Improved Solid-Phase Extraction Method for Systematic Toxicological Analysis in Biological Fluids

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Abstract

A method for the simultaneous qualitative and quantitative determination of drugs of abuse (opiates, cocaine, or amphetamines) and prescribed drugs (tricyclic antidepressants, phenotiazines, benzodiazepines, etc.) in biological fluids—blood, urine, bile, and gastric contents—was developed. This procedure involves solid-phase extraction with Bond-Elut Certify columns followed by analysis by gas chromatography–nitrogen-phosphorus detection (GC–NPD) and confirmation by gas chromatography–mass spectrometry (GC–MS), after derivatization, when necessary. Pretreatment was performed on all samples: sonication for 15 min plus enzymatic hydrolysis with β-glucuronidase in urine. With respect to the internal standards, nalorphine and trihexylamine were used for basic substances, allobarbital for acidic drugs, and prazepam for benzodiazepines. Acidic and basic compounds were extracted from different aliquots of samples at different pH levels: 6–6.5 for the acidic and neutral and 8–8.5 for the basic and the benzodiazepines. Several areas of experimental design were considered in the process of method optimization. These included internal standards, pH, sonication, flow rate and washing solvents. It was found that systematic analysis could be reliably performed using optimized extraction conditions. The recovery rates for the compounds tested were always higher than 61.02%.

Introduction

One of the main requirements in systematic toxicological analysis is the performance of a screening analysis. The majority of the cases analyzed are blind, that is, the substances causing death are unknown. A wide variety of compounds can be found, ranging from highly lipophilic to moderately polar in nature, and exhibiting basic, acidic, or neutral properties.

Extraction of analytes from biological matrices is one of the most tedious and time-consuming steps in systematic toxicological analysis. In addition, the extraction step is required for several reasons: to eliminate possible substance interference, to concentrate and stabilize the analytes that may be in the sample, and finally to take the sample to the optimal conditions for instrumental analysis. Nevertheless, because there have been few advances in the quality of the used extraction procedures, variable analytical results have been consistently produced. Choosing the adequate sample preparation method, therefore, is of utmost important in systematic toxicological analysis.

Traditionally, liquid–liquid extraction (LLE) has been routinely used in most toxicology laboratories. However, in the past several years solid-phase extraction (SPE) has become a popular technique in the preparation of samples for analysis and has been increasingly used for extracting drugs from biological matrices. Among the several advantages that SPE offers over LLE are higher selectivity, cleaner extracts, more reproducibility, and the avoidance of emulsion formation (1).

There are numerous publications on SPE for biological samples, but most methods deal with the extraction of single drugs or groups of related drugs (2–8). Other authors propose the use of a pair of SPE columns, one to extract the acidic compounds and the other to extract the neutral and basic compounds (9). Some papers have been geared towards systematic toxicological analysis of acidic, basic, and neutral compounds using only one SPE column (10–16). Franke and de Zeeuw (17) reviewed all of them in an overview of screening procedures in SPE.

Bond Elut Certify is a mixed-bed chromatographic support containing a mixture of short alkyl chains and strong cation exchange moieties. It has been especially designed for the SPE of drugs of abuse because it is able to retain acidic, neutral, and basic drugs under the proper extraction conditions. The effectiveness of the SPE method depends on several parameters, such as the cartridge stationary adsorbent, pH, sample pretreatment, solvents used for washing and eluting, and the flow rate during the different steps. The aim of the study was the development and optimization of an SPE procedure for toxicological screening focusing on the selection of adequate internal standards and optimal extraction parameters.

Reagents and Materials

Chemicals

All chemicals and solvents were of analytical grade (Merck,

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Barcelona, Spain) with the exception of ammonia (33%), which was of extra-pure quality (Merck).

Bond Elut Certify columns (300 mg sorbent mass/10-mL column volume) and a Vac-Elut vacuum manifold system were purchased from Varian Sample Preparation Products (Harbor City, CA).

