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ANALYSIS OF BENZODIAZEPINES AND TRICYCLIC ANTIDEPRESSANTS IN SERUM USING A COMMON SOLID-PHASE CLEAN-UP AND A COMMON MOBILE PHASE

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SUMMARY

A single, rapid and specific solid-phase clean-up procedure was developed for the analysis of benzodiazepines and tricyclic antidepressants using a carefully selected wash step and specific sequential elution. Benzodiazepines were eluted from the solid-phase column using a mixture of water–methanol–acetonitrile (2:3:3) followed by the elution of tricyclic antidepressants with methanol containing 0.6% diethylamine. A 30% solution of acetonitrile in phosphate buffer containing dimethyloctylamine was used as a common isocratic mobile phase for the analysis of benzodiazepines and tricyclic antidepressants on a reversed-phase column and detection was carried out at 242 nm. The sensitivity limit of the assay for benzodiazepines and tricyclic antidepressants was 25 ng/ml in serum with recoveries of 95–105% for benzodiazepines and 76–95% for tricyclic antidepressants. The results were linear for benzodiazepines over the range 50–2000 ng/ml and for tricyclic antidepressants over the range 25–500 ng/ml. Analysis for benzodiazepines and tricyclic antidepressants gave good precision, with a coefficient of variation of less than 5.0%. The method described here will be suitable for use in a clinical setting, where there is a concomitant use of benzodiazepines and tricyclic antidepressants.

INTRODUCTION

Since the discovery of diazepam (Valium) and chlordiazepoxide (Librium) more than two decades ago, several new benzodiazepines have been introduced for the treatment of a wide spectrum of clinical disorders. They are used as hypnotics, tranquilizers, anticonvulsants or muscle relaxants [1] and rank as the most frequently prescribed class of drugs [2]. More recently, a new gen-

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eration of benzodiazepines belonging to the class of triazolobenzodiazepines have gained widespread use due to their potent action at low doses [3]. Alprazolam and triazolam are the prominent members of this class. Alprazolam also shows antidepressant activity in addition to the pharmacological action common to other benzodiazepines [4]. Extensive use of benzodiazepines has lead to overuse and abuse, resulting in toxicity. Furthermore, the unrestricted use of these drugs concomitantly with other drugs warrants careful monitoring to avoid the toxic effects of drug interactions. Many of the benzodiazepines are also used in combination with antidepressants in the management of depression [5]. Tricyclic antidepressants (TCAs) are the most popular class of drugs in the treatment of depression. Prominent members of this class (imipramine, desipramine, amitriptyline and nortriptyline) accounted for a 46% share of TCA prescriptions in the U.S.A. in 1986 [6]. The efficacy of monitoring TCAs to assess compliance, toxicity, clinical response and drug interactions is well documented [7]. Concurrent administration of benzodiazepines and other drugs along with TCAs is common and can affect the rate of metabolism and serum levels of TCAs. In view of the above considerations, a versatile analytical procedure is needed to assay both classes of drugs, particularly in a setting where a patient is taken them concurrently.

Methods presently utilized for the analysis of benzodiazepines are immunoassay (IA) [8,9], thin-layer chromatography (TLC) with densitometry [10,11], gas chromatography (GC) [12,13] and high-performance liquid chromatography (HPLC) [14,15]. Procedures involving IA techniques lack specificity, while TLC methods are not sensitive, and the technique is tedious to adapt for routine analysis. The thermal instability of some of these drugs complicates GC analysis. Measurement of benzodiazepines by HPLC is preferred due to its sensitivity and specificity, and the methods do not require derivatization. Several papers [16–19] concerning the analysis of individual benzodiazepines and their metabolites have been published as well as a few methods [20,21] applicable for quantitation of these drugs as a group by HPLC. A majority of the methods use laborious solvent extraction techniques to separate the drug from the serum. More recently solid-phase sample preparation has been employed using methanol as an elution solvent [22,23].

A variety of methods involving IA [24,25], GC [26,27], gas chromatography–mass spectrometry (GC–MS) [28,29] and HPLC [30–32] have been applied for the determination of TCAs. The merits of HPLC and the limitations of other methods for the analysis of TCAs were recently discussed [33,34]. The HPLC methods have evolved as most useful because of assay specificity and the possibility of analysing both the parent drug and its metabolites simultaneously. Sample preparations for HPLC analysis of TCAs involve solvent extraction or solid-phase clean-up [35,36]. Such procedures are not specific for the TCAs and have the potential of coextraction of any benzodiazepines that might be present in the sample. In order to obtain a preferential extraction

of benzodiazepines or TCAs by a solid-phase method, a very specific sequential eluent is needed. The current method describes a single, simple and a selective solid-phase sample clean-up procedure for both benzodiazepines and TCAs and the subsequent analysis on a reversed-phase column using a common isocratic mobile phase.

