Xiao-Hua Chen,[1] *B.S.,; Jaap Wijsbeek,*[1]*; Jan-Piet Franke,*[1] *Ph.D.; and Rokus A. de Zeeuw,*[1] *Ph.D.*

A Single-Column Procedure on Bond Elut Certify for Systematic Toxicological Analysis of Drugs in Plasma and Urine

REFERENCE: Chen, X.-H., Wijsbeek, J., Franke, J.-P., and de Zeeuw, R. A., "**A Single-Column Procedure on Bond Elut Certify for Systematic Toxicological Analysis of Drugs in Plasma and Urine,**" *Journal of Forensic Sciences*, JFSCA, Vol. 37, No. 1, Jan. 1992, pp. 61–71.

ABSTRACT: A single-column solid-phase extraction procedure was developed for the screening of acidic, neutral, and basic drugs from plasma. The recoveries of all 25 tested drugs exceeded 82%. After the plasma had been diluted with phosphate buffer (pH 6.0), the drugs were extracted using a single Bond Elut Certify column. The acidic and most of the neutral drugs were eluted by acetone/chloroform (1:1) and the basic drugs were eluted by 2% ammoniated ethyl acetate. Some neutral drugs appeared in both fractions. The two fractions were collected separately and evaporated until approximately 100 μL of solvent remained in the tube. Both fractions were analyzed separately on a gas chromatograph equipped with a wide-bore capillary column and a flame ionization detector. The procedure could also be used for urine samples.

KEYWORDS: toxicology, extraction, drug identification, blood, solid-phase extraction, plasma, urine, systematic toxicological analysis

Over the past decade, solid-phase extraction (SPE) has emerged as a powerful technique for the pretreatment of biological samples for clinical and toxicological drug analysis [*1–4*]. SPE offers several distinct advantages over traditional liquid-liquid extraction. The main advantages are higher selectivity, since a large number of sorbents and solvents can be chosen for various applications; cleaner extracts, since the analytes can be selectively retained on and eluted from the column; and more reproducible results, since SPE is based on specific molecular interactions. In addition, the easy use of the columns allows large numbers of samples to be processed, and the procedure can be automated. The majority of publications have been geared toward the extraction of individual drugs or groups of related drugs [*5–14*]. In systematic toxicological analysis (STA), however, one of the main purposes is screening analysis. It is, therefore, desirable to develop an SPE procedure that can be used for the extraction of a wide range of drugs. So far, very few reports on the screening of various classes of drugs have been published.

Received for publication 16 March 1991; revised manuscript received 3 June 1991; accepted for publication 4 June 1991.

[1]Research associates, senior research associate, and professor of toxicology, respectively, Department of Analytical Chemistry and Toxicology, University Centre for Pharmacy, Groningen, The Netherlands.

Recently, a procedure has been developed for the screening of acidic, neutral, and basic drugs in urine by using a single copolymeric SPE column [*15*]. Unfortunately, data for benzodiazepines, an important class of drugs in both forensic science and clinical cases, were not given. Bond Elut Certify columns, which contain a proprietary bonded silica sorbent that exhibits a unique hydrophobic ion-exchange extraction mechanism, was originally developed for extraction of drugs of abuse from urine, using separate procedures for such different classes of drugs as amphetamines, barbiturates, and opiates [*16,17*]. In this study, the use of Bond Elut Certify columns was investigated for the extraction of a broad selection of acidic, neutral, and basic drugs from plasma within one run. The drugs were eluted into two groups, according to their physical properties, and analyzed subsequently by gas chromatography. The method described here is a continuation of an earlier study by the authors of this paper with a limited number of drugs [*18*]. It was prompted by the fact that the earlier method gave recoveries that were too low and irreproducible when the selection of tested drugs was extended.

Materials and Methods

Materials

All chemicals and solvents were of analytical grade (Merck, Darmstadt, Germany) with the exception of ammonia (33%), which was of extra-pure quality (Merck). Bond Elut Certify columns with a capacity of 10 mL were obtained from Analytichem International (Harbor City, California), and the Baker-10 SPE vacuum manifold system, which can accept ten SPE columns simultaneously, was purchased from J. T. Baker (Phillipsburg, New Jersey). A UniVap centrifuge concentrator purchased from UniEqip (Martinsried, Germany) was equipped with a vacuum pump (GeneVac, Ipswich, England). Phosphate buffer (0.1*M*) was prepared by dissolving 6.81 g of potassium dihydrogen phosphate into 450 mL of deionized water, adjusting the pH to 6.0 ($\pm$0.1) with 1.0*M* potassium hydroxide, and making the total volume up to 500 mL with deionized water. The 0.01*M* acetic acid solution was prepared by mixing 57.5 μL of glacial acetic acid with 100 mL of deionized water. Ammoniated ethyl acetate (2%) was prepared daily by adding 1 mL of ammonia to 49 mL of ethyl acetate. The mixture was sonicated for 5 min before use. A chromatographic standard solution was prepared by dissolving the appropriate amount of prazepam in methanol/ethyl acetate (1:1) to make the solution equivalent to 200 μg/mL.

