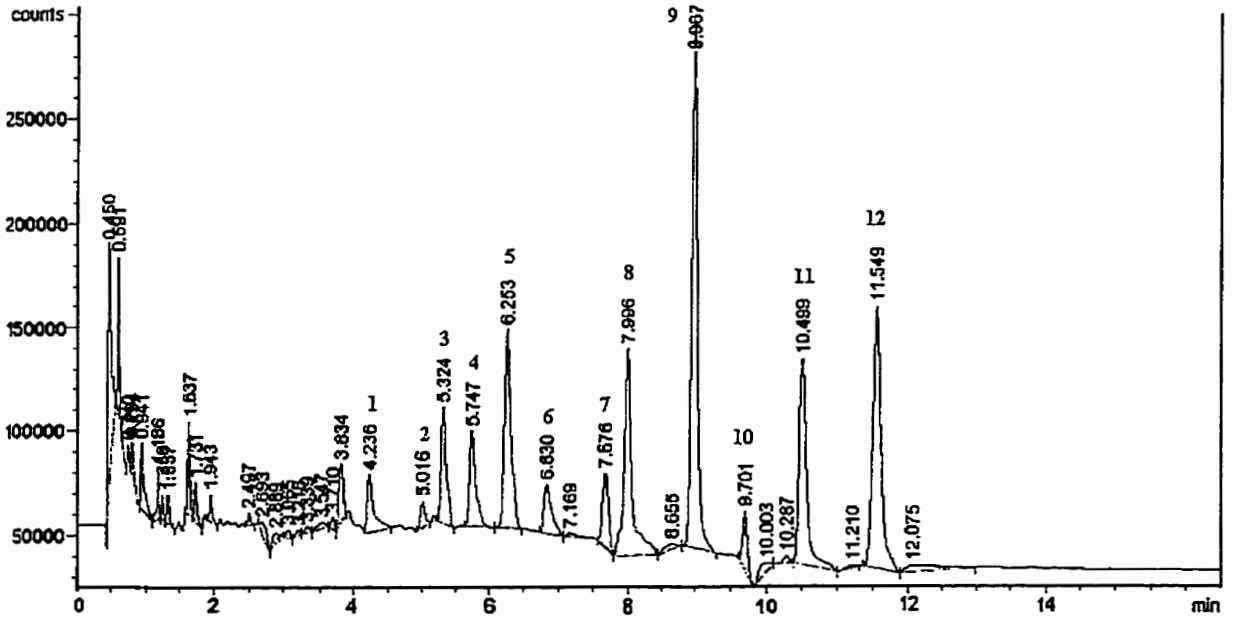

Figure 6 Benzodiazepine Basic Elution Fraction. Peak Identification: (1) oxazepam, (2) lorazeapm, (3) diazepam, (4) desalkylflurazepam, (5) nordiazepam, (6) temazepam, (7) methylnitrazepam (ISTD), (8) temazepam breakdown product, (9) nitrazepam, (10) clonazepam, (11) alprazolam, (12) triazolam

Percent recoveries for the benzodiazepines were not calculated due to the inability to find a suitable internal standard. A standard curve could have been used to determine percent recovery, however, the larger variability of the results with this method was unacceptable for forensic toxicological work (RCMP Laboratory Toxicology Section Methods Guide, Revised July 1995).

The benzodiazepine elution extracts were visually dirty when reconstituted. The acidic fractions were dark orange with a fine brown precipitate while the basic fractions were a light orange color. GC maintenance, i.e. changing the liner, septum and/or column to maintain good chromatographic conditions and to maximize sensitivity, was required after each set of analyses indicating that these extracts would be unsuitable for routine analysis.

7. Discussion

7.1 Oasis HLB® Method

Codeine was chosen as the test drug because it had one of the highest occurrence rates of those drugs commonly found in forensic casework. A concentration of 80 μg%, greater than the therapeutic level (10 μg%), was used since it was important to readily detect the drug while optimizing the method.

7.1.1 Sample Preparation

Column clogging was the most common problem encountered when attempting to optimize sample preparation. To reduce the amount of particulate matter in the samples, sonication was used to hemolyze red blood cells. Previous studies evaluating different methods of sample preparation found that sonication provided the best recovery of compounds (11,24). After sonication, the samples were centrifuged to settle particulate matter. Only the supernatant was loaded onto the column.

Samples prepared with 1 mL of diluted porcine blood were successfully extracted. Diluting the blood by 25% using water decreased the viscosity but still kept the hemoglobin within the acceptable range (7.4 to 11.2 mM) (25). All other volumes of blood, both diluted and undiluted, caused clogging of the column and were unsuitable for routine use.

For best recovery, drugs have to be strongly retained on the sorbent during the loading and washing steps. Use of different buffer pH's for sample preparation may

enhance retention by altering the ionization of sample compounds. Codeine, with a pKa of 8.2, is completely ionized at pH 4 and 6, 94% ionized at pH 7 and approximately 14% ionized at pH 9. The formula used to calculate percent ionization was:

$$\% \text{ ionization} = \frac{100}{1 + \text{antilog}\,(\text{pH} - \text{p}K_a)}$$

Samples prepared using pH 4 buffer clogged the column. This was possibly due to blood protein precipitation. These samples were not suitable for analysis. Preparation with pH 6 buffer resulted in a loss of codeine and nalorphine in the pH adjustment and drying step with the codeine being eluted into the acid/neutral elution fraction. Use of pH 9 buffer did not cause a loss of drugs in any fraction; the codeine was eluted only in the basic elution fraction. However, since the elution fractions were very dirty this buffer was unsuitable for sample preparation. The pH 7 buffer did not cause a loss of drugs but the codeine and nalorphine were eluted into the acidic/neutral elution fraction, not the basic fraction. To compromise between cleanliness and drug retention, pH 7 buffer was chosen for sample preparation.

From this small study of different buffer pHs, it appears that the Oasis HLB® columns retain nonionized compounds more strongly than ionized compounds (i.e. drug was lost with pH 6 buffer and retained with pH 9 buffer).

7.1.2 Wash Solvents

The most effective way of removing undesired compounds from the sorbent is by washing the column with a solvent or solvent mixture. A mixture of organic solvents in

water increased the lipophilicity of the solution and the potential to remove from the column slightly lipophilic compounds that are insoluble in water. The polarity of a solvent or solvent mixture is expressed by a P′ value (polarity). The lower the value, the more lipophilic the solvent or solvent mixture. A list of the P′ values for the wash solvents used for the Oasis HLB® and the Oasis MCX® columns is presented in Table 13.

Another way of grouping solvents is by their ability to participate in hydrogen bonding or dipole interactions (26). Solvents with similar bonding abilities and structures are placed into the same group. Methanol, ethyl acetate, and acetonitrile are from different solvent groups (II, VIa, VIb, respectively) and have different P′ values suggesting that they may have different selectivities for different compounds.

In general, solvent wash mixtures with P′ values below 9.91 caused a loss of codeine and/or nalorphine. Water/ethyl acetate (97:3) (P′=10.02) caused elution of codeine and nalorphine but water/methanol (96:4) (P′=9.996) did not cause loss of drug. This discrepancy may reflect the fact that ethyl acetate has a stronger dipole than methanol (26). Therefore, ethyl acetate may have a greater potential than methanol to interact with retained compounds through dipole-dipole interactions and elute them from the column.

The recoveries of codeine from the different wash solvents are presented in Table 5. The recovery of codeine from water/methanol (96:4) was significantly higher than from water/methanol (97:3) ($p < 0.05$, ANOVA). It is not known why the recovery of codeine would be significantly lower with the use of water/methanol (97:3) as compared to water/methanol (96:4) since there is not much difference in polarity between the two

mixes. There was no significant difference in the recovery of codeine between water and water/methanol (96:4) ($p > 0.05$, ANOVA).