Internal standards (allobarbital, nalorphine, prazepam, and trihexylamine) were obtained from Sigma (Madrid, Spain). Drug-of-abuse standards, morphine, codeine, 6-acetylmorphine (6-MAM), methadone, cocaine, benzoylecgonine (BE), amphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), *N*-ethyl-3,4-methylenedioxyamphetamine (MDEA), were purchased from Radian (Austin, TX). Standards for the different drugs, carbamazepine, 7-aminoflunitrazepam, chlormethiazole, diazepam, lidocaine, lorazepam, lormetazepam, mepivacaine, nordiazepam, olanzapine, pentobarbital, phenobarbital, phenytoin, and thiopental, were kindly provided by the corresponding pharmaceutical laboratories, and all of them were of pharmaceutical quality. *N,O*-Bis-trimethylsilyl-trifluoroacetamide (BSTFA)/trimethylchlorosilane (TMCS) (99:1) was obtained from Supelco (Bellefonte, PA). β-Glucuronidase, isolated from *Patella vulgata*, was obtained from Sigma Chemical Co. (catalog # G-8132, Madrid, Spain). A working solution of 5000 units/mL was prepared in 0.1M acetate buffer (pH 4).

Standard solutions

Individual stock solutions (1000 mg/L) were prepared by dissolving the appropriate amount of drug in methanol. Then the solution was stored in closed glass tubes and maintained at 4°C.

Internal standard solutions were prepared by diluting the stock solutions with methanol to reach a concentration of 100 mg/L for allobarbital and nalorphine, and for trihexylamine (THA) and prazepam the concentration was 10 mg/L. Phosphate buffer (0.1M, pH 6) was prepared by dissolving 6.81 g of potassium dihydrogen phosphate in 450 mL of deionized water, adjusting the pH to 6.0 (± 0.1) with 1.0M potassium hydroxide, and making the total volume 500 mL with deionized water. Phosphate buffer (2M, pH 8) was prepared by dissolving 174 g of dipotassium hydrogen phosphate in 450 mL of deionized water, adjusting the pH to 8.0 (± 0.1) with phosphoric acid (85%), and making the total volume 500 mL with deionized water. Acetate buffer (0.1M, pH 4) was prepared by mixing 570 µL of glacial acetic acid with 80 mL of deionized water and 1.6 mL of 1.0M potassium hydroxide. If necessary the pH was adjusted to 4.0 and the volume was brought to 100 mL with the addition of deionized water. Ammoniated chloroform/isopropanol (80:20) (2%) was prepared daily.

Table I. Sample Pretreatment

	Acidic substances	Basic substances
Volume	2.5 mL (urine and blood) 1 mL (bile and gastric content)	2.5 mL (urine and blood) 1 mL (bile and gastric content)
I.S.	0.1 mL allobarbital	0.1 mL nalorphine, 0.1 mL THA, 0.1 mL prazepam
Dilution	1 mL buffer pH 6	1 mL deionized water
Hydrolysis	—	β-glucuronidase (urine)
Sonication	10 min	10 min
pH	6–6.5	8–8.5
Sonication	10 min	10 min
Centrifugation	10 min	10 min

Analytical Method

Sample pretreatment

Acidic and basic fractions from whole blood, urine, bile, and gastric content were obtained separately, not sequentially, from the sample. Pretreatment was also different for both fractions (Table I).

Pretreatment before extracting acidic and neutral substances. Whole blood and urine (2.5 mL) or 1 mL of bile and gastric content were diluted with 1 mL of phosphate buffer (pH 6), and 0.1 mL of I.S. solution (allobarbital 100 mg/L) was added. The sample was sonicated in an ultrasonic bath (P-Selecta) for 10 min. The pH was adjusted to 6.0–6.5 with 1M KOH or 1M HCl. The sample was again sonicated for 10 min, and the pH was checked and adjusted when necessary. Finally, specimens were centrifuged at 2500 rpm for 10 min. The supernatant was used for further extraction.

Pretreatment before extracting basic substances. In this case pretreatment for urine is different from that for blood, bile and gastric content samples.

Blood (2.5 mL) or bile and gastric content (1 mL) were diluted with 1 mL of distilled water and 0.1 mL each of I.S. solution (nalorphine at 100 mg/L and trihexylamine and prazepam, both at 10 mg/L). The sample was sonicated in an ultrasonic bath for 10 min at room temperature, and the pH was adjusted to 8–8.5 with phosphate buffer (pH 8). A new sonication was performed for 10 min, and pH was checked and adjusted when necessary. The sample was then centrifuged as previously described for the acidic compounds.

