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# Chromatographic method for the determination of diazepam, pyridostigmine bromide, and their metabolites in rat plasma and urine

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**Abstract**

This study describes a chromatographic method for the determination of diazepam, an anxiolytic drug that is also used as an antidote against nerve agent seizures, its metabolites *N*-desmethyldiazepam, and temazepam, the anti-nerve agent drug pyridostigmine bromide (PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide) and its metabolite *N*-methyl-3-hydroxypyridinium bromide in rat plasma and urine. The compounds were extracted using $C_{18}$ Sep-Pak Vac 3cc (500 mg) cartridges and separated using isocratic mobile phase of methanol, acetonitrile and water (pH 3.2) (10:40:50) at a flow-rate of 0.5 ml/min in a period of 12 min, and UV detection ranging between 240 and 280 nm. The limits of detection for all analytes ranged between 20 and 50 ng/ml, while limits of quantitation were 100 ng/ml. Average percentage extraction recoveries of five spiked plasma samples were 79.1±7.7, 83.5±6.4, 83.9±5.9, 71.3±6.0 and 77.7±5.6, and from urine 79.4±7.9, 83.1±6.9, 73.6±7.7, 74.3±7.1 and 77.6±5.9 for diazepam, *N*-desmethyldiazepam, temazepam, pyridostigmine bromide, and *N*-methyl-3-hydroxypyridinium bromide, respectively. The relationship between peak areas and concentration was linear over the range between 100 and 1000 ng/ml. This method was applied to determine the above analytes following a single oral administration in rats as a tool to study the pharmacokinetic profile of each compound, alone and in combination. 

*Keywords:* Diazepam; Pyridostigmine bromide; *N*-Desmethyldiazepam; Temazepam

## 1. Introduction

Diazepam is an anxiolytic drug [1], and it is used for the treatment of convulsions caused by nerve agents and organophosphate poisoning [2–6]. Pryidostigmine bromide (PB) is used in the treatment of myasthenia gravis patients [7,8], and has been used during the Gulf War to protect soldiers against possible attack by nerve agents such as sarin and soman [9,10]. Diazepam is metabolized into two major metabolites, *N*-desmethyldiazepam and temazepam, which are excreted mainly in urine [11,12], while PB has been reported to be absorbed into plasma, and metabolized mostly into *N*-methyl-3-hydroxypyridinium bromide which is excreted in urine following an oral or intravenous dose in rats [13–17], man [18,19], and dogs [20].

Several methods have been reported for the de-

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termination of diazepam, its metabolites, pyridostigmine bromide, and its metabolite when applied alone in biological matrices. These methods included using high-performance liquid chromatography [12,13,16,21–26], gas chromatography [27–30], gas chromatography–mass spectrometry [31], and radioimmunoassay technique [32].

Military personnel were treated with diazepam or pyridostigmine bromide before and after nerve agent attacks [2,33]. We hypothesized that such treatment might result in toxic interactions. To assess possible pharmacokinetic interactions between these compounds, a method was needed to determine concentrations of the parent and metabolites of both compounds. In this study we present a simple method for simultaneous determination of diazepam, pyridostigmine bromide, and their metabolites in rat plasma and urine using solid-phase extraction coupled with reversed-phase high-performance liquid chromatography.

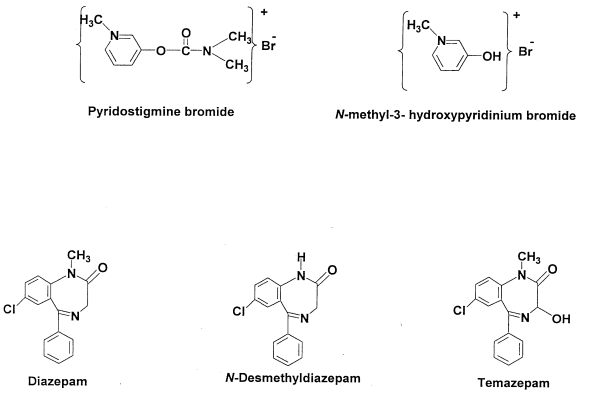


Fig. 1. Structures of diazepam, *N*-desmethyldiazepam, temazepam, pyridostigmine bromide and *N*-methyl-3-hydroxypyridinium bromide.