Water/methanol (96:4) was chosen as the wash solvent because: 1) it gave a higher recovery of codeine than water/methanol (97:3) and 2) in theory, it should be better at removing undesirable compounds from the sorbent than water due to a lower P′ value.

Table 13 P′ Values of Wash Solvents used in the Oasis HLB® and Oasis MCX® Method Development

Solvent Mix		P' value
water		10.2
water/MeOH	90:10	9.69
	95:5	9.95
	96:4	10.00
	97:3	10.05
water/EtOA	95:5	9.91
	97:3	10.02
water/ACN	90:10	9.76
water/acetone	95:5	9.95

ACN = acetonitrile, EtOA = ethyl acetate, MeOH = methanol

7.1.3 pH Adjustment

It was thought that using either a strong acid (0.01 M HCl) or eliminating the pH adjustment step would enhance retention of the codeine thereby preventing it from being eluted in the acidic/neutral fraction. Optimization of the pH adjustment step was performed before the final buffer pH was chosen. Therefore, it was not known if the

retention of codeine was enhanced at higher pHs. Samples used in this part of the study were prepared with pH 7 buffer. Interestingly, the use of 0.01 M acetic acid (pH 3.4) or 0.01 M HCl (pH 2) did not cause a loss of codeine. Perhaps the volume of acid used was not large enough to cause elution.

Eliminating the pH adjustment step resulted in very dirty fractions suggesting that the acid was functioning as another wash step, further cleaning the sample. Hydrochloric acid was chosen instead of acetic acid because a lower pH was assumed to remove a greater number of undesirable compounds from the sorbent although this was not tested.

7.1.4 Drying Solvent Volume

To obtain a clean elution fraction it is important to dry the sorbent thoroughly before elution to remove any leftover wash solvent. The volume of drying solvent should be sufficient to fill all pores and spaces in the sorbent. Based on the pore volume of the Oasis HLB® sorbent (1.4 mL/g), 150 mg of sorbent requires at least 210 μL of drying solvent. This volume of methanol was found to be insufficient to dry the sorbent possibly because the pore volume of the polyethylene frits above and below the sorbent were not taken into account. The drying solvent volume chosen for the final procedure was 250 μL since it dried the sorbent thoroughly and did not cause elution of the drugs.

7.1.5 Elution Solvents

A number of elution solvent mixes were tested to determine which gave the cleanest fractions with the best recovery of codeine. Methanol was recommended by

Waters Corporation in their Application Manual as the elution solvent for the Oasis HLB® columns (8). The majority of the other solvent mixtures tested were successfully used in other procedures developed for reversed phase and mixed mode columns (27-31).

The mixtures which produced favorable results, with respect to cleanliness and recovery, were: hexane/ethyl acetate (50:50 and 75:25), hexane/ethyl acetate/methanol (67.5:22.5:10), ethyl acetate/ammonium hydroxide (98:2), and ethyl acetate/methanol/ ammonium hydroxide (88:10:2).

A final solvent mix for the Oasis HLB® procedure was not chosen because the Oasis MCX® columns became available which were capable of sequential elution of acidic, neutral and basic drugs.

7.2 Oasis MCX® Method

Many of the conditions chosen for the Oasis MCX® columns were based on the results from the Oasis HLB® columns.

7.2.1 Sample Preparation

Since the Oasis MCX® sorbent required that the basic drugs be ionized for optimal retention, a buffer of approximately pH 4.4 was used for sample dilution. At this pH almost all of the drugs in Appendix A would be 100% ionized as they have pK_a's greater than 6.4. Since the analysis of the load fractions did not contain any drugs, other pH's were not tested.

7.2.2 Wash Solvents

The three step wash procedure for the Oasis MCX® columns consisted of 2 mL each of water, 0.1 M HCl, and water/methanol (95:5). Unlike the Oasis HLB® columns, the use of water/methanol (95:5) did not cause elution of drugs. This is likely due to the retention of basic drugs on the sorbent by ionic interactions. For acidic drugs, retention was due to strong hydrophobic interactions. Increasing the volume of water and acid to 3 mL each did not cause a loss of drugs, although 3 mL of water/methanol (95:5) did result in drug loss. Of the wash solvents tested, use of 3 mL water, followed by 3 mL 0.1 M HCl then 2 mL water/methanol (95:5) produced the cleanest extracts and, therefore, was chosen as the final wash sequence.

7.2.3 Elution Solvents

7.2.3.1 Acidic Elution Solvents

Use of 2 mL acetonitrile/methanol (70:30), as suggested by Waters Corporation, did not cause total elution of the barbiturates into the acidic fraction; some were detected in the basic fraction. Recoveries were increased when 4 mL of elution solvent was used. Negligible amounts of barbiturates were eluted into the basic fraction. The increased volume of elution solvent allowed more contact with the sorbent permitting the barbiturates to be completely eluted.

Elution solvents containing no ammonium hydroxide which produced favorable results on the Oasis HLB® columns were tested as acidic elution solvents on the Oasis

MCX® columns. Elution with hexane/ethyl acetate (75:25) and hexane/ethyl acetate/ methanol (68:22:10) produced good recoveries from the Oasis HLB® columns but did not elute tolylbarbital (acidic ISTD) from the Oasis MCX® columns. The use of hydrophobic solvent mixtures such as these have been reported to cause incomplete elution of acidic and neutral compounds from silica based columns (31).

Previous studies using acetone/chloroform (1:1) as an elution solvent for mixed-mode silica based columns found that this mixture resulted in higher recoveries than other solvents (14). When this solvent mix was tested on the Oasis MCX® columns significantly higher recoveries were also found as compared to acetonitrile/methanol (70:30). The increase in recovery was the basis for the selection of this mixture as the acidic/neutral elution solvent.

7.2.3.2 Basic Elution Solvents

Two basic elution solvent mixtures were recommended by Waters Corporation for the Oasis MCX® columns: 5% ammonium hydroxide in ethyl acetate and 5% ammonium hydroxide in ethanol (Waters Corporation, personal communication).

Elution with 5% ammonium hydroxide in ethyl acetate resulted in good drug recoveries but the elution extracts were too basic in nature to be used for routine GC analysis. Column damage has been reported with as little as 3% ammonium hydroxide in ethyl acetate (14). Use of 2% ammonium hydroxide in ethyl acetate did not harm the GC column yet produced recoveries similar to that obtained with 5% ammonium hydroxide. Reducing the percentage of ammonium hydroxide to 1% resulted in very low recoveries.

This elution solvent was not strong enough to neutralize the positively charged basic drugs allowing them to be eluted from the column.

Since 5% ammonium hydroxide was unacceptable, 2% ammonium hydroxide *in ethanol* was tried. The elution extracts were darkly colored and contained a precipitate suggesting that some of the blood pigments were eluted from the sorbent. This elution solvent was not suitable.

The only elution solvent mixture that produced good recoveries and acceptable cleanliness, with no damage of the GC column was 2% ammonium hydroxide in ethyl acetate. This mixture was chosen as the basic elution solvent mixture.

This elution solvent mixture resulted in the elution of all the extractable basic drugs except for some benzodiazepines.

7.2.4 Benzodiazepines

7.2.4.1 Splitting Between Elution Fractions

The benzodiazepines, a group of weakly basic drugs, were found to split between the two elution fractions. Clonazepam, lorazepam, oxazepam and temazepam were eluted into the acidic/neutral elution fraction; alprazolam, desalkylflurazepam, diazepam, nordiazepam and triazolam were eluted into the basic fraction. Nitrazepam was eluted mainly in the basic fraction but a small percentage was eluted in the acidic fraction in some instances.