EXPERIMENTAL

Materials

Benzodiazepines (norchlordiazepoxide, chlordiazepoxide, oxazepam, desalkylflurazepam, nordiazepam and diazepam) and TCAs (nordoxepin, doxepin, desipramine, imipramine, nortriptyline, amitriptyline and trimipramine) were purchased from Alltech–Applied Science (State College, PA, U.S.A.). Alprazolam and triazolam were kindly supplied by Upjohn (Kalamazoo, MI, U.S.A.). Fludiazepam was synthesized in house by methylation of desalkylflurazepam with methyl iodide in alkaline conditions and purified by repeated crystallization from methanol. The melting point corresponded to the value reported in the literature and the compound showed a single peak on analysis by HPLC. HPLC-grade solvents (methanol and acetonitrile) and ACS-certified pure chemicals (potassium dihydrogenphosphate, diethylamine, potassium bicarbonate and potassium hydroxide) were purchased from Fisher. Drug-free serum and serum controls for benzodiazepines and TCAs were obtained from Utak (Canyon Country, CA, U.S.A.) and Bio-Rad Labs. ECS Division (Anaheim, CA, U.S.A.). N,N-Dimethyloctylamine was purchased from Aldrich (Milwaukee, WI, U.S.A.) and water used was purified in house using an ion-exchange system. Bonded-phase C_{18} extraction columns with 100 mg capacity and 40 μm particle size were obtained from Bio-Rad Labs.

The first reagent for conditioning the solid-phase columns was prepared by dissolving 600 μl of diethylamine in 100 ml of HPLC-grade methanol and was stored at 4 °C, protected from light and moisture. The same reagent is also used for the elution of TCAs. The second conditioning agent was prepared by dissolving 1 g of potassium bicarbonate in 100 ml of 10% acetonitrile in water. The wash reagent was prepared by the addition of 20 ml of acetonitrile to 80 ml of distilled water. The benzodiazepines' elution reagent was made by mixing 20 ml of distilled water with 30 ml of methanol and 30 ml of acetonitrile.

Standards and controls

A 1 mg/ml solution of individual drugs in methanol was prepared by dissolving 10 mg in HPLC-grade methanol. The working composite standards of different concentrations of benzodiazepines (major) containing norchlordiazepoxide, oxazepam, chlordiazepoxide, desalkylflurazepam, nordiazepam and diazepam was prepared by combining an aliquot of each stock solution and diluting with drug-free serum. A similar procedure was adopted for the TCA

standards containing nordoxepin, doxepin, desipramine, imipramine, nortriptyline and amitriptyline. A mixture containing different concentrations of clonazepam, alprazolam and triazolam was also prepared by the same procedure. These composite standards of various concentrations in drug-free serum were used for linearity studies. Two different standards were prepared to be used regularly as calibrators for the assay. The first standard contained the major benzodiazepines [oxazepam (500 ng/ml), chlordiazepoxide (500 ng/ml), desalkylflurazepam (200 ng/ml), nordiazepam (500 ng/ml), diazepam (500 ng/ml)] and all the TCAs listed above in a 200 ng/ml concentration in drug-free serum. The second standard contained 100 ng/ml each of clonazepam, alprazolam and triazolam and 200 ng/ml of all the above listed TCAs in drug-free serum. The standards were lyophilized, stored at 4 °C and used after reconstitution with distilled water.

Internal standards

From a 1 mg/ml methanolic solution of fludiazepam (internal standard for benzodiazepines) and trimipramine (internal standard for TCAs) was prepared a working internal standard solution in 20% acetonitrile solution containing a mixture of fludiazepam (5 μg/ml) and trimipramine (3.75 μg/ml).