Pretreatment in urine samples was similar to that previously described, with the exception of an enzymatic hydrolysis, which is required for possible glucuronide cleavage. To do that, 2 mL of working β-glucuronidase solution (5000 units/mL) was added to 2.5 mL of sample, and pH was then adjusted to 4–4.5. The tubes were capped, vortex mixed, and incubated in a water bath and agitated at 60–65°C. After 2 h, the pH was adjusted to 8–8.5 with buffer pH 8. The rest of the procedure is the same as for blood samples.

SPE

Extraction was performed with a vacuum manifold assembled with Bond Elut Certify columns.

SPE of acidic substances. The column was preconditioned with 2 mL of methanol, followed by 2 mL of 0.1M phosphate buffer (pH 6). The vacuum was turned off as soon as the buffer reached the top of the sorbent bed to prevent column drying (flow rate 2 mL/min) (step 1).

Pretreated samples were transferred to the column. The Vac-Elut valve was loosened to reduce vacuum, and specimen was drawn slowly through the column. At least 2 min are required in order to pass the sample through the column (step 2).

The column was rinsed by passing through it sequentially: (a) 1 mL 0.1M phosphate buffer (pH 6)/methanol (80:20), then the column was dried under full vacuum (≈ 5 in. Hg, flow rate 1.5 mL/min) for 5 min; (b) 1 mL 1.0M acetic acid (1.5 mL/min), and the column was once again dried under full vacuum for 2 min; (c) 1 mL *n*-hexane (1.5 mL/min), and the column was once again dried under full vacuum for 2 min (step 3).

The column was released from the manifold and 4 mL of methylene chloride were passed through it and collected gravitationally (step 4).

Methylene chloride was then evaporated under nitrogen at room temperature and reconstituted in 100 µL of methanol (Fraction A) (step 5).

SPE of basic substances (including benzodiazepines). First, the column was preconditioned by passing 2 mL of methanol and 2 mL of deionized water through it sequentially. The vacuum was turned off to prevent column drying (step 1).

The pretreated sample was poured into the column reservoir. The Vac-Elut valve was loosened to reduce vacuum. At least 2 min are required in order to pass the sample through the column (step 2).

Two milliliters of deionized water (2 mL/min) and 2 mL of acetate buffer pH 4 at a flow rate of 1 mL/min were passed through the column sequentially (step 3).

The column was removed from the main manifold, and 2 mL of acetonitrile were passed through it and collected gravitationally. The acetonitrile was evaporated under nitrogen at room temperature and reconstituted in 100 µL of methanol (Fraction C, which contains benzodiazepine compounds) (step 4).

The column was returned to the manifold and dried under full vacuum for 2 min (step 5).

The column was removed from the manifold, once again, and 4 mL of ammoniated chloroform/isopropanol (80:20) (2%) were passed through it and collected gravitationally (step 6).

Finally, 0.1 mL of acidified methanol (0.1 mL HCl in 100 mL methanol) was added to the fraction, evaporated under nitrogen at room temperature, and reconstituted in 100 µL of methanol (Fraction B) (step 7).

Derivatization

The confirmation of some substances, such as opiates or benzoylecgonine (BE), by gas chromatography–mass spectrometry (GC–MS) requires Fraction B to be derivatized. This is performed by heating with 50 µL BSTFA/TMCS (99:1) at 70°C for 20 min. The extract is directly injected in the GC–MS system in the selected ion monitoring (SIM) mode.

Instrumentation

Screening and first identification of the compounds was performed on a Varian CP-3800 GC with a nitrogen-phosphorus detector (NPD) and equipped with a Varian CP-8200 automatic sampler. The column was an HP-1MS crosslinked methyl siloxane capillary column (25-m length, 0.2-mm i.d., 0.33-µm film thickness). The oven temperature was held at 60°C for 2 min, then increased at a rate of 12°C/min to 280°C, and held at this final temperature for 14 min. Injector and detector temperatures were 280°C and 300°C, respectively. The injector was set in the splitless mode, and the helium carrier gas flow was 1 mL/min.