In general, the drugs with pK_a's of 1.3 to 1.8 were eluted into the acidic fraction and drugs with pK_a's of 3.2 to 3.4 were eluted into the basic fraction. Exceptions to this were triazolam (pK_a 1.3) and desalkylflurazepam (pK_a 1.5) which eluted into the basic fraction.

A possible structural requirement (see Figure 7 and Table 14) for elution into the acidic/neutral fraction may be a hydroxyl substituent (-OH) at position R3 (as seen in lorazepam, oxazepam, and temazepam).

Nitrazepam has the same substituents as clonazepam but with a hydrogen at R2' instead of a chlorine atom. This structural difference may explain why some of the nitrazepam was eluted into the acidic/neutral fraction.

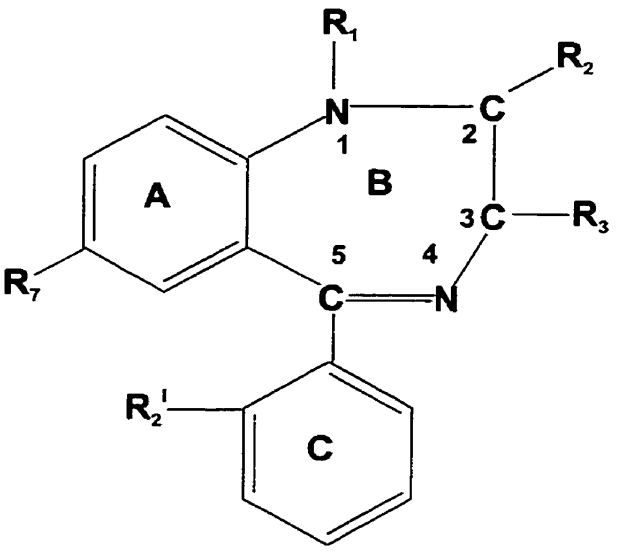

Figure 7 General Structure of the Benzodiazepines

Table 14 Benzodiazepine Structures

Benzodiazepine	R1	R2	R3	R7	R2'
alprazolam	fused triazolo ring		-H	-Cl	-H
clonazepam	-H	=O	-H	$-NO_2$	-Cl
desalkylflurazepam	-H	=O	-H	-Cl	-F
diazepam	$-CH_3$	=O	-H	-Cl	-H
lorazepam	-H	=O	-OH	-Cl	-Cl
nitrazepam	-H	=O	-H	$-NO_2$	-H
nordiazepam	-H	=O	-H	-Cl	-H
oxazepam	-H	=O	-OH	-Cl	-H
temazepam	$-CH_3$	=O	-OH	-Cl	-H
triazolam	fused triazolo ring		-H	-Cl	-Cl

7.2.4.2 Internal Standard Search

To calculate recoveries for the benzodiazepines an internal standard for each elution fraction was required. A number of possibilities were tried based on their detectability, structural similarity to the benzodiazepines, and availability.

CBP, (1-(4-chlorobenzhydryl)-piperazine), a benzophenone structurally similar to the benzodiazepines, was not extracted despite using a concentration greater than that used in routine analysis.

Carbamazepine is not structurally similar to the benzodiazepines but has been detected by the electron capture detector despite its lack of halogens. This drug was also not extracted.

Demoxepam, halazepam, lormetazepam and prazepam, uncommonly used benzodiazepines, were not suitable as internal standards for reasons previously discussed.

Although the recoveries of the benzodiazepines were not calculated, it was determined from the retention times of peaks from the chromatograms that they are extractable at therapeutic concentrations.

7.2.5 Recovery

Of the 98 drugs tested, 80 were successfully extracted. Of the remainder, 7 were not extracted from spiked water samples, 8 were not extracted from spiked blood samples, and 3 were not extractable from ethanol standards. Since many of the unextractable drugs were tested at low concentrations (below 10 μg%) it was possible that they were extracted but were below the detection limit of the GC. Volatile drugs, such as amphetamine, may have been extracted but lost during the evaporation step. Structures and the pKa of the unextracted drugs were not likely an influence since they had a wide range of pKa values (2.1 to 9.9) and varying structures.

Percent recoveries were calculated for 63 of the 80 drugs extracted. For the remaining 17 drugs, recoveries were not calculated, either because a suitable internal standard was not found, the drugs coeluted with another peak, or the drug degraded into multiple peaks.

Of the 63 drugs, more than two-thirds had recoveries of approximately 70% or greater with relative standard deviations of less than 10%. Thus, our primary goal was achieved. The range of recoveries were 28 to 136% with a percent relative standard deviation of 3 to 47. Drugs with recoveries above 100% were tested at a low concentration, which produced a small peak area on the chromatogram. Any artifact from the blood eluting at the same retention time as the drug may have caused a higher peak area than expected. This may have contributed to the high recoveries observed. With the exception of barbital, all recoveries were above 55%.

7.2.6 Advantages of the Oasis MCX® Procedure

The Oasis MCX® procedure can more easily be transferred to an automated extraction system, than can liquid-liquid extractions. The Oasis MCX® procedure once automated greatly increases efficiency as the analyst can concentrate on other work while the samples are being extracted.

Another advantage of the Oasis MCX® procedure is that it successfully extracted morphine. At present, a time-consuming specialized screening method is used for the

extraction of morphine. With the automated Oasis MCX® procedure there is less manual labor required, thus, decreasing the chances of error.

The presence of cholesterol in the acidic/neutral elution fraction was confirmed by GC/MS. Having cholesterol eluted into this fraction rather than the basic fraction was an advantage when screening for basic drugs. Since more basic than acidic drugs were eluted at the same retention time as cholesterol, the potential for masking them was greater (11). The elution of cholesterol into the acidic/neutral fraction lessened the chance of obscuring a basic drug. GC/MS chromatograms of the basic fraction were also cleaner.

While the Oasis MCX® procedure was optimized for screening for a wide range of basic drugs, it can also be optimized for a particular drug. With the knowledge of the drug's pKa, the pH of the sample and the column can be changed to enhance retention and elution of the drug.

Recoveries from the Oasis MCX® procedure were compared to those obtained from the liquid-liquid extraction method currently used. Thus, these results were compared to those from a previous liquid-liquid extraction study performed at the RCMP Laboratory in Winnipeg (In House Data. RCMP Forensic Laboratory, Winnipeg, Manitoba). It was found that liquid-liquid extraction and solid-phase extraction methods were each able to extract drugs that the other method missed. More drugs were extractable by the liquid-liquid extraction method. However, the concentrations of some drugs used in that study were higher than that used in the Oasis® study. Student's t-tests were performed for each drug comparing recoveries from the two extraction methods. Of

the 24 drugs extracted by both methods, 16 had higher recoveries with the solid-phase extraction procedure ($p < 0.05$, t-test) and one had a higher recovery with liquid-liquid extraction ($p < 0.05$, t-test). There were no statistically significant differences in recovery for seven drugs. These results are summarized in Table 15. The comparison shows that solid-phase extraction can be used to complement other screening methods as each method has its own strengths and weaknesses.