Solid-phase extraction

Solid-phase columns fitted with a reservoir were positioned in luerlock fittings on the cover of a vacuum box. The columns were conditioned by washing with 3 ml of the first conditioning reagent under a vacuum of 254 mmHg. A 3-ml solution of the second conditioning reagent was applied and allowed to drain under vacuum. A 1-ml volume of standard, control or patient serum was mixed with 200 μl of working internal standard, diluted with 1 ml of distilled water and vortex-mixed for 20 s. Only 50 μl of internal standard solution were used for the analysis of alprazolam. The serum samples were poured on the column and allowed to drain under a vacuum of 254 mmHg. The columns were washed three times with 1 ml of wash reagent. After the last wash the columns were left under vacuum for 2 min. The reservoirs were disconnected and the undersurface of the vacuum box cleaned with water. The benzodiazepines were eluted with two 400-μl portions of benzodiazepines elution reagent. The combined eluates were mixed with 1 ml of distilled water and vortexed, and 50 μl were injected on the column. TCAs which were retained on the column were subsequently eluted with two 300-μl portions of TCA elution reagent. The combined eluates were diluted with 300 μl of distilled water and mixed, and 50 μl were injected on the column.

Mobile phase preparation

Potassium dihydrogen phosphate (1.36 g, 0.01 *M*) was dissolved in 1000 ml of distilled water and 100 μl of N,N-dimethyloctylamine were added while stir-

ring. The pH of the solution was adjusted to 6.4 by the careful addition of 2 *M* potassium hydroxide. Mobile phases was prepared by mixing 700 ml of this buffer with 300 ml of acetonitrile.

HPLC instrumentation

The chromatographic system was from Bio-Rad Labs. and was composed of a Model 1330 dual-piston pump, a Model 1306 variable-wavelength UV detector, a column heater and a Model AS-48 autosampler with a 50-μl fixed loop. A Model HP3392 integrator (Hewlett Packard, Avondale, PA, U.S.A.) was used for the analysis of the data collected by monitoring the eluent at 242 nm. A 100×2.1 mm I.D., 3 μm particle size C_8 analytical column (Bio-Rad Labs.) with a 30×2.1 mm I.D., 10 μm particle size, guard cartridge was used for the chromatographic separation.

HPLC conditions

For the analysis of the major benzodiazepines the analytical and guard columns were maintained at 55°C with a flow-rate of 0.3 ml/min and the UV detector was set at 242 nm with 0.02 a.u.f.s. Alprazolam was chromatographed while maintaining the column temperature at 35°C with a flow-rate of 0.3 ml/min and the detector set at 242 nm with 0.01 a.u.f.s. The TCAs were run with

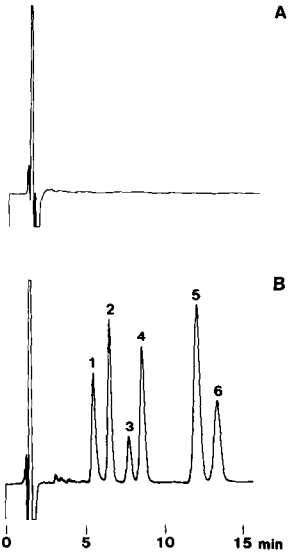

Fig. 1. Chromatograms of 50 μl of (A) extracted drug-free serum and (B) extracted benzodiazepine-spiked serum. Peaks: 1=oxazepam (500 ng/ml); 2=chlordiazepoxide (500 ng/ml); 3=desalkylflurazepam (200 ng/ml); 4=nordiazepam (500 ng/ml); 5=fludiazepam (internal standard, 1000 ng/ml); 6=diazepam (500 ng/ml). Flow-rate 0.3 ml/min at 55°C. Detection, 242 nm (0.02 a.u.f.s.).

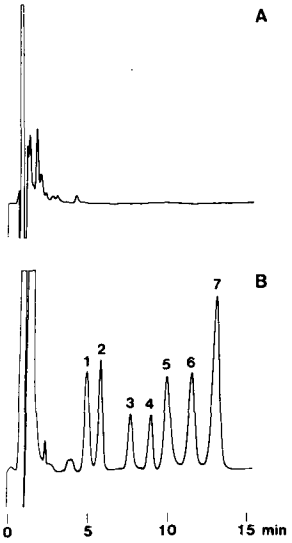

Fig. 2. Chromatograms of (A) extracted drug-free serum and (B) extracted TCA-spiked serum. Peaks: 1 = nordoxepin (200 ng/ml); 2 = doxepin (200 ng/ml); 3 = desipramine (200 ng/ml); 4 = imipramine (200 ng/ml); 5 = nortriptyline (200 ng/ml); 6 = amitriptyline (200 ng/ml); 7 = trimipramine (750 ng/ml). Flow-rate, 0.6 ml/min at 35 °C. Detection, 242 nm (0.01 a.u.f.s.).