The GC–MS system was a Hewlett-Packard 6890 series GC coupled to a 5973 MS and equipped with a Hewlett-Packard 6890 series injector. The electron multiplier voltage was set at 200 eV above the autotune voltage. The MS was autotuned daily with perfluorotributylamine. Column, oven, and helium gas flow conditions were the same as previously reported for GC–NPD. Injector and GC–MS interface temperatures were 250°C and 280°C, respectively.

Confirmation and identification of unknown compounds were performed with GC–MS in the total ion mode. Mass spectra were then compared with the Hewlett-Packard libraries Wiley 275 and PMW_toxr. Confirmation was achieved when the matched quality values for the compared spectra were higher than 80%. Opiates and BE required a confirmation by GC–MS in the selected ion monitoring mode (SIM) after derivatization, as described. The selected ions were 455, 440, 414 (nalorphine); 182, 303 (cocaine); 361, 240 (BE); 196, 317 (ethylbenzoylecgonine or cocaethylene); 371, 356, 343 (codeine); 429, 401, 414 (morphine); and 399, 340 (6-MAM).

Results

In order to evaluate the method, 25 drugs were selected from among the substances most frequently found in the autopsy cases in our lab. Other contributing factors were that they had

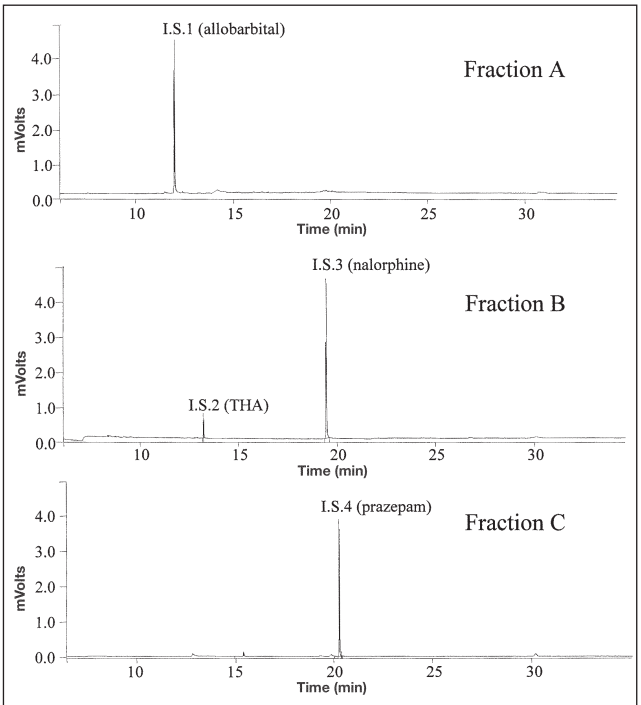

Figure 1. GC–NPD chromatograms of the three fractions, acidic (A), basic (B), and benzodiazepine (C), obtained from a blank blood sample.

various characteristics, were of various classes—acidic, basic, and neutral drugs were included—and covered a relatively wide range on the chromatogram.

Figure 1 shows the chromatograms of the three different fractions obtained from a blank blood sample. They were free of interferences.

Table II shows the fraction in which each drug eluted, the internal standards used for quantitation, and the limits of detection (LOD) for the 25 drugs considered in this study. The LOD for each drug was calculated at a signal-to-noise ratio of 3. All of them were lower than 0.1 mg/L, with the exception of phenobarbital and lormetazepam, which had LODs of 0.106 and 0.108 mg/L, respectively.

Table III shows the ranges of recoveries and the between-day precision of the method. Absolute extraction recoveries were determined by comparing the peak areas of extracted samples—five aliquots of each sample, blood, urine, bile, and gastric content—with the peak areas of methanolic standards. The concentrations studied were 0.1 and 0.5 mg/L. The recoveries ranged from 61.02% for MDA to 118.67% for 6-MAM at 0.1 mg/L and from 72.35% for lormetazepam to 117.33% for 6-MAM at 0.5 mg/L.

Precision was calculated by analyzing four aliquots of both the four samples with final concentrations of 0.1 and 0.5 mg/L weekly (on four different days) during one month. The results of the interassay studies show an acceptable precision with relative standard deviation (RSD) values between 14.36% and 0.56% for both concentrations in the fourth types of matrices.