Table 15 Recoveries from Liquid-Liquid Extraction and Solid-Phase Extraction

Drug	Liquid-Liquid Extraction Recovery	Solid-Phase Extraction Recovery	Statistical Significance
amitriptyline	43.5 ● 3.7	97.4 ± 6.2	ss
benzocaine	69.4 ± 5.5	74.0 ± 5.4	nss
chlorpheniramine	77.5 ± 2.8	121.8 ± 7.4	ss
clemastine	36.3 ± 4.4	68.1 ± 3.9	ss
codeine	70.3 ± 1.2	86.7 ± 7.4	ss
diphenhydramine	69.9 ● 3.4	87.7 ± 4.8	ss
diphenoxylate	11.8 ± 0.3	73.6 ± 10.4	ss
flunarizine	10.0 ± 0.6	55.9 ± 6.6	ss
lidocaine	84.3 ± 3.8	95.5 ± 4.9	ss
MDA	105.3 ± 8.9	58.2 ± 14.8	ss
meperidine	82.4 ± 5.2	91.6 ± 6.9	nss
methaqualone	85.0 ± 2.8	86.6 ± 3.2	nss
methylecgonine	46.0 ± 9.9	75.3 ± 8.1	ss
methylphenyldate	84.3 ± 8.3	133.6 ± 25.2	ss
nefazodone	59.9 ± 0.8	78.4 ± 7.7	ss
pentazocine	79.4 ± 2.9	96.1 ± 5.0	ss
phencyclidine	43.5 ± 1.1	100.4 ± 7.6	ss
pheniramine	88.9 ± 4.9	89.3 ± 3.9	nss
phenyltoloxamine	58.3 ● 1.8	122.6 ± 16.2	ss
pseudoephedrine	48.3 ± 3.2	136.4 ± 28.0	ss
sertraline	56.1 ± 9.9	58.2 ± 5.6	nss
strychnine	81.8 ± 8.4	83.1 ± 20.4	nss
trazodone	83.9 ± 9.6	87.0 ± 8.1	nss
trimipramine	34.9 ± 2.1	90.9 ± 6.5	ss

nss = not statistically significant, $p > 0.05$, MDA = methylenedioxyamphetamine,
ss = statistically significant, $p < 0.05$

7.2.7 Disadvantages of the Oasis MCX® Procedure

The elution fractions were not as clean as anticipated. Despite the use of different wash solvents and volumes, the acidic/neutral elution fractions were slightly orange in color. After evaporation, some of the reconstituted fractions contained a fine precipitate. The presence of cholesterol in the acidic elution fraction may also contribute to the dirtiness of the fraction as well as potentially obscuring some acidic drugs. Occasionally, the basic fractions would contain a small amount of precipitate but the solvent was not as darkly colored as the acidic/neutral extracts. Routine analyses of the acidic elution extracts may lead to increased GC maintenance, time, and cost.

Unfortunately, the Oasis MCX® procedure was unsuitable for the extraction of the benzodiazepines due to very dirty elution extracts and the difficulty in finding a suitable internal standard. An alternative method would have to be used to screen for benzodiazepines.

While many drugs were extractable by this procedure, some were not, including some drugs of abuse (e.g. amphetamine). It is important to recognize this limitation. Therefore, for comprehensive drug screening this solid-phase extraction procedure should be used in conjunction with another technology such as immunoassay.

8. Conclusion

The Oasis HLB® columns were not suitable for the differential elution of acidic, neutral and basic drugs. Basic drugs eluted into the acidic elution fraction. The procedure may work for the extraction of basic/neutral drugs but more work is needed to validate the methodology.

The Oasis MCX® procedure was capable of differential elution of acidic, neutral and basic drugs. Many drugs were extractable from whole blood and detectable at therapeutic concentrations. Good recoveries and clean extracts were achieved. Automation of the procedure greatly reduced the amount of manual labor required and potentially the amount of time required to extract a large number of samples. This also reduced the chances of human error in multi-procedure techniques. The successful extraction of morphine eliminated the use of a second, time-consuming extraction procedure. Unfortunately, the Oasis MCX® procedure was unable to extract all drugs (e.g. benzodiazepines). This suggests that other techniques, such as immunoassay, should be used along with the Oasis MCX® procedure to obtain the most comprehensive drug screening.

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Appendix A Drugs Commonly Found in Forensic Casework in Manitoba

alprazolam	fluoxetine	orphenadrine
amitriptyline	flurazepam	oxazepam
amoxapine	fluvoxamine	paroxetine
azacyclonal	haloperidol	pentazocine
azatadine	hydrocodone	perphenazine
benztropine	hydroxyzine	phencyclidine
brompheniramine	imipramine	pheniramine
chlordiazepoxide	levorphan	phenylpropanolamine
chlorpheniramine	lidocaine	prochlorperazine
chlorpromazine	lorazepam	procyclidine
chlorprothixene	loxapine	propoxyphene
clemastine	maprotiline	propranolol
clomipramine	meperidine	pseudoephedrine
clonazepam	methadone	quinidine
cocaine	methamphetamine	sertraline
codeine	methaqualone	strychnine
desalkylflurazepam	methotrimeprazine	temazepam
desipramine	methylecgonine	thioridazine
dextromethorphan	methylenedioxyamphetamine	trazodone
dextrorphan	methylphenidate	triazolam
diazepam	methyprylon	trifluoperazine
dicyclomine	metoprolol	trimipramine
diethylpropion	midazolam	triprolidine
diltiazem	nitrazepam	verapamil
diphenhydramine	nordiazepam	zopiclone
ephedrine	nordoxepin	
flunarizine	nortriptyline	

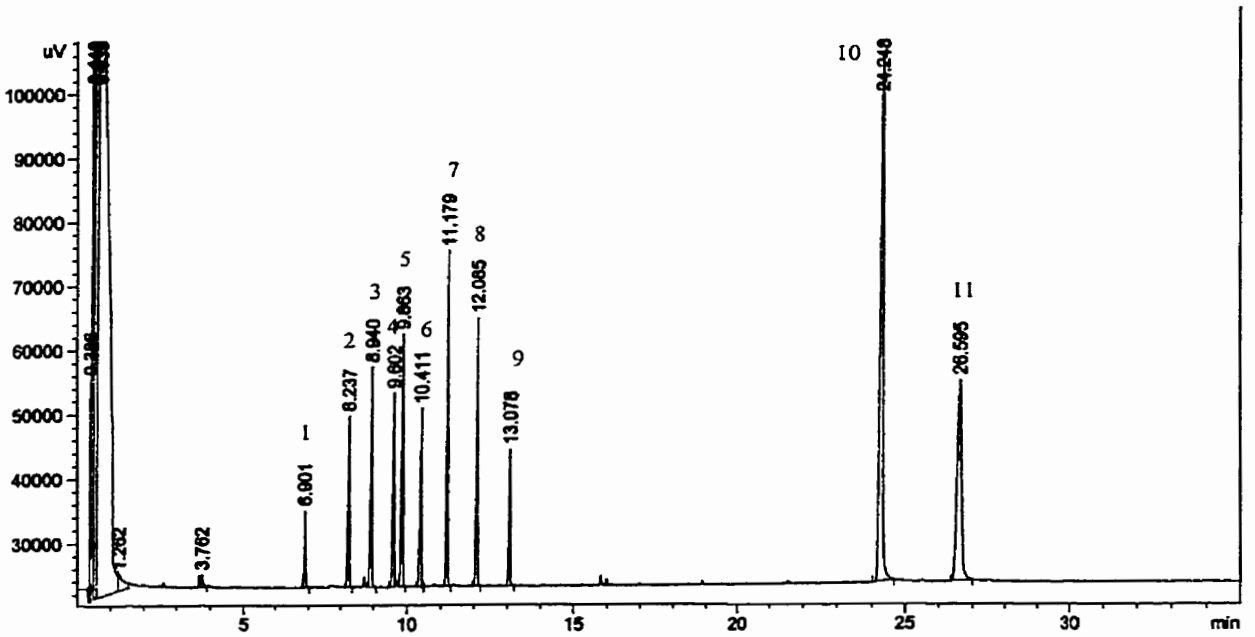
Appendix B Source of Drugs used in Project