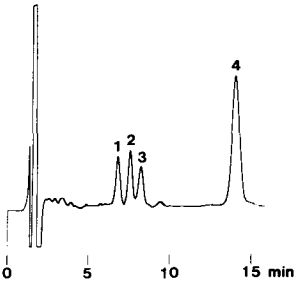

Fig. 3. Chromatogram of clonazepam (100 ng/ml, peak 1), alprazolam (100 ng/ml, peak 2), triazolam (100 ng/ml, peak 3) and fludiazepam (250 ng/ml, peak 4) extracted from serum. Flow-rate, 0.3 ml/min at 35 °C. Detection, 242 nm (0.01 a.u.f.s.).

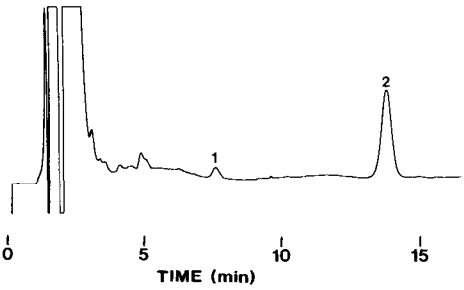

Fig. 4. Chromatogram of a patient serum containing alprazolam (14 ng/ml). Peaks: 1 = alprazolam; 2 = fludiazepam (internal standard).

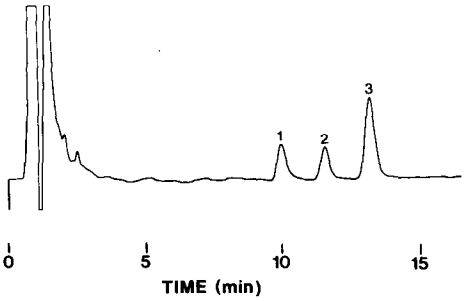

Fig. 5. Chromatogram of a patient serum containing nortriptyline (140 ng/ml) and amitriptyline (120 ng/ml). Peaks: 1 = nortriptyline; 2 = amitriptyline; 3 = trimipramine (internal standard).

the column temperature at 35 °C with a flow-rate of 0.6 ml/min and monitored at 242 nm with 0.01 a.u.f.s. The chromatograms obtained are presented in Figs. 1–3. Chromatograms from two different patient samples containing alprazolam and TCAs are presented in Figs. 4 and 5.

RESULTS

The assay for major benzodiazepines was linear in the range 50–2000 ng/ml and for alprazolam in the range 10–200 ng/ml. Regression analysis was performed between the concentration and the peak-height ratio and showed a correlation coefficient greater than 0.999. TCAs were assessed for linearity over the range 25–500 ng/ml. A strong linear relationship was observed by the comparison of concentration to peak-height ratio. The correlation coefficient for all the tricyclic antidepressants was above 0.999.

Recovery studies were conducted at two different concentrations for major benzodiazepines (50 and 500 ng/ml) and for alprazolam (10 and 50 ng/ml); the recovery ranged from 97 to 103% based on the comparison of peak heights of extracted spiked serum samples against unextracted aqueous standards. The recoveries for TCAs were determined similarly at two levels (50 and 500 ng/ml) and were lower (76–95%) than for the benzodiazepines. Tables I and II show the recovery data.

Within-run precision studies of the major benzodiazepines were performed on the two levels of commercial controls. The data showed coefficients of variation (C.V.) of less than 5% (Table III). TCAs were measured in replicates at two levels using commercial serum controls for the six components and showed a variation of less than 4.3% as presented in Table IV.

A number of drugs were evaluated for potential interferences. Serum standards (1 ml) containing benzodiazepines were spiked with 10 μg of each potentially interfering drug and carried through the extraction and chromatographic procedure. No interference was detected from these compounds as

TABLE I

RECOVERY OF BENZODIAZEPINES ($n=4$)

Drug	Amount spiked (ng/ml)	Amount measured (ng/ml)	Recovery (%)	Amount spiked (ng/ml)	Amount measured (ng/ml)	Recovery (%)
Norchlordiazepoxide	50.0	50.0	100	500	506	101
Oxazepam	50.0	49.3	99	500	497	99
Chlordiazepoxide	50.0	51.6	103	500	509	102
Desalkylflurazepam	50.0	48.3	97	500	505	101
Nordiazepam	50.0	48.5	97	500	504	101
Diazepam	50.0	49.9	100	500	515	103
Alprazolam	10.0	10.0	100	50	49	98