## 2. Experimental

### *2.1. Chemicals and materials*

Pyridostigmine bromide (98% PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide), diazepam (99% 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodazepin-2-one), *N*-desmethyldiazepam (7-chloro-1,3-dihydro-5-phenyl-3H-1,4-benzodiazepin-2(1H)-one, and temazepam (7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 1) were purchased from Sigma (St. Louis, MO, USA); 95% *N*-methyl-3-hydroxypyridinium bromide was prepared in our laboratory following the method of Somani et al. [18]. Water (HPLC grade) and cetonitrile were obtained from Mallinckrodt Baker (Paris, KY, USA). $C_{18}$ Sep-Pak$^{R}$ cartridges were obtained from Waters (Milford, MA, USA).

### *2.2. Animals*

Rats (Sprague–Dawley) were purchased from Zivic Miller (Zelienople, PA, USA). The animals were kept in plastic metabolic cages. Five rats were treated with a combination of a single oral dose of 5 mg/kg of diazepam and 10 mg/kg of pyridostigmine bromide. (Pyridostigmine bromide dose has been recommended by US Department of Defense, personal communication.) Five untreated control rats were treated with an oral dose of water. The animals were held in metabolic cages to allow for the collection of urine samples. Urine samples were collected from treated and control rats after 12 h of dosing. The animals were anesthetized with halothane and sacrificed by heart exsanguination. Blood was collected immediately after the animals were killed (12 h after dosing) via heart puncture with a heparinized syringe and centrifuged at 2400 rpm for 15 min at 5°C to separate plasma. Urine and plasma samples were stored at −20°C prior to analysis.

### *2.3. Instrumentation*

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E Multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector, and a Waters 2487 Dual λ absorbance detector (Waters). A guard column (Supelco, 2 cm×4.0 mm, 5 μm (Supelco Park, Bellefonte, PA), and a reversed-phase $C_{18}$ column μBondapak™ $C_{18}$ 125 A°, 10 μm, 3.9×300 mm were used (Waters).

### 2.4. Preparation of spiked samples

Urine (1 ml), and plasma (0.5 ml) samples from untreated rats were spiked with concentrations of diazepam, *N*-desmethyldiazepam, temazepam, pyridostigmine bromide and *N*-methyl-3-hydroxypyridinium bromide. Stock solution of 1 mg/ml of each compound was prepared. Then diluted to 2 μl/ml (2000 ng/ml). From this solution, 1, 0.5, 0.4, 0.2, and 0.1 ml was added to a 1-ml urine sample, and the final volume was adjusted to 2 ml. The final concentration of spiked urine samples was 1000, 500, 400, 200, and 100 ng/ml, respectively. For preparation of plasma samples, 500, 250, 125, 63, and 31 μl of the diluted solution (2000 ng/ml) were added to 0.5 ml plasma, and the final solution was adjusted to 1 ml. Spiked and treated samples were acidified with 1 N acetic acid (pH 5.0). Disposable $C_{18}$ Sep-Pak Vac 3cc (500 mg) cartridges (Waters, Milford, MA) were conditioned with 3 ml of acetonitrile, then equilibrated using 3 ml of water prior use. The spiked urine and plasma samples were vortexed for 30 s, centrifuged for 5 min at 1000 *g*, and the supernatant was loaded into the disposable cartridges, then washed with 3 ml of water, and eluted 2 times by 2 ml of methanol, then 2 times by 1 ml of acetonitrile,. The total collection was reduced to 500 μl using a gentle stream of nitrogen, prior to analysis by HPLC.

### 2.5. Accuracy and precision

Intra-day precision and accuracy of the method were determined in plasma and urine samples spiked with the analytes. For intra-day accuracy and precision, plasma and urine samples ($n=5$) spiked at concentration of 100, 200, 400, 500 and 1000 ng/ml were analyzed. The accuracy was determined as the mean of [(concentration found–concentration added)/concentration added]×100%. For the determination of the precision, the coefficient of variation (C.V.) was used.