Drug	Manufacturer
allobarbital	Sigma
alprazolam	Upjohn
amitriptyline	Merck Sharp & Dohme
amobarbital	Lilly
amoxapine	Lederle
azacyclonal	Merrell Dow
azatadine	Schering
barbital	Sigma
benztropine	Merck
brompheniramine	Robins
butabarbital	May & Baker
carbamazepine	Geigy
CBP (1-(4-chlorobenzhydryl)-piperazine)	Aldrich
chlordiazepoxide	Roche
chlorpheniramine	Smith Kline & French
chlorpromazine	Poulenc
chlorprothixene	Roche
clemastine	Sandoz
clomipramine	Geigy
clonazepam	Roche
cocaine	BDH
codeine	BDH
demoxepam	Alltech
desalkylflurazepam	Roche
desipramine	Geigy
dextromethorphan	Upjohn
dextrorphan	Roche
diazepam	Roche
dicyclomine	Merrell
diethylpropion	Merrell
diltiazem	Nordic
diphenhydramine	Parke-Davis
doxapram	Robins
ephedrine	Sigma
flunarizine	Janssen
fluvoxamine	Solvay
halazepam	Schering
haloperidol	McNeil
hydrocodone	Health Protection Branch
hydroxyzine	Pfizer
imipramine	Geigy
ketamine	Parke-Davis
levorphan	Roche
lidocaine	Pfaltz & Bauer
lorazepam	Novopharm
lormetazepam	Wyeth
loxapine	Lederle
maprotiline	Ciba

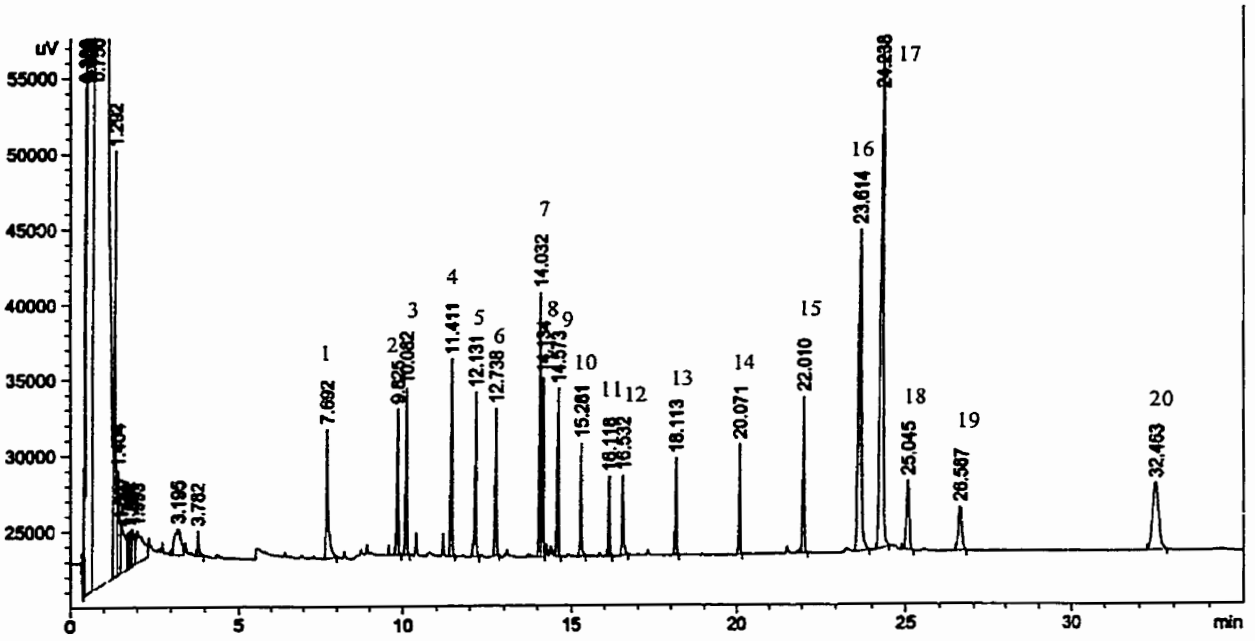
Appendix B (continued) Source of Drugs used in Project

Drug	Manufacturer
meperidine	Winthrop
methadone	Dow
methamphetamine	Health Protection Branch
methaqualone	ICN
methotrimeprazine	Poulenc
methylecgonine	Carleton
methylenedioxyamphetamine	Radian
methylnitrazepam	Hoffman-La Roche
methylphenidate	Ciba
methylprylon	Sigma
metoprolol	Astra
midazolam	Roche
nalorphine	Frosst
nitrazepam	Roche
nordiazepam	Roche
nordoxepin	Pfizer
nortriptyline	Lilly
orphenadrine	Pfaltz & Bauer
oxazepam	Wyeth
paroxetine	SmithKline Beecham
pentazocine	Winthrop
pentobarbital	Abbott
perphenazine	Schering
phencyclidine	Parke-Davis
pheniramine	Pfaltz & Bauer
phenobarbital	Smith Kline & French
phenylpropanolamine	Smith Kline & French
prazepam	Warner-Chilcott
prochlorperazine	Rhone-Poulenc
procyclidine	Burroughs Wellcome
propoxyphene	Nova
propranolol	Ayerst
pseudoephedrine	Burroughs Wellcome
quinidine	Burroughs Wellcome
secobarbital	Lilly
sertraline	Pfizer
strychnine	Pfaltz & Bauer
temazepam	Anca
thiopental	Abbott
thioridazine	Sandoz
tolylbarbital	Aldrich
trazodone	Bristol-Myers Squibb
triazolam	Upjohn
trifluoperazine	Smith Kline & French
trimipramine	Poulenc
triprolidine	Burroughs Wellcome
verapamil	Knoll
zopiclone	Rhone-Poulenc

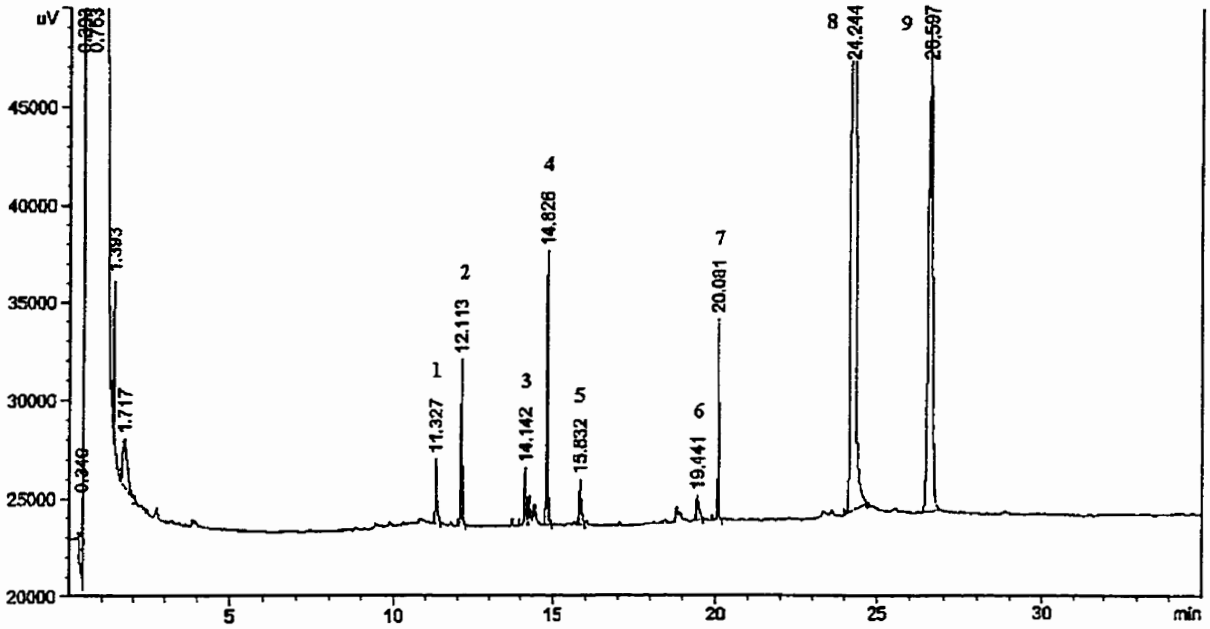
Appendix C Chromatograms of Extracted Drugs Using the Oasis MCX® Method