TABLE II

RECOVERY OF TRICYCLIC ANTIDEPRESSANTS ($n=4$)

Drug	Amount spiked (ng/ml)	Amount measured (ng/ml)	Recovery (%)	Amount spiked (ng/ml)	Amount measured (ng/ml)	Recovery (%)
Nordoxepin	50	46.5	93	500	415	83
Doxepin	50	47	94	500	410	82
Desipramine	50	44	88	500	380	76
Imipramine	50	44	88	500	405	81
Nortriptyline	50	47.5	95	500	390	78
Amitriptyline	50	44	88	500	400	80

TABLE III

WITHIN-RUN PRECISION DATA FOR BENZODIAZEPINES

Drug	Mean concentration (ng/ml)	n	C.V. (%)	Mean concentration (ng/ml)	n	C.V. (%)
Norchlordiazepoxide	903	9	2.0	1889	9	1.5
Oxazepam	940	9	4.7	1899	9	2.4
Chlordiazepoxide	1067	9	1.7	2121	9	1.6
Nordiazepam	405	9	2.1	787	9	1.8
Diazepam	983	9	1.6	1855	9	0.8
Desalkylflurazepam	114	10	3.7	195	10	1.2
Alprazolam	20	10	3.9	54	10	2.3

TABLE IV

WITHIN-RUN PRECISION DATA FOR TRICYCLIC ANTIDEPRESSANTS

Drug	Mean concentration (ng/ml)	*n*	C.V. (%)	Mean concentration (ng/ml)	*n*	C.V. (%)
Nordoxepin	103	9	2.9	385	9	4.3
Doxepin	102	9	2.4	382	9	3.4
Desipramine	103	10	3.7	416	9	2.1
Imipramine	96	10	3.6	387	9	2.6
Nortriptyline	101	10	3.0	413	9	2.0
Amitriptyline	78	10	2.0	384	9	1.5

TABLE V

RETENTION TIMES OF BENZODIAZEPINES AND RELATED DRUGS

HPLC conditions: flow-rate, 0.3 ml/min; column temperature, 55 °C.

Compound	Retention time (min)	Compound	Retention time (min)	Compound	Retention time (min)
Bromazepam	3.28	Lorazepam	5.81	Clobazam	8.47
Demoxepam	3.67	Chlordiazepoxide	6.10	Fludiazepam	11.35
Carbamazepine	3.92	Alprazolam	6.63	Midazolam	12.29
Norchlordiazepoxide	4.19	Flunitrazepam	6.50	Diazepam	12.55
Methaqualone	4.46	Triazolam	7.20	Flurazepam	15.49
Nitrazepam	4.69	Desalkylflurazepam	7.28	Medazepam	31.75
Oxazepam	5.14	Temazepam	7.97	Halazepam	33.27
Clonazepam	5.34	Nordiazepam	8.07		
Desmethylclobazam	5.47				

evidenced by the lack of change in the concentration of benzodiazepine standards when compared with the standards analyzed without these compounds. The retention times of the drugs tested for interference are presented in Table V.

TCA serum standards (1 ml) were similarly evaluated with spiked samples of 10 μg of some potentially interfering drugs. Methadone was found to coelute with nortriptyline, a methadone metabolite coeluted with doxepin, and propoxyphene coeluted with amitriptyline. Hydroxy metabolites of TCAs were extracted along with the parent compounds. However, the metabolites had shorter retention time and did not interfere with the analysis. The calibrators which contained both benzodiazepines and tricyclic antidepressants did not show any carry-over of TCAs into benzodiazepine extracts or benzodiazepines

TABLE VI

RETENTION TIMES OF TRICYCLIC ANTIDEPRESSANTS AND RELATED DRUGS

HPLC conditions: flow-rate, 0.6 ml/min; column temperature, 35 °C.

Compound	Retention time (min)	Compound	Retention time (min)	Compound	Rete time (mi
Quinidine	2.02	Trazodone	4.69	Methadone	9.0
10-Hydroxynortriptyline	2.06	Nordoxepin	4.94	Imipramine	9.2
2-Hydroxydesipramine	2.51	Doxepin	5.90	Nortriptyline	10.1
Propranolol	2.73	Methadone metabolite[a]	5.93	Propiomazine	11.2
Disopyramide	2.84	Desipramine	7.71	Amitriptyline	12.0
2-Hydroxyimipramine	2.90	Amoxapine	7.71	Propoxyphene	12.0
10-Hydroxyamitriptyline	3.10	Cyclobenzaprine	7.88	Thiothixene	12.6
Benztropine	4.62	Maprotiline	9.02	Chlorpromazine	12.9
				Trimipramine	13.9

[a]2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.

into TCA extracts. Extracted drug-free serum did not show any endogenous peaks interfering with either the benzodiazepines or the TCA analyses. The retention times are shown in Table VI.