Figures 2 and 3 show the chromatograms of blood, urine, bile, and gastric content from forensic casework samples.

Table II. Eluting Fraction, Internal Standard (I.S.) Used for Quantitation, and Limit of Detection (LOD) for 25 Selected Drugs

Compound	Fraction	I.S.	LOD (mg/L)
Alprazolam	B+C	Nalorphine + Prazepam	0.008
7-Aminoflunitrazepam	B	Nalorphine	0.035
Benzoylecgonine	B	Nalorphine	0.105
Carbamazepine	A	Allobarbital	0.019
Chlormethiazole	A	Allobarbital	0.008
Cocaethylene	B	Nalorphine	0.006
Cocaine	B	THA	0.006
Codeine	B	Nalorphine	0.006
Diazepam	C	Prazepam	0.005
Lidocaine	B	THA	0.005
Lorazepam	C	Prazepam	0.003
Lormetazepam	C	Prazepam	0.108
6-MAM	B	Nalorphine	0.014
MDA	B	THA	0.095
MDEA	B	THA	0.045
MDMA	B	THA	0.097
Mepivacaine	B	THA	0.009
Methadone	B	Nalorphine	0.006
Morphine	B	Nalorphine	0.013
Nordiazepam	B+C	Nalorphine + Prazepam	0.015
Olanzapine	B	Nalorphine	0.041
Pentobarbital	A	Allobarbital	0.097
Phenobarbital	A	Allobarbital	0.106
Phenytoin	A	Allobarbital	0.089
Thiopental	A	Allobarbital	0.093

Discussion

During the last few years, several papers have appeared in the literature on the screening of acidic, neutral, and basic drugs in biological matrices using a single SPE column (10–16). For this reason, we tried to apply the previously published methodologies when we started to develop our method. Unfortunately, data such as recovery and reproducibility were not good enough; they were very low for the greater part of the compounds tested. In addition, the extracts were dirtier than those

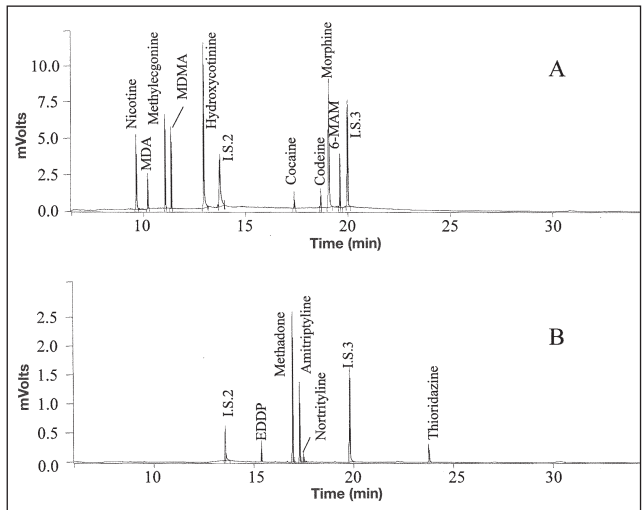

Figure 2. GC–NPD chromatograms of basic fractions obtained from real casework samples: urine from a drug abuser of opiates, cocaine, and amphetamines (A) and blood from a person who committed suicide with medical drugs (B).

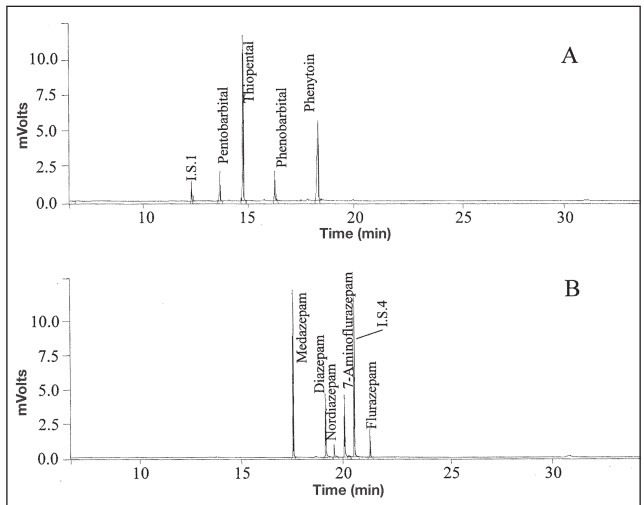

Figure 3. GC–NPD chromatograms obtained from real forensic cases: Fraction A from a gastric content where barbiturates and antiepileptics were involved (A) and Fraction C from a bile sample containing benzodiazepines (B).

obtained when extracting both fractions separately. As a consequence, we chose to use two separate SPE columns.