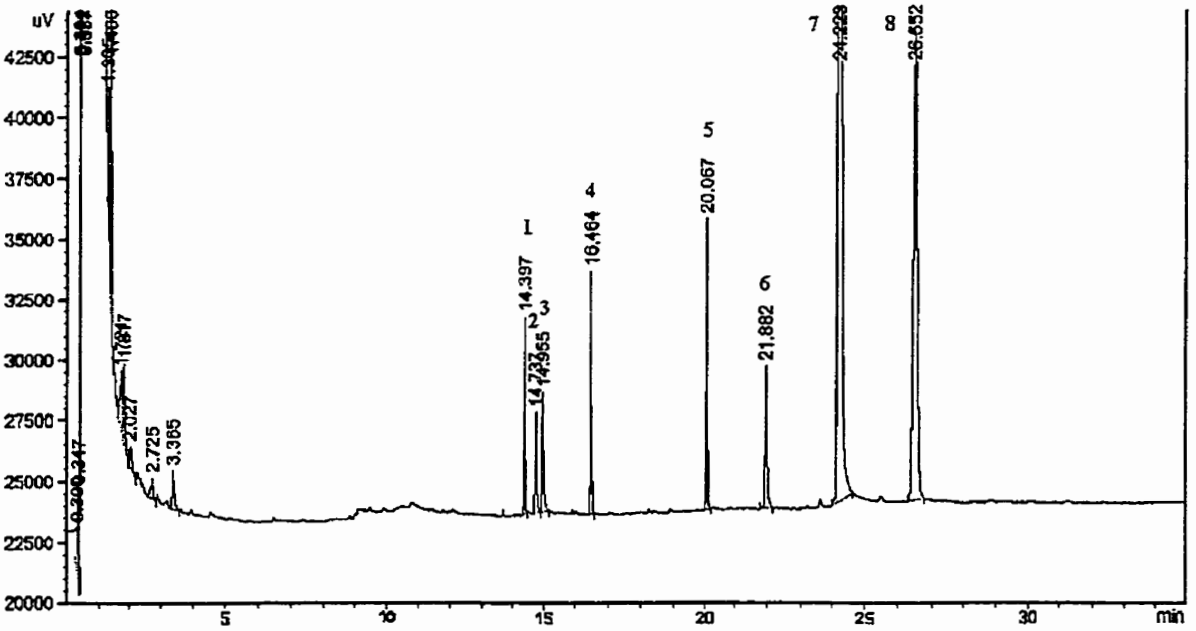
Extracted Barbiturate Mix, Acidic Fraction. Peak Identification: (1) barbital, (2) allobarbital, (3) butabarbital, (4) amobarbital, (5) pentobarbital, (6) secobarbital, (7) thiopental, (8) phenobarbital, (9) tolylbarbital (ISTD), (10) and (11) ethyl acetate artifacts



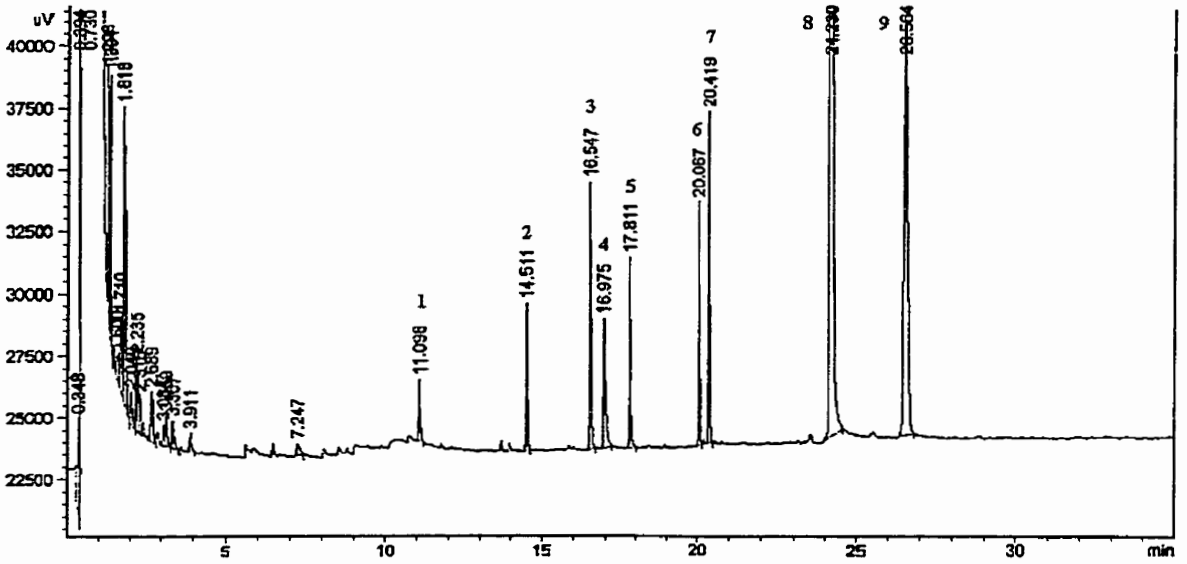
Extracted NPQC Mix, Basic Fraction. Peak Identification: (1) benzocaine, (2) methylphenidate, (3) meperidine, (4) lidocaine, (5) phenyltoloxamine, (6) chlorpheniramine, (7) methaqualone, (8) methadone, (9) amitriptyline (10) pentazocine, (11) codeine, (12) morphine, (13) heroin, (14) doxapram (ISTD), (15) strychnine, (16) trazodone, (17) ethyl acetate artifact, (18) diphenoxylate, (19) ethyl acetate artifact, (20) nefazodone



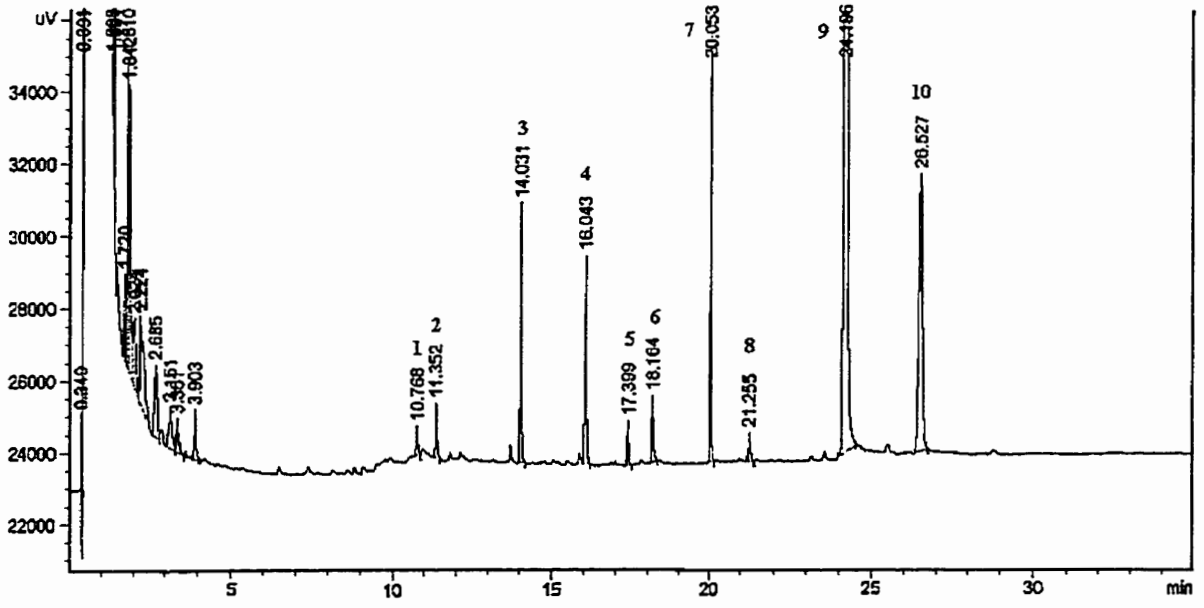
Extracted Drug Mix 1, Basic Fraction. Peak Identification: (1) fluoxetine, (2) orphenadrine, (3) propranolol, 4) imipramine, (5) maprotiline, (6) quinidine, (7) doxapram (ISTD), (8) and (9) ethyl acetate artifacts