Instrumentation

The analysis was performed with a Hewlett-Packard Model 5880 gas chromatograph (Avondale, Pennsylvania), equipped with a Hewlett-Packard 7671 A automatic sampler, a 5880 A gas-chromatograph (GC) terminal, and a flame ionization detector (FID). The column was a 30-m HP-1, fused-silica wide-bore capillary column (0.53 mm in inside diameter, with a film thickness of 0.88 μm, by Hewlett-Packard). The oven temperature program was 2 min at 80°C, 20°C/min to 215°C, 5°C/min to 285°C, and 2 min at 285°C. The injector and detector temperatures were 275 and 310°C, respectively. The injection port was in the splitless mode.

Extraction Procedure

Citrated calf plasma (2 mL) or urine (2 mL), which had been spiked with the appropriate drugs (10 μg/mL), was diluted with phosphate buffer (6 mL), and the mixture was

vortexed to ensure homogeneity. The concentration of 10 μg/mL was chosen for analytical convenience as well as to represent toxicologically relevant levels for most of the drugs. The extraction procedure is described in Fig. 1.

Results and Discussion

Column Preconditioning

The column was preconditioned with 2 mL of methanol, followed by 2 mL of phosphate buffer to solvate the sorbent of the column and prepare it to receive an aqueous sample. It must be mentioned that the column must not become dry before application of the sample, or low and irreproducible recoveries will result.

Sample Application

In order to prevent clogging of the column, the plasma was diluted with phosphate buffer. For purposes of screening, the flow of the sample through the column should not be too fast in order to ensure that the drug present in the sample is retained on the column completely. In the experiment, the flow rate was controlled at approximately 1.5 mL/min.

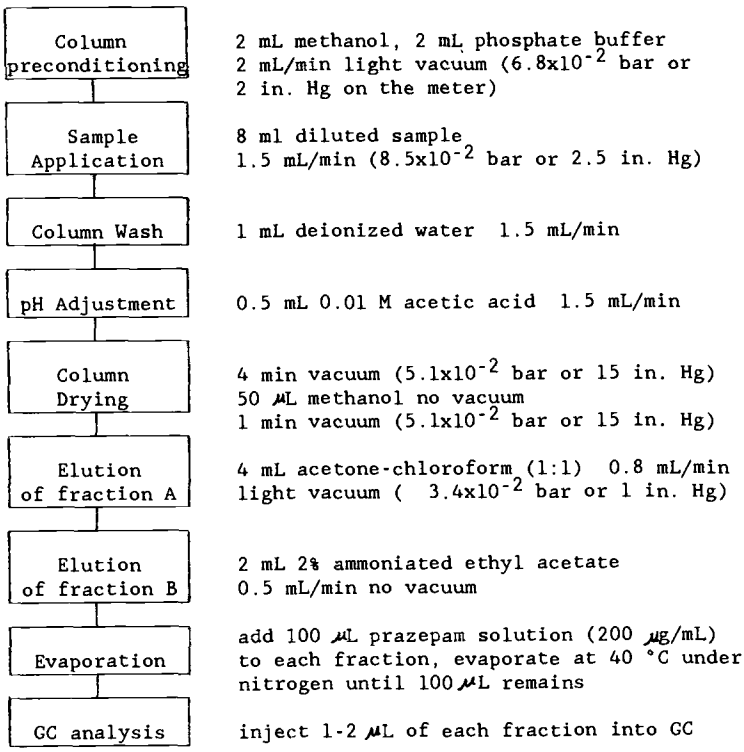

FIG. 1—*Extraction scheme for plasma on Bond Elut Certify columns.*

Column Wash

Washing with 1 mL of deionized water was found to be adequate to remove interferences from the column without affecting drug recoveries. Figure 2 (*top*) illustrates the cleanliness of blank plasma extracts, where Graph *a* represents the acetone/chloroform fraction (Fraction A) and Graph *b* the ammoniated ethyl acetate fraction (Fraction B). The small peak present in the chromatogram of Fraction A was due to plasma cholesterol, and the small peak present in the chromatogram of Fraction B was also caused by plasma or by urine, but has not been identified yet.