Several parameters were optimized in our procedure.

Choice of adequate internal standards

Internal standards are required for a correct quantitation in order to avoid, or at least minimize, possible errors due to surface adsorption and losses during extraction, solvent evaporation, or derivatization processes. Another reason for using an internal standard is the need to check chromatographical behavior.

Internal standards should ideally be closely related structural analogues of the analytes with similar retention times and behavior. In our case, as we have to perform screening analyses, it was not possible to use suitable internal standards for each one of the compounds that may be present. Consequently, we had to choose one or two for each group of substances.

For basic compounds we considered the special characteristics of opiates (above all morphine, which is one of the analytes encountered most frequently in our lab). For this reason we chose nalorphine as an internal standard because its chromatographic and extractive properties are similar to morphine. However, many substances (antidepressants, phenotiazines, local anesthetics, etc.) do not have these problems, so we used THA for their quantitation. Another reason for the application of two different internal standards for basic compounds is the retention times. They elute at the beginning (THA) and near the end (nalorphine) of the chromatogram, thus covering the whole range.

For acidic and benzodiazepine compounds we applied only one internal standard each: allobarbital (acidic) and prazepam (benzodiazepines). This is a consequence of the fact that both the physicochemical and the chromatographic properties of the compounds found in each group are more similar to each other than they are in the basic group.

An important point considered when choosing the internal standard was that none of them could be used either as a drug of abuse or as a prescribed drug.

Influence of pH and sonication

The behavior of drugs in the mixed-mode column (e.g., Bond Elut Certify) is dependent on the compound pK_as and on the extraction system pH. For this reason, pH shifts during the extraction are very important to ensure both the extraction of all possible drugs during the toxicological screening and, at the same time, the removal of matrix compounds and interferences in order to make instrumental analysis easier.

The majority of drugs show strong plasma protein binding. Several authors have demonstrated sonication and dilution of blood samples before application to the column to be effective in disrupting these unions and in enhancing the extraction recovery (14,18,19). Therefore, we have found that sonication plays a very important part in the pH stabilization during the sample pretreatments.

For all the previous reasons and after several experiments, we were able to verify that the order of the sequence we propose for sample pretreatment, which includes the repetition of sonication and a pH check after the first pH adjustment, is of utmost importance, especially with blood samples where some pH fluctuations, which affected the results, were noticeable after the second sonication. In the case of urine, this problem was of a lower incidence because the density in these samples is lower than that in blood. Consequently, it was easier to achieve homogenization.

Choice of washing solvents

In the extraction of acidic substances (step 3a), the column is washed with 0.1M phosphate buffer (pH 6)/methanol (80:20), which partially disrupts ionic and weak hydrophobic interactions between sorbent and analytes. As demonstrated by Nguyen et al. (20), this mixture is able to eliminate both strong and weak basic substances while the acidic substances of interest remain in the column. In the next step (3b), 1.0M acetic acid is applied to remove a variety of impurities and does not affect the recovery of acidic compounds. Lastly, washing with *n*-hexane (step 3c) was found to be effi-

Table III. Ranges of Recoveries and Between-Day Precision for Some Selected Drugs in the Four Spiked Samples—Urine, Blood, Bile, and Gastric Contents