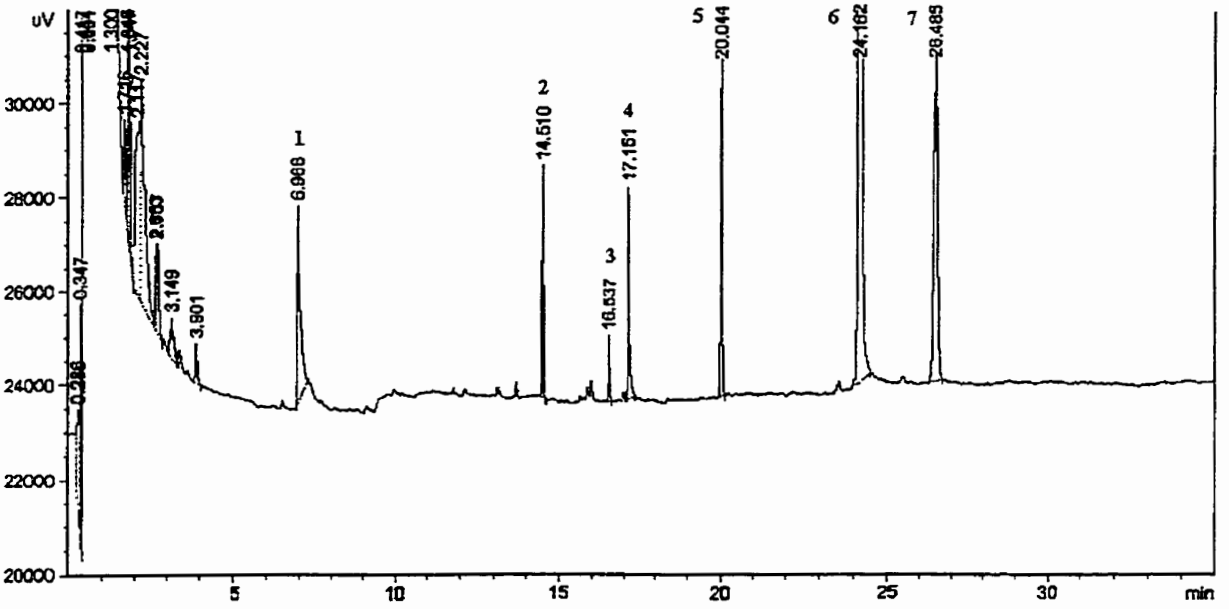
Extracted Drug Mix 2, Basic Fraction. Peak Identification: (1) procyclidine, (2) dextrophan, (3) desipramine, (4) clomipramine, (5) doxapram (ISTD), (6) thioridazine, (7) and (8) ethyl acetate artifacts



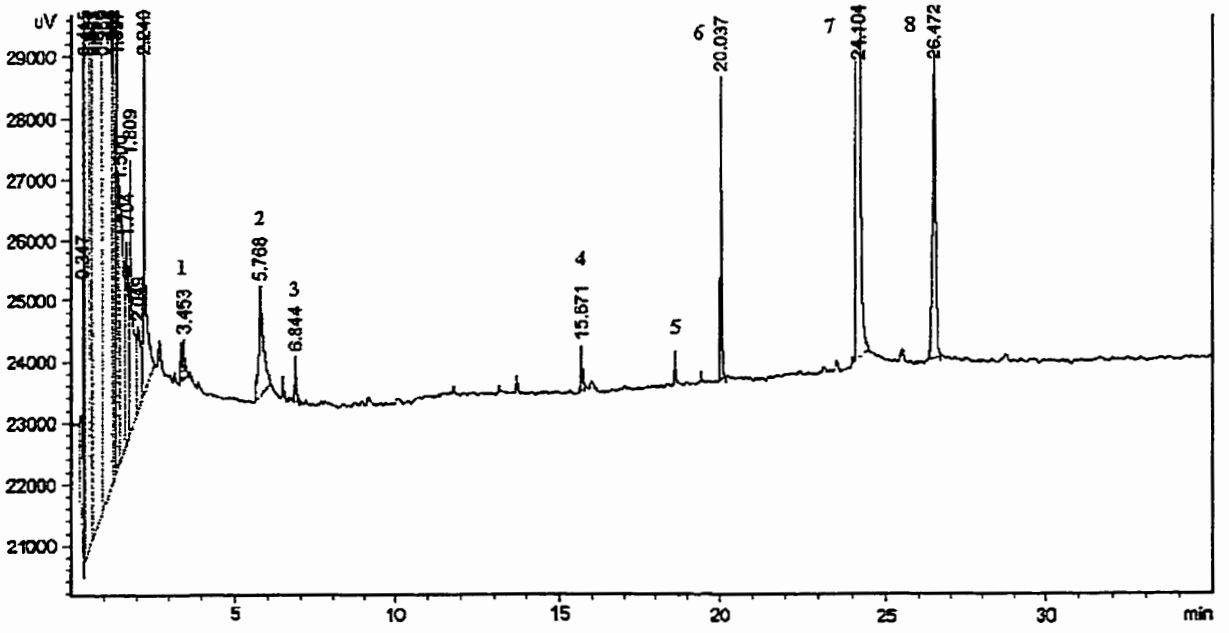
Extracted Drug Mix 3, Basic Fraction. Peak Identification: (1) ketamine, (2) cocaine, (3) diazepam, (4) nordiazepam, (5) midazolam, (6) doxapram (ISTD), (7) diltiazem, (8) and (9) ethyl acetate artifacts