pH Adjustment

Many toxicologically relevant drugs are acids or bases, and it can hence be anticipated that their column retention and elution behavior can be affected by the pH of the extraction system. Based on this principle, an attempt was made to elute the drugs selectively from the column by adjusting the pH. It was observed that, because of the different pK_a values of the drugs, at one pH value, the tested drugs were difficult to separate entirely into two groups. However, it appeared possible to select a pH value for the extraction system so that most of the drugs could be classified. Various pH values for the system were tested, and it was found that 0.5 mL of 0.01*M* acetic acid (pH 3.3) was a suitable choice. Under this pH condition, acidic and neutral drugs behave as relatively nonpolar compounds, which are retained on the column by the hydrophobic groups of sorbent, while basic drugs behave as charged compounds, which are absorbed by the negative ionic groups of the sorbent.

Column Drying

In our primary study, 1 mL of hexane was used for displacing traces of absorbed water from the column [*18*]. Although this worked well for nitrazepam, we found that another benzodiazepine, lorazepam, was lost when the column was dried with hexane. Therefore, methanol (50 μL) was employed for removing the residual water, which resulted in a "dry" eluate without inappropriate elution of benzodiazepines.

Elution of Fraction A

Several solvents, including methylene chloride, chloroform, acetone, and mixtures of compounds were tested for optimal elution efficiency for acidic and neutral drugs. Acetone/chloroform mixtures were found to be the most efficient. Figure 3 presents the relationship between the recoveries of some barbiturates and benzodiazepines and the percentage of acetone in chloroform. The recoveries show that, using 30 to 70% of acetone in chloroform, more than 89% of each tested drug was recovered. Therefore, 50% was chosen as the percentage of acetone in chloroform. The elution curves of the barbiturates and benzodiazepines by various volumes of 1:1 acetone/chloroform are shown in Fig. 4. It can be seen that 3 to 5 mL of elution solvent are adequate for eluting the drugs, with recoveries of the tested drugs higher than 93%. A volume of 4 mL was selected for the elution solvent for acidic and neutral drugs.

Elution of Fraction B

It was found that there was too much water present in Fraction B when ethyl acetate contained more than 3% ammonia. This damaged the gas chromatograph column. Therefore, 2% ammoniated ethyl acetate was used as the elution solvent for basic drugs. Figure