### 2.6. Chromatographic conditions

A 10-μl solution of plasma or urine residues was injected into HPLC. The mobile phase was isocratic methanol, acetonitrile, water (adjusted to pH 3.20 using 1 N acetic acid) (10:40:50). The flow-rate was 0.5 ml/min. The eluents were monitored by UV detection (240 nm) for diazepam, *N*-desmethyldiazepam, temazepam, and (280 nm) for pyridostigmine bromide, *N*-methyl-3-hydroxypyridinium bromide. The chromatographic analysis was performed at ambient temperature.

### 2.7. Calibration procedures

Five different calibration standards of a mixture of diazepam, *N*-desmethyldiazepam, temazepam, pyridostigmine bromide and *N*-methyl-3-hydroxypyridinium bromide were prepared in acetonitrile. Their concentrations were 100, 200, 400, 500 and 1000 ng/ml. Linear calibration curves were obtained by plotting the average peak areas of five replicates of each chemical against concentration, using GraphPad Prism program for windows (GraphPad Software, San Diego, CA, USA). The standard curves were used to determine recovery of the analytes from plasma and urine samples.

### 2.8. Limits of detection (LOD) and limits of quantitation (LOQ)

Limits of detection and limits of quantitation were determined at the lowest concentration to be detected, taking into consideration a 1:3 and 1:10 baseline noise:calibration point ratio, respectively. The LOQ was repeated 5 times for confirmation.

## 3. Results

### 3.1. Standard calibration curves

The standard calibration curves of average peak area against actual concentration of diazepam, *N*-desmethyldiazepam, temazepam, pyridostigmine bromide, *N*-methyl-3-hydroxypyridinium bromide are shown in Fig. 2. Linearity of the calibration curves for the compounds was achieved at concentrations of 100, 200, 400, 500 and 1000 ng/ml for each compound. Values of the peak area of each con-