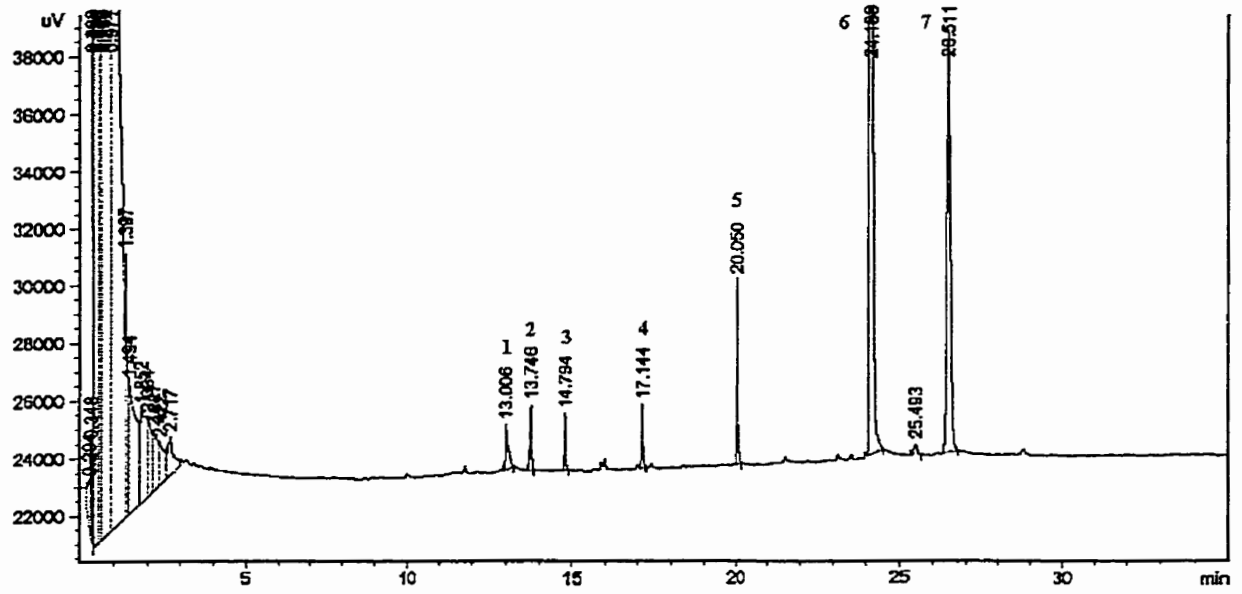
Extracted Drug Mix 4, Basic Fraction. Peak Identification: (1) pheniramine, (2) diphenhydramine, (3) dextromethorphan, (4) sertraline, (5) methotrimeprazine, (6) amoxapine, (7) doxapram (ISTD), (8) flunarizine, (9) and (10) ethyl acetate artifacts



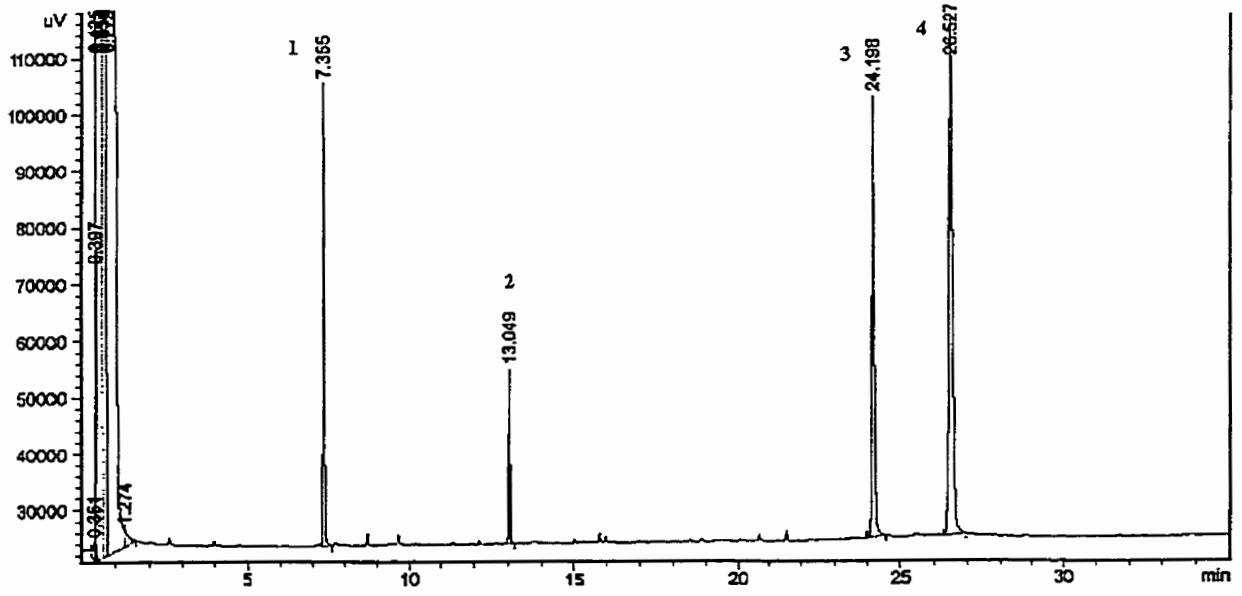
Extracted Drug Mix 5, Basic Fraction. Peak Identification: (1) methylenedioxyamphetamine (MDA), (2) propoxyphene, (3) clemastine, (4) chlorpromazine, (5) doxapram (ISTD), (6) and (7) ethyl acetate artifacts



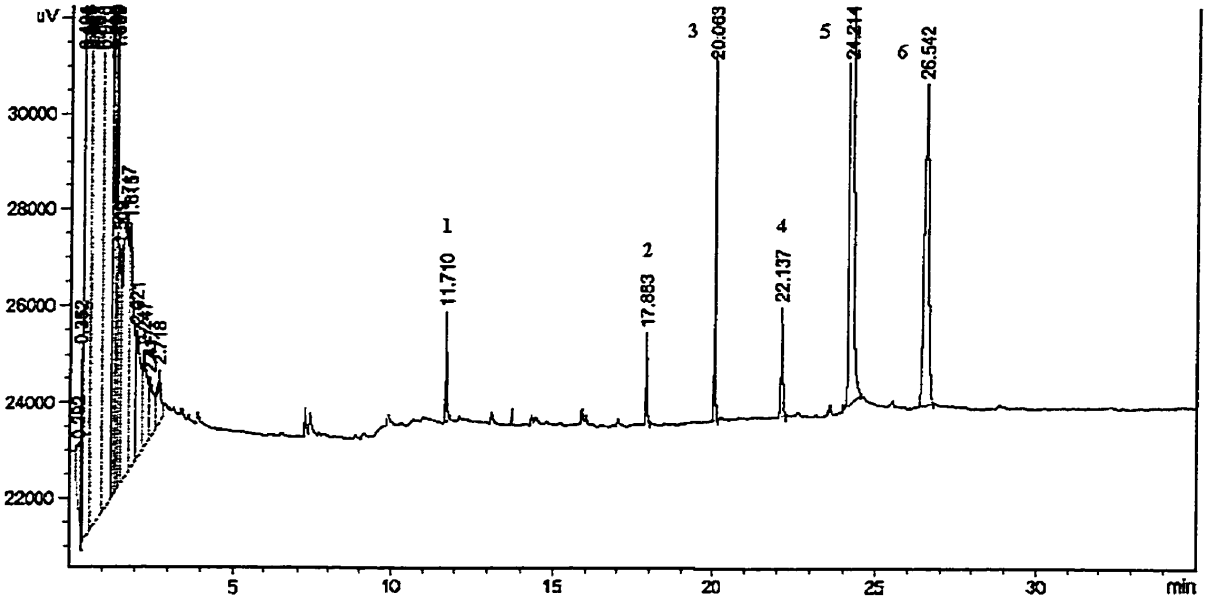
Extracted Drug Mix 6, Basic Fraction. Peak Identification: (1) methamphetamine, (2) pseudoephedrine, (3) methylecgonine, (4) benztropine, (5) trifluoperazine, (6) doxapram (ISTD), (7) and (8) ethyl acetate artifacts



Extracted Drug Mix 7, Basic Fraction. Peak Identification: (1) metoprolol, (2) dicyclomine, (3) trimipramine, (4) chlorprothixene, (5) doxapram (ISTD), (6) and (7) ethyl acetate artifacts



Extracted Drug Mix 8, Acidic Fraction. Peak Identification: (1) methyprylon, (2) tolylbarbital (ISTD), (3) and (4) ethyl acetate artifacts.



Extracted Drug Mix 8, Basic Fraction. Peak Identification: (1) phencyclidine, (2) loxapine, (3) doxapram (ISTD), (4) verapamil, (5) and (6) ethyl acetate artifacts.