	Recovery (%)		Between-day precision RSD (%)	
Compound	0.1 mg/L	0.5 mg/L	0.1 mg/L	0.5 mg/L
Alprazolam	100.75–97.08	99.78–89.3	4.03–2.72	4.57–1.41
7-Aminoflunitrazepam	95.62–85.70	100.12–88.58	2.91–2.70	2.99–2.41
Benzoylecgonine	99.05–93.70	101.16–91.87	4.18–2.85	4.72–0.98
Carbamazepine	97.36–80.50	95.61–81.73	0.87–0.74	0.92–0.56
Chlormethiazole	100.71–90.31	100.87–95.74	2.76–1.94	3.09–0.79
Cocaethylene	99.71–97.42	102.50–92.51	3.86–2.92	4.24–1.60
Cocaine	104.67–100.0	97.95–82.02	12.72–8.73	14.36–3.10
Codeine	106.67–95.42	102.31–74.01	11.16–7.21	13.07–3.26
Diazepam	101.08–97.50	100.72–89.97	3.59–2.13	4.18–2.30
Lidocaine	112.66–86.67	110.66–99.70	7.01–5.71	7.59–3.83
Lorazepam	99.35–97.03	100.66–93.85	3.85–2.76	4.29–1.23
Lormetazepam	89.75–68.71	92.37–72.35	2.57–2.21	2.71–1.77
6-MAM	118.67–99.72	117.33–95.02	3.32–2.04	3.84–0.97
MDA	113.33–61.02	99.79–73.33	9.13–8.70	9.74–8.52
MDEA	100.99–95.32	101.05–97.71	4.01–2.02	4.82–2.90
MDMA	102.13–72.43	98.37–85.51	9.24–5.94	10.59–1.29
Mepivacaine	112.67–97.31	99.41–88.33	7.85–6.34	9.99–5.71
Methadone	109.33–74.33	100.95–77.42	4.02–2.12	6.70–1.34
Morphine	109.51–98.90	103.65–91.76	5.04–4.64	5.20–4.08
Nordiazepam	97.32–87.50	99.42–90.07	1.81–1.61	1.87–1.41
Olanzapine	104.03–89.99	101.01–95.70	4.03–3.08	4.42–1.74
Pentobarbital	98.31–80.62	95.15–85.71	3.59–2.91	3.87–1.95
Phenobarbital	102.69–73.33	112.22–79.20	5.44–4.15	7.26–3.62
Phenytoin	99.75–87.32	93.37–90.55	2.28–1.57	4.96–1.82
Thiopental	90.07–79.70	90.89–82.50	1.97–1.27	2.25–0.97

cient in displacing any trace of adsorbed water. This is very important before eluting drugs because residual water in the column may negatively affect that elution and the evaporation time may be prolonged. Another reason to choose this solvent instead of any other is that *n*-hexane, because of its apolar character, may remove fats present in biological fluids—especially blood and bile—and they may interfere in the instrumental analysis.

In the extraction of basic drugs, the column is first washed with water (step 3) in order to eliminate endogenous impurities, while the analytes of interest remain in the column, none of them being prematurely eluted. Then a pH 4 is achieved in the system by passing acetate buffer pH 4 through the column. In these conditions, benzodiazepines are in the non-ionized form and can be eluted in the next washing step with organic solvent (step 4).

Two solvents, methanol and acetonitrile, were tested in step 4, just before the elution of basic analytes. First, we washed with methanol as recommended by Varian Sample Preparation in the Bond Elut Certify instruction manual, as well as by other authors (17) in previously published papers. However, a low BE recovery rate (30%) was noticeable, but improved considerably, reaching at least 91%, when we changed the washing solvent to acetonitrile.

A possible explanation for this phenomenon may have to do with the polarity of both solvents and analytes. Methanol, a highly polar solvent, removes BE, which is also very polar. For this reason, we tried to wash with a less-polar solvent, acetonitrile, and the result is shown in Figure 4. The experiment was performed with a blank urine sample spiked with BE, morphine, 6-MAM, and nalorphine, the latter as the internal standard. Figures 4A and 4B show the chromatograms after washing with methanol and acetonitrile, respectively. In both cases derivatization was performed with BSTFA. A higher ratio BE/nalorphine (drug/internal standard) is clearly noticeable in Figure 1B.

Influence of flow rate

When extracting basic analytes, it is of fundamental importance that the acetate buffer flow rate (step 3) be 1 mL/min. The objective of this step is to acidify the column to enable the acetonitrile used in the next step to remove all of the acidic, neutral, and weak base substances present in the sample while retaining the basic substances of interest in the column. For this reason, the described specific buffer flow rate is required to achieve a perfect acidification of the column.