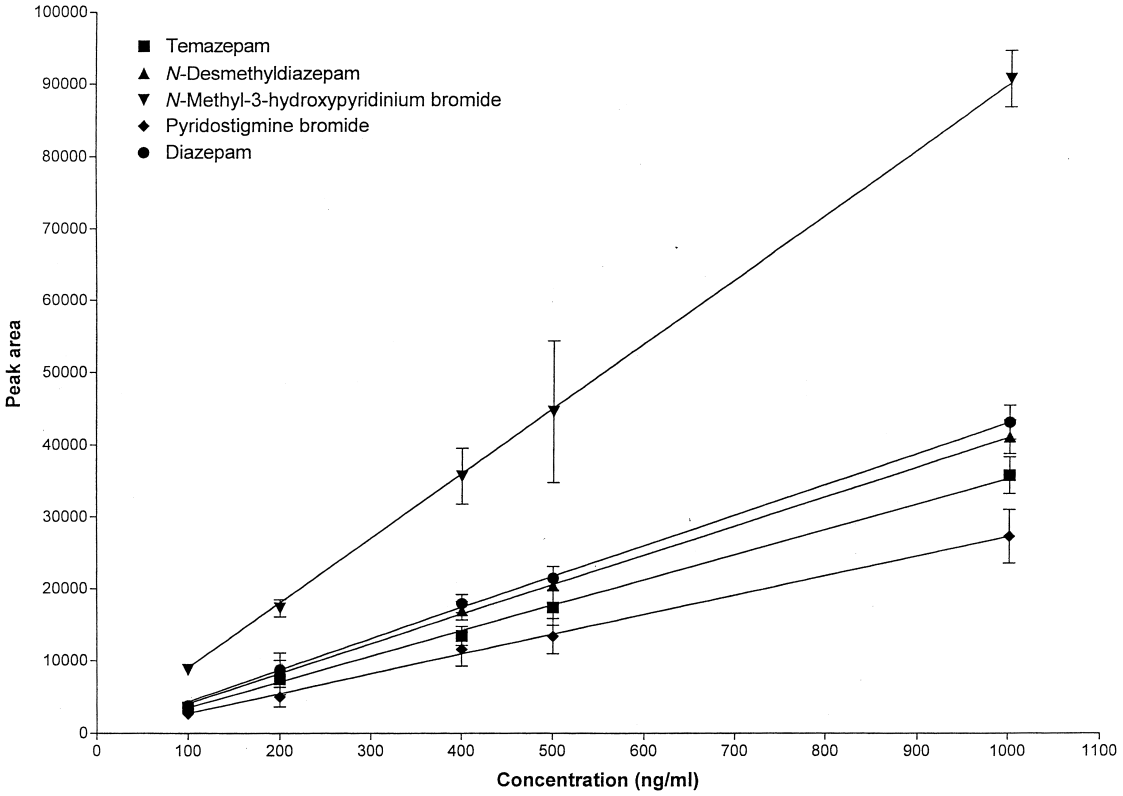


Fig. 2. Standard calibration curves of diazepam, *N*-desmethyldiazepam, temazepam, pyridostigmine bromide and *N*-methyl-3-hydroxypyridinium bromide. Values are expressed as mean±SD of five injections for each concentration.

centration were expressed as mean±SD of five injections ($n=5$).

### 3.2. Chromatogram

Chromatographic profiles were obtained for rat spiked plasma and urine samples with 500 ng/ml of each compound, after solid-phase extraction using $C_{18}$ Sep Pak$^{R}$cartridges under HPLC conditions, described above (Figs. 3 and 4). Retention times were 6.1, 6.8, 7.6, 8.8, and 10.1 min for *N*-methyl-3-hydroxypyridinium bromide, pyridostigmine bromide, *N*-desmethyldiazepam, diazepam, and temazepam, respectively. The total run time was 12 min. The chromatograms show no interference from endogenous substances in plasma and urine samples. This suggests an efficient sample preparation and clean up method using the described solid-phase extraction.

### 3.3. Extraction efficiency and recovery

The average extraction recoveries of diazepam, *N*-desmethyldiazepam, temazepam, pyridostigmine bromide and *N*-methyl-3-hydroxypyridinium bromide were determined at concentrations ranged between 100 and 1000 ng/ml (Tables 1 and 2). Spiked plasma and urine samples were extracted and analyzed for each concentration in five replicates. Average percentage recovery of five spiked plasma samples were 79.1±7.7, 83.5±6.4, 83.9±5.9, 71.3±6.0, and 77.7±5.6, and from urine 79.4±7.9, 83.1±6.9, 73.6±7.7, 74.3±7.1 and 77.6±5.9 for diazepam, *N*-desmethyldiazepam, temazepam,

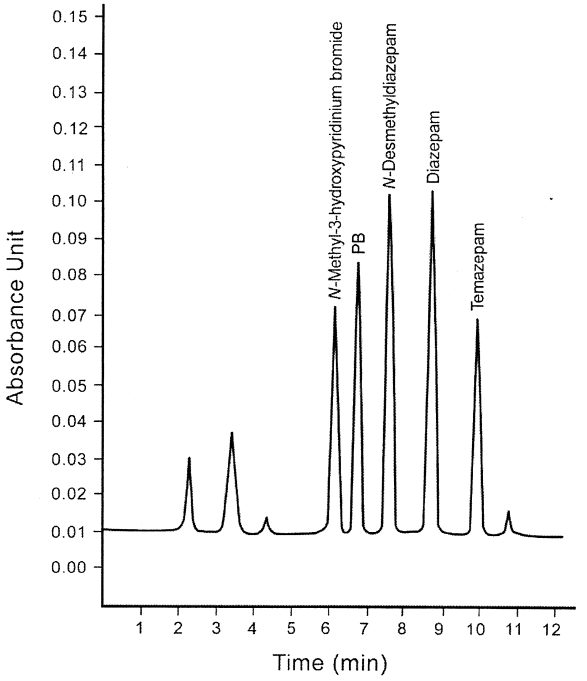


Fig. 3. Chromatogram of spiked plasma sample with 500 ng/ml of *N*-methyl-3-hydroxypyridinium bromide, pyridostigmine bromide, *N*-desmethyldiazepam, diazepam, and temazepam under established HPLC conditions.