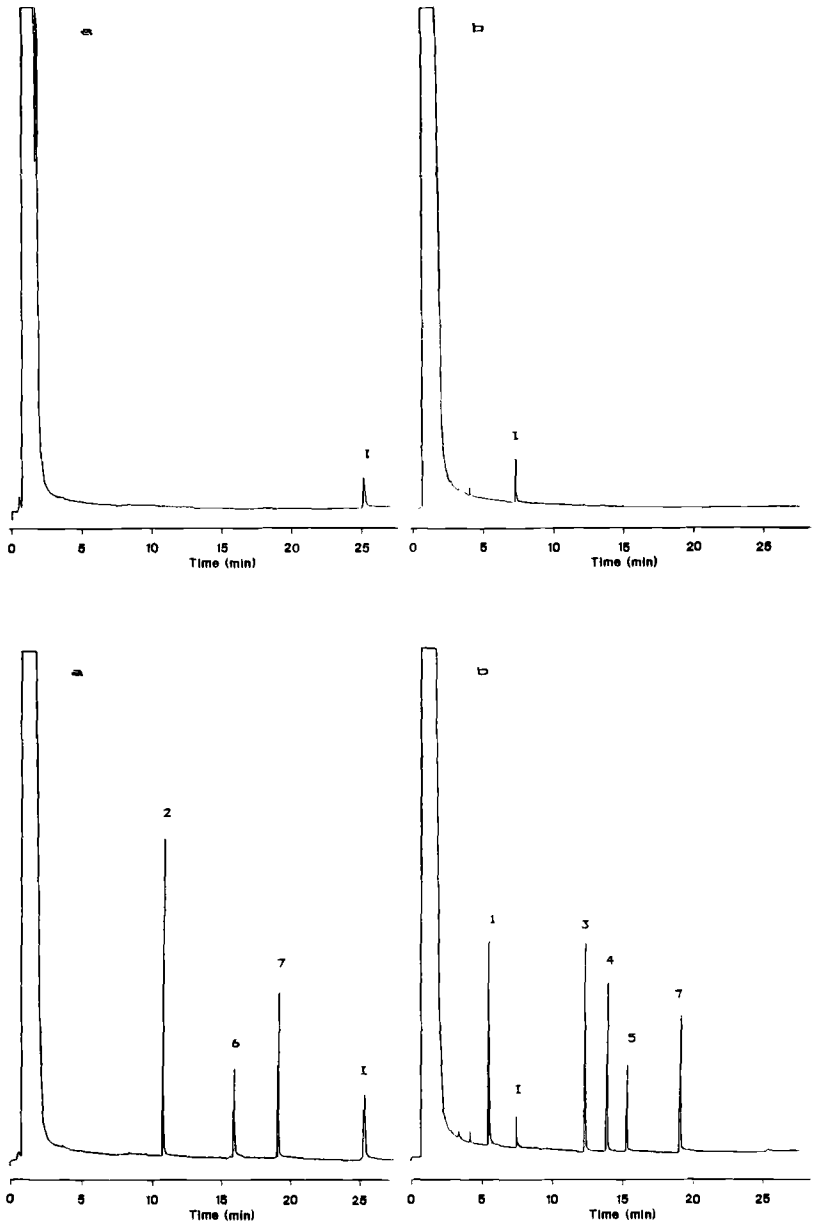

FIG. 2—*Chromatograms of* (top) *blank calf plasma and* (bottom) *spiked calf plasma, 10 μg/mL of each drug:* (a) *Fraction A,* (b) *Fraction B. Key: 1 = methamphetamine; 2 = hexobarbital; 3 = mepivacaine; 4 = trimipramine; 5 = levallorphan; 6 = lorazepam; 7 = prazepam (chromatographic standard); and I = impurity.*

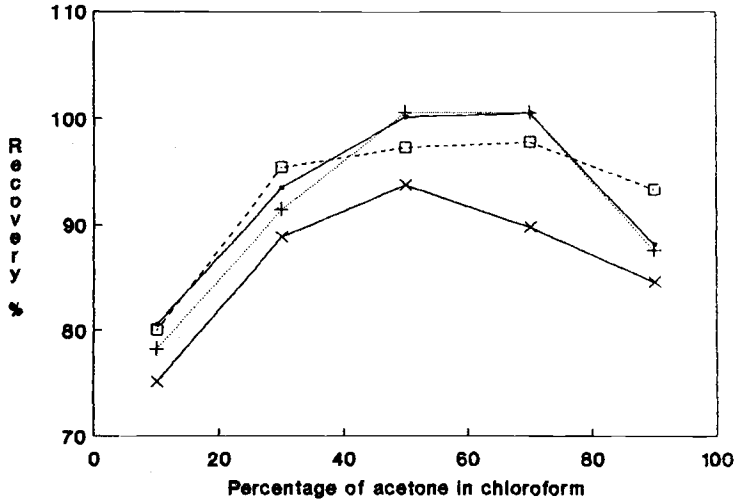

FIG. 3—*Influence of the percentage of acetone in chloroform on the recovery of some acidic and neutral drugs from spiked plasma; 4 mL of elution solvent was used. Key:* ● = *pentobarbital;* + = *secobarbital;* □ = *oxazepam; X = lorazepam.*

5 shows the recoveries of some basic drugs eluting with various volumes of 2% ammoniated ethyl acetate. Although 2 to 4 mL of elution solvent can be used for eluting methamphetamine, cocaine, and imipramine, 2 mL is the best choice, since the recovery of amphetamine decreased when more than 2 mL of elution solvent was used.

Because of the strong absorption of morphine on the Bond Elut Certify column, two applications, each of 2 mL of ammoniated ethyl acetate were required for eluting morphine completely. Figure 6 illustrates the elution of morphine from a Bond Elut Certify

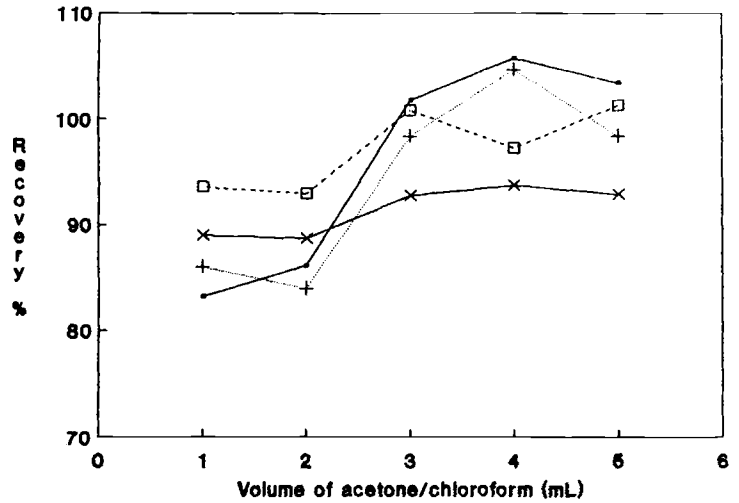

FIG. 4—*Relationship between the recovery of some acidic and neutral drugs from spiked plasma and the volume of 1:1 acetone/chloroform. Key:* ● = *pentobarbital;* + = *secobarbital;* □ = *oxazepam; X = lorazepam.*