DISCUSSION

The method described here for the analysis of benzodiazepines and TCAs involves the combination of two steps. The first step was the selective adsorption of these drugs on a bonded-phase column, removal of hydrophilic and hydrophobic impurities from the serum and a very selective elution of the benzodiazepines and TCAs. Several combinations of wash mixtures containing different concentrations of methanol–water or acetonitrile–water were evaluated for removing endogenous impurities and salts to obtain a clean solvent front. A 20% solution of acetonitrile proved specific for washing the impurities which would appear in the HPLC solvent front. The total volume of wash was optimized to 3 ml and the number of washes to three of 1 ml each. Larger wash volumes affected the recovery of benzodiazepines and smaller volumes gave a large solvent front and late-eluting peaks. The benzodiazepines and TCAs retained on the bonded silica due to non-polar interaction (benzodiazepines) and a combination of non-polar and polar interaction (strong hydrogen bonding interaction between the amino side-chain of the TCAs with the free silanol group of the silica) with the solid phase presented a challenge for selective elution. Ternary mixtures are frequently used with great success in the TLC separation of complex mixtures of compounds. We tried this approach with different combinations of water, methanol and acetonitrile. A solution water–

acetonitrile–methanol (2:3:3) gave complete elution of all the benzodiazepines with the total retention of TCAs on the column. The TCAs were subsequently eluted by methanol containing diethylamine. Methanol alone gave incomplete elution of TCAs from the solid-phase column and use of tertiary amines, such as triethylamine or dimethyloctylamine in methanol, gave a very poor elution of tricyclic drugs containing secondary amine structure.

Dilution of the benzodiazepine eluates with water was essential and dilution with less than 1 ml of water gave broader peaks. Maximum peak compression and optimum resolution was obtained when the eluates were diluted with 1 ml of water. However, dilution of TCA eluates with more than 300 μl of water did not have any effect on either resolution or peak compression.

Separation of benzodiazepines and their metabolites have been achieved by a number of workers on a reversed-phase column utilizing a variety of solvent combinations. Similarly, there is an abundance of literature on the separation of TCAs by reversed-phase chromatography. Recently, Minder et al. [37] reported a common isocratic mobile phase for the separation of both benzodiazepines and antidepressants with photodiode array detection. We have developed a common mobile phase adaptable for the separation of major benzodiazepines and TCAs in a clinical setting with the run time of 15 min which will be very convenient for routine analysis.

We evaluated the effect of various chromatographic conditions on separation by injecting three test mixtures: one containing six major benzodiazepines and metabolites with internal standard, another containing six TCAs and internal standard and a third mixture containing clonazepam, alprazolam, triazolam and internal standard. Variations in the composition of the mobile phase, pH, ionic strength and the concentration of amine as well as the column temperature were examined. Higher concentrations of acetonitrile have a predominant effect on the separation of benzodiazepines with very little effect with pH variations of mobile phase. Again variation in ionic strength did not make any notable difference. At a column temperature of 55°C the resolution and peak shapes were better than at lower temperatures. However, better separation was achieved at 35°C of alprazolam from clonazepam and triazolam. TCA separation was more susceptible to pH changes and amine concentration. Lower pH decreased the resolution of imipramine and nortriptyline and an increase in the pH decreased the resolution of nordoxepin and doxepin. Use of other amines such as nonylamine did not give a good resolution between the parent tertiary amines and their desmethyl metabolites. At temperatures higher than 35°C there was incomplete resolution between nordoxepin and doxepin and between imipramine and nortriptyline.

CONCLUSION

The method presented permits the analysis of common benzodiazepines and their metabolites along with the commonly used TCAs. In a clinical situation

of concomitant use of these drugs, patient samples can be monitored by a single procedure. We think the simplicity, specificity and sensitivity of this method will have wide applications in clinical settings for routine or immediate analysis. Furthermore, the method may also be useful for the determination of alprazolam at therapeutic concentrations.

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