Final fractions for acidic (Fraction A), benzodiazepines (Fraction C), and basic (Fraction B) were obtained by gravitationally collecting the different solvents. The main reason for this procedure was the avoidance of possible contamination. At the same time, we were able to demonstrate that the flow rate achieved in these conditions allowed recoveries higher than 60%.

Extraction of benzodiazepines

Benzodiazepines are one of the most difficult families of drugs to extract from biological matrices, especially when performing systematic toxicological analysis. The main reason is that they possess different pK_as, which range from ≈ 1.9 for flunitrazepam to > 12 for clorazepate. Some of them have amphoteric behavior with two different pK_as (bromazepam, clonazepam, flurazepam, lorazepam, nitrazepam, nordiazepam, oxazepam, etc.).

We initially spiked blank urine with several benzodiazepines covering the pK_a range. The extraction was performed following both protocols for acidic and basic drugs. When applying the method for acidic substances, our recovery rates in Fraction A were close to 0%. Nevertheless, with the protocol for basic drugs, the recovery rates in Fraction B were increased, especially for those of more basic character (nordiazepam, bromazepam, etc.).

To learn in which step these compounds were lost, we analyzed the washing fractions before final elution in both methods. On the one hand, when analyzing the *n*-hexane from the acidic fraction (step 3c), we did not find any benzodiazepine. Consequently, we re-extracted and analyzed the 1.0M phosphate buffer (pH 6)/methanol (80:20) fraction wash (step 3a), finding recoveries lower than 25%. On the other hand, when analyzing the acetonitrile from the basic fraction (step 4), we were able to find the benzodiazepines previously spiked. In addition, the recoveries were acceptable. The explanation for this is the column pH (4 and 6 in basic and acidic protocols, respectively) plus the different solvent polarities, as previously explained. Another contributing factor in the choice of acetonitrile as against the mixture buffer/methanol is that the latter requires a re-extraction before injecting in the GC system, and acetonitrile needs only to be evaporated.

Taking into account that benzodiazepines are analyzed in the acetonitrile fraction (Fraction C), this extract should be as clean as possible. For this reason it is very important to be sure that the 2 mL of acetate buffer (step 3) reach the end of the column

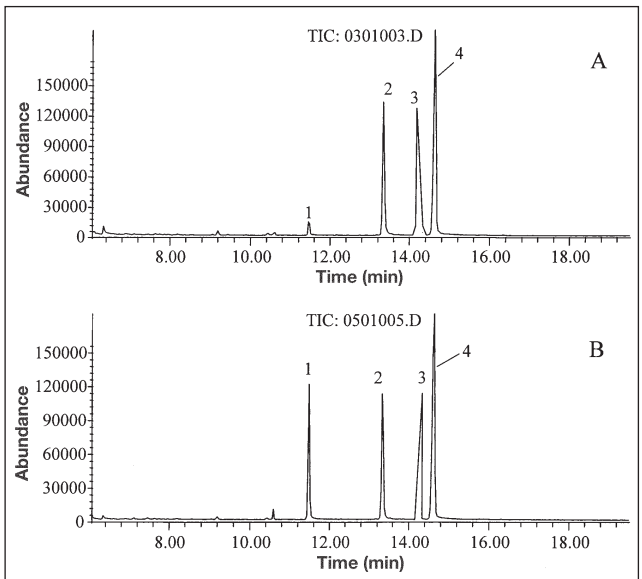

Figure 4. Influence of washing solvent in basic fraction. Selected ion chromatograms of urine samples fortified with benzoylecgonine (1), morphine (2), 6-monoacetylmorphine (3), and nalorphine (4) after washing with methanol (A) or with acetonitrile (B). Derivatization was performed with 50 mL of bis-trisilylmethyl-trifluoroacetamide (BSTFA) by heating at 70°C for 20 min.

before adding the acetonitrile. This is achieved by increasing the vacuum in the manifold, but, at the same time, trying to prevent the column from drying out.

Conclusions

The results indicate that the developed and optimized procedure is an effective extraction method in systematic toxicological analysis and particularly suitable for screening in toxicology laboratories. It offers good recoveries and reproducibility.

The method has been used routinely in our laboratory over the last two years. It has demonstrated to be successful not only with the substances included in this study, but also with a wide range of compounds of toxicological interest.

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