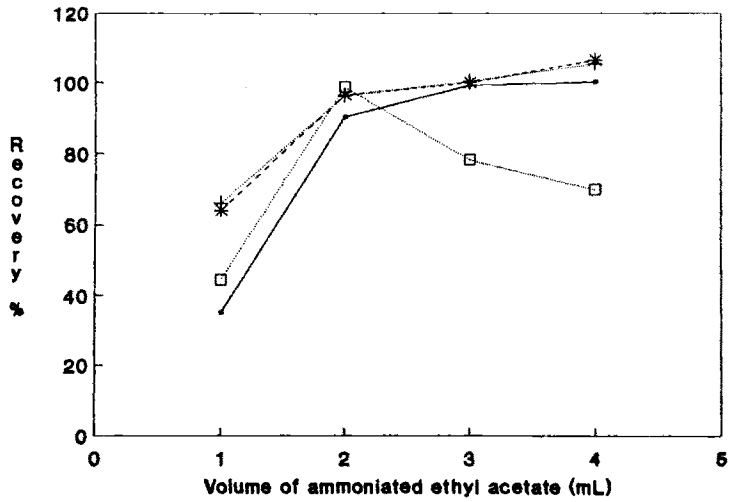

FIG. 5—*Relationship between the recoveries of basic drugs from spiked plasma and the volume of 2% ammoniated ethyl acetate. Key:* □ = *amphetamine;* ● = *methamphetamine;* + = *cocaine;* * = *imipramine.*

column. Only 62% of the morphine was obtained when using 2 mL of ammoniated ethyl acetate once. The recovery was improved by increasing the volume of the elution solvent. The best way was using 2 mL of ammoniated ethyl acetate twice, and a recovery of 97.8% was obtained. It is interesting to note that 2 mL of elution liquid used twice yielded higher recovery than 4 mL used once. It may be anticipated that other polar basic substances will also require two applications of 2 mL of ammoniated ethyl acetate.

The time needed for the preconditioning of the columns plus the elution is approximately 30 min when ten columns are being used simultaneously.

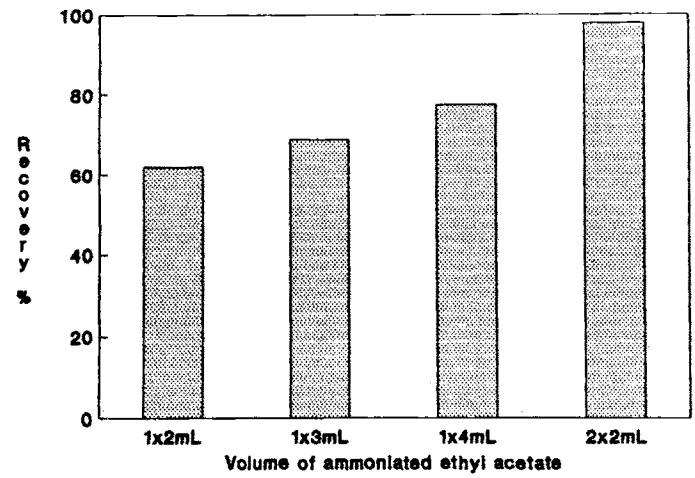

FIG. 6—*Recovery of morphine from spiked calf plasma on Bond Elut Certify column by various of elution methods. The concentration of morphine in plasma was 10 μg/mL.*

Evaporation

It must be mentioned that neither fraction should be evaporated to dryness, otherwise some of the more volatile drugs, such as amphetamines, metharbital, and probarbital, will be lost in the evaporation step. Therefore, the evaporation was stopped when approximately 100 μL of eluate remained in the tube. The time needed for evaporation was about 25 min.

It was found that some of the benzodiazepines, such as lorazepam and oxazepam, disappeared during the evaporation under alkaline condition when the two fractions were collected together, according to our primary procedure [*18*]. The problem was overcome by keeping the two fractions separated (see Table 1).