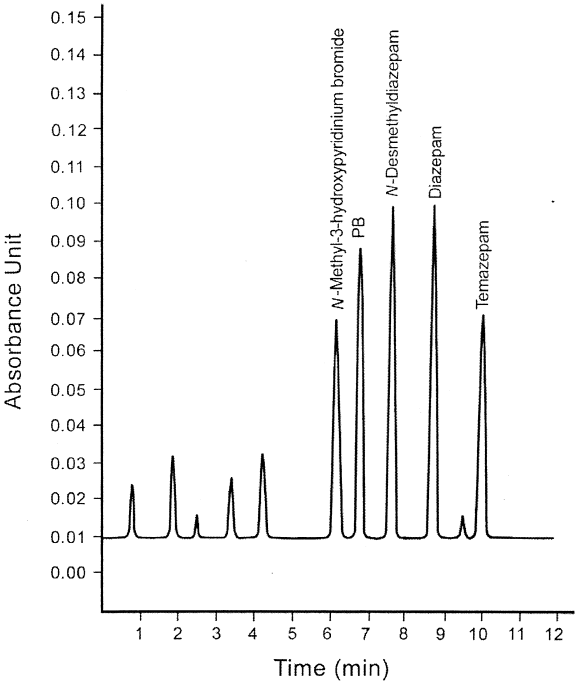


Fig. 4. Chromatogram of spiked urine sample with 500 ng/ml of *N*-methyl-3-hydroxypyridinium bromide, pyridostigmine bromide, *N*-desmethyldiazepam, diazepam, and temazepam under established HPLC conditions.

pyridostigmine bromide, and *N*-methyl-3-hydroxypyridinium bromide, respectively.

### *3.4. Accuracy and precision*

Results of the intra-day accuracy and precision were as described in Section 2. The average accuracy for all concentrations in plasma was 2.1±.6, 4.1±2.0, 2.9±1.2, 4.2±1.4 and 3.1±1.5%, and in urine was 3.6±1.2, 4.8±1.6, 3.0±1.6, 3.9±1.5 and 2.7±1.2% for diazepam, *N*-desmethyldiazepam, temazepam, pyridostigmine bromide and *N*-methyl-3-hydroxypyridinium bromide, respectively. Intra-day precision (C.V.) for plasma and urine samples ranged between 1.8±1.0 and 2.9±1.2%.

### *3.5. Limits of detection (LOD)*

Blank plasma and urine samples from untreated rats were used as references for plasma and urine collections. Limits of detection were calculated from a peak signal-to-noise ratio of 3:1. The resulting detection limits were 50, 50, 50, 50, and 20 ng/ml for diazepam, *N*-desmethyldiazepam, temazepam, pyridostigmine bromide, and *N*-methyl-3-hydroxypyridinium bromide, respectively.

### *3.6. Limits of quantitation (LOQ)*

Limits of quantification from plasma and urine were determined to be 100 ng/ml for diazepam, *N*-desmethyldiazepam, temazepam, pyridostigmine bromide, and *N*-methyl-3-hydroxypyridininum bromide, respectively.

### *3.7. Application of the method to biological samples*

In order to validate the method, it was applied for determination of the parent and their metabolites

Table 1
Percent recovery[a] of diazepam, pyridostigmine bromide and their metabolites from rat plasma

| Concentration (ng/ml) | Diazepam | *N*-Desmethyl-diazepam | Temazepam | Pyridostigmine bromide | *N*-methyl-3-hydroxypyridinium bromide |
|---|---|---|---|---|---|
| 1000 | 82.9±7.1 | 88.2±5.2 | 90.4±3.7 | 72.3±8.6 | 80.1±7.2 |
| 500 | 83.2±6.3 | 86.5±7.2 | 85.1±6.1 | 75.2±4.9 | 82.2±5.6 |
| 400 | 75.6±9.7 | 83.2±6.3 | 87.3±4.8 | 70.4±7.4 | 78.1±6.5 |
| 200 | 78.6±8.4 | 81.9±5.9 | 80.2±6.7 | 71.3±5.2 | 75.6±4.9 |
| 100 | 75.1±7.0 | 77.8±7.6 | 76.7±8.2 | 67.1±3.9 | 72.5±3.8 |