In our study, the possibility of using a UniVap centrifuge concentrator for evaporation was investigated. The concentration was performed at 30°C under vacuum until approximately 100 μL of eluate remained in the tube, which took about 20 min. The results are listed in Table 2. Compared with those in Table 1, the recoveries of the tested drugs tend to be lower with the UniVap centrifuge concentrator. However, the results are still acceptable. The UniVap concentrator is especially useful for larger numbers of samples.

TABLE 1—*Recoveries for 25 drugs after extraction from calf plasma by means of Bond Elut Certify columns.*

Drug	Recovery (n = 5), %		Total Recovery, %	Relative Standard Deviation
	Fraction A	Fraction B		
Metharbital	95.1	ND[a]	95.1	4.5
Probarbital	82.4	ND	82.4	10.2
Pentobarbital	100.2	ND	100.2	6.3
Butalbital	93.3	ND	93.3	5.4
Secobarbital	100.6	ND	100.6	3.8
Hexobarbital	96.1	ND	96.1	5.0
Heptabarbital	100.5	ND	100.5	2.7
Oxazepam	91.1	ND	91.1	5.4
Lorazepam	93.8	ND	93.8	7.0
Diazepam	83.9	14.6	98.5	1.9
Clonazepam	87.1	ND	87.1	3.8
Nitrazepam	58.6	35.2	93.8	7.2
Methaqualone	87.0	11.8	98.8	4.9
Meprobamate	99.2	ND	99.2	5.5
Amphetamine	ND	98.9	98.9	8.9
Methamphetamine	ND	90.2	90.2	6.8
Mepivacaine	ND	103.7	103.7	4.2
Trimipramine	ND	105.5	105.5	3.5
Levallorphan	ND	101.6	101.6	4.0
Procaine	ND	98.6	98.6	5.0
Promethazine	ND	95.6	95.6	5.3
Cocaine	ND	96.2	96.2	6.2
Imipramine	ND	94.4	94.4	3.5
Codeine	ND	92.3	92.3	2.5
Morphine	ND	97.8[b]	97.8	5.2

[a]ND = not detected.

[b]Eluted with two times 2 mL of 2% ammoniated ethyl acetate. The first 2 mL of solvent eluted 61.9% of the morphine, and the second eluted 35.9% of the morphine.

TABLE 2—*Recoveries for 11 drugs after extraction from calf plasma by means of Bond Elut Certify columns and evaporated by a UniVap concentrator centrifuge.*

Drug	Recovery (n = 3), %		Total Recovery, %	Relative Standard Deviation
	Fraction A	Fraction B		
Butalbital	86.1	ND[a]	86.1	4.0
Pentobarbital	87.4	ND	87.4	3.5
Secobarbital	98.2	ND	98.2	6.2
Hexobarbital	95.6	ND	95.6	5.3
Heptabarbital	98.5	ND	98.5	3.2
Clonazepam	99.6	ND	99.6	6.8
Methamphetamine	ND	88.2	88.2	4.7
Mepivacaine	ND	103.0	103.0	3.4
Cocaine	ND	90.2	90.2	2.9
Imipramine	ND	95.1	95.1	6.6
Levallorphan	ND	95.4	95.4	5.4

[a]ND = not detected.

GC Analysis

The recovery data for 25 tested drugs are presented in Table 1. The range of recoveries is between 82.4 and 105.5%, with a relative standard deviation of less than 10%. All of the acidic and most of the neutral drugs were present in Fraction A, while the basic drugs were present in Fraction B. Yet, three of the neutral drugs, methaqualone, diazepam, and nitrazepam (the pK_a values of which are 2.5, 3.3, and 3.2, respectively), with pK_a values close to the pH of the acidic extraction system (3.3) were present in both Fractions A and B.

Thus, by employing Bond Elut Certify column, most of the drugs can be eluted into two groups. This classification into two groups according to the chemical characteristics of the substances is caused by the special character of the Bond Elut Certify material, which cannot be obtained with a column material with a single functional group sorbent.

Figure 2 (*bottom*) presents the typical chromatograms of spiked plasma, where Graph *a* represents the extract of Fraction A and Graph *b* represents that of Fraction B. The results in Table 1 indicate that, in the screening of an unknown sample, the drug present in Fraction A should be an acidic or neutral one and that in Fraction B should be a basic one. When a drug is found in both fractions with the same retention index (RI), this substance is likely to be a drug whose pK_a is close to the pH of the extraction system.

The advantage of two separate fractions is that two drugs that have similar retention times but different physical properties can be separated into two fractions. For instance, the retention indices of heptabarbital and mepivacaine are 2059 and 2071, respectively [*19*], and their peaks will overlap or coincide under usual gas chromatography conditions. As shown in Table 1, heptabarbital was present in Fraction A, while mepivacaine was present in Fraction B, so no interference occurred. Another pair of drugs is oxazepam and levallorphan, their retention indices being 2336 and 2360, respectively [*19*].

Although the extraction procedure was developed primarily for use with plasma specimens, it could also be used for human urine without any modification. The recoveries from spiked human urine and the relative standard deviations for a number of drugs are shown in Table 3. The recoveries of the tested drugs were over 97%, with good reproducibility. The chromatograms were as clean as those produced by the extracts from plasma (Fig. 7).

TABLE 3—*Recoveries for seven drugs after extraction from human urine by means of Bond Elut Certify columns.*

Drug	Recovery (n = 4), %		Total Recovery, %	Relative Standard Deviation
	Fraction A	Fraction B		
Pentobarbital	97.0	ND[a]	97.0	4.5
Secobarbital	97.1	ND	97.1	2.0
Hexobarbital	99.2	ND	99.2	1.6
Diazepam	65.5	32.5	98.0	5.0
Methamphetamine	ND	97.9	97.9	5.7
Mepivacaine	ND	104.3	104.3	2.7
Imipramine	ND	101.7	101.7	1.9

[a]ND = not detected.

Conclusions

The results of this study indicate that the developed procedure is an effective extraction method in STA. According to their physical properties, the drugs were classified into two groups: acidics and neutrals on the one hand, and basics on the other hand. The acidic and most of the neutral drugs were present in Fraction A, while basic drugs were eluted in Fraction B. A drug present in both fractions should be one whose pK_a is close to the pH of the system. Clean extracts were obtained as well as excellent recoveries. Evaluation of this procedure for lower concentrations is in progress.

Acknowledgment

The authors would like to thank Analytichem International Inc. for supplying the Bond Elut Certify columns.

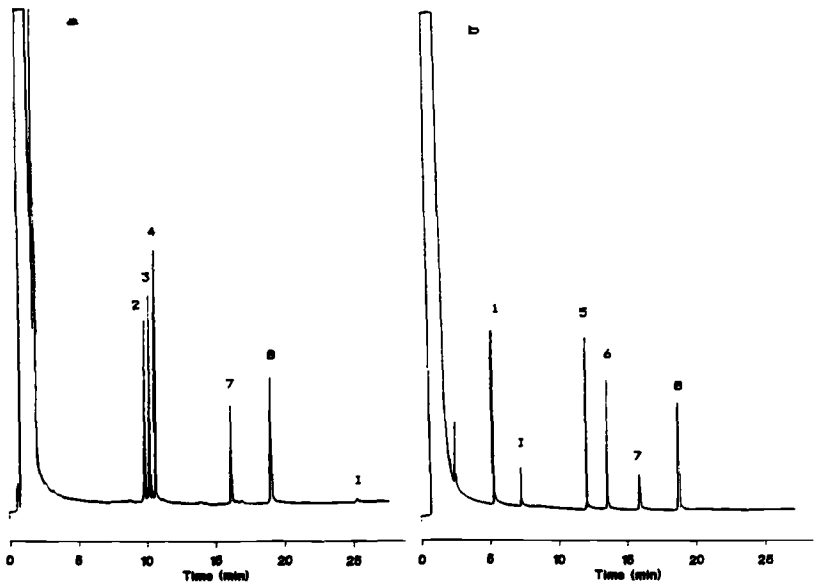

FIG. 7—*Chromatograms of spiked human urine, 10 μg/mL of each drug:* (a) *Fraction A,* (b) *Fraction B. Key: 1 = methamphetamine; 2 = pentobarbital; 3 = secobarbital; 4 = hexobarbital; 5 = mepivacaine; 6 = imipramine; 7 = diazepam; 8 = prazepam (chromatographic standard); and I = impurity.*

References

[1] Johnson, E. L., Reynolds, D. L., Wright, D. S., and Pachla, L. A., "Biological Sample Preparation and Data Reduction Concepts in Pharmaceutical Analysis," *Journal of Chromatographic Science*, Vol. 26, No. 8, Aug. 1988, pp. 372–379.

[2] McDowall, R. D., "Sample Preparation for Biomedical Analysis," *Journal of Chromatography*, Vol. 492, Aug. 1989, pp. 3–58.

[3] Van Horne, K. C., *Sorbent Extraction Technology*, Analytichem International Inc., Harbor City, CA, 1985.

[4] Zief, M. and Kiser, R., *Solid Phase Extraction for Sample Preparation*, J. T. Baker, Phillipsburg, NJ, 1988.

[5] Patel, R. M., Benson, J. R., Hometchko, D., and Marshall, G., "Polymeric Solid Phase Extraction of Organic Acids," *International Laboratory*, April, 1990, pp. 38–45.

[6] Patterson, S., "Determination of Temazepam in Plasma and Urine by High-Performance Liquid Chromatography Using Disposable Solid-Phase Extraction Columns," *Journal of Pharmaceutical and Biomedical Analysis*, Vol. 4, No. 2, 1986, pp. 271–274.

[7] Inoue, T. and Suzuki, S. I., "High-Performance Liquid Chromatographic Determination of Triazolam and Its Metabolites in Human Urine," *Journal of Chromatography*, Vol. 422, No. 1, 1987, pp. 197–204.

[8] Suzuki, O., Seno, H., and Kumazawa, T., "Rapid Isolation of Benzodiazepines with Sep-Pak C_{18} Cartridges," *Journal of Forensic Sciences*, Vol. 33, No. 5, Sept. 1988, pp. 1249–1253.

[9] Roberts, G. M. and Hann, C. S., "A Rapid and Reliable Method for Extraction and Assay of Tricyclic Antidepressant Drugs in Serum or Plasma," *Biomedical Chromatography*, Vol. 1, No. 2, 1986, pp. 49–52.

[10] Musch, G. and Massart, D. L., "Isolation of Basic Drugs from Plasma Using Solid-Phase Extraction with a Cyanopropyl-Bonded Phase," *Journal of Chromatography*, Vol. 43, 1988, pp. 209–222.

[11] Mazhar, M. and Binder, S. R., "Analysis of Benzodiazepines and Tricyclic Antidepressants in Serum Using a Common Solid-Phase Cleanup and a Common Mobile Phase," *Journal of Chromatography*, Vol. 497, 1989, pp. 201–212.

[12] Evenson, M. A. and Engstrand, D. A., "A SepPak HPLC Method for Tricyclic Antidepressant Drugs in Human Vitreous Humor," *Journal of Analytical Toxicology*, Vol. 13, No. 6, Nov./Dec. 1989, pp. 322–325.

[13] Logan, B. K., Stafford, D. T., Tebbett, I. R., and Moore, C. M., "Rapid Screening for 100 Basic Drugs and Metabolites in Urine Using Cation Exchange Solid-Phase Extraction and High-Performance Liquid Chromatography with Diode Array Detection," *Journal of Analytical Toxicology*, Vol. 14, No. 3, May/June, 1990, pp. 154–159.

[14] Cordonnier, J., Van den Heede, M., and Heyndrickx, A., "A Rapid Isolation of Acidic and Basic Drugs from Human Tissues by Coupled Chem Elute™ Extraction Columns," *International Analyst*, Vol. 3, May 1987, pp. 28–34.

[15] Thompson, B. C., Kuzmack, J. M., Law, D. W., and Winslow, J. J., "Copolymeric Solid-Phase Extraction for Quantitating Drugs of Abuse in Urine by Wide-Bore Capillary Gas Chromatography," *LC-GC International*, Vol. 3, No. 2, 1990, pp. 55–58.

[16] *Bond Elut Certify Instruction Manual*, Analytichem International Inc., Harbor City, CA, 1988.

[17] Dino, J., Jr., Anderson, G. R., Masuda, K. R., and Foley, T. J., "Studies of Multiple Drug Extractions Through Bond Elut Certify™ SPE Columns," presented at the 42nd Annual Meeting of American Academy of Forensic Sciences, Philadelphia, PA, Feb. 1990.

[18] Chen, H. X., Wijsbeek, J., Van Veen, J., Franke, J. P., and de Zeeuw, R. A., "Solid Phase Extraction for the Screening of Acidic, Neutral, and Basic Drugs in Plasma Using a Single Column Procedure on Bond Elut Certify," *Journal of Chromatography*, Vol. 529, No. 1, 1990, pp. 161–166.

[19] Moffat, A. C., *Clarke's Isolation and Identification of Drugs*, The Pharmaceutical Press, London, 1986.

Address requests for reprints or additional information to
Xiao Hua Chen
Department of Analytical Chemistry and Toxicology
University Centre for Pharmacy
Antonius Deusinglaan 2
NL-9713 AW Groningen
The Netherlands