[a] Values are expressed as mean±SD of five replicates.

following combined single oral dose in rats. Following administration of 5 mg/kg of diazepam and 10 mg/kg of pyridostigmine bromide, the rats were sacrificed 12 h after dosing. In plasma diazepam, *N*-methyl-3-hydroxypyridininum bromide, and pyridostigmine bromide were detected. Their levels were 196±32, 926±218, and 572±264 ng/ml for diazepam, *N*-methyl-3-hydroxypyridinium bromide, and pyridostigmine bromide, respectively. *N*-Desmethyldiazepam, and *N*-methyl-3-hydroxypyridinium bromide were identified in rat urine. Their concentrations were 297±53 and 1239±340 ng/ml, respectively.

## 4. Discussion

Recoveries of the parent and metabolites were suitable for application of the method for analysis of treated samples for parent compounds and their metabolites. In our method, recoveries of the analyzed analytes were between 74 and 86%. This range lies within the reported values in the literature, taking into consideration simultaneous analysis of the parent and their metabolites. This is in agreement with a previous study that reported a recovery of diazepam and its metabolites in human plasma and urine to be ranging from 88 to 95% [12]. In another study, Chiba et al. [24] reported that recoveries of diazepam and metabolites from human urine were >87% using liquid–liquid extraction. The low recovery for pyridostigmine bromide might have resulted from the use of solvent system that was not quite suitable for extracting pyridostigmine bromide. However, this system was needed for extracting and analyzing the other chemicals under similar conditions. Also hydrolysis of pyridostigmine bromide from buffer solutions, plasma and blood during the extraction is possible [8]. Hennis et al. [20] reported a recovery of 50% of *N*-methyl-3-hydroxypyridinium from dog plasma and urine, while Chan et al. [34] reported a recovery of 82% of pyridostigmine bromide. Robustness of the method was assessed by determining the intra-day accuracy and precision in plasma and urine samples. The results showed that the method was not affected by dilution as relate to spike concentration analyzed.

The limits of detection reported in this study allow

Table 2
Percent recovery[a] of diazepam, pyridostigmine bromide and their metabolites from rat urine

| Concentration (ng/ml) | Diazepam | *N*-Desmethyl-diazepam | Temazepam | Pyridostigmine bromide | *N*-methyl-3-hydroxypyridinium bromide |
|---|---|---|---|---|---|
| 1000 | 81.9±6.2 | 86.2±6.2 | 77.2±6.2 | 75.6±8.2 | 82.5±5.3 |
| 500 | 83.7±8.2 | 85.3±8.2 | 76.2±7.9 | 78.2±6.9 | 84.2±7.2 |
| 400 | 78.5±11.2 | 83.2±6.3 | 74.6±8.1 | 73.1±7.4 | 75.1±5.9 |
| 200 | 78.4±5.9 | 81.0±6.5 | 69.7±9.1 | 72.3±7.8 | 73.6±6.8 |
| 100 | 74.6±8.2 | 80.0±7.3 | 70.2±7.4 | 72.1±4.7 | 72.8±4.2 |

[a] Values are expressed as mean±SD of five replicates.

the analysis of samples from treated animals. Pyridostigmine bromide dose was determined by the US Department of Defense (personal communication). The maximum dose was determined to be 13 mg/kg. The ability to detect parent compounds and metabolites in plasma and urine after 12 h of dosing is evidence of the method utility. Limits of detection and quantification depended upon the nature of the matrix, rate of application, and method of analysis [12,34]. In a previous study, limits of quantitation of diazepam and its metabolites in human plasma and urine were 10 ng/ml, using HPLC–UV [12], and were 2 ng/ml in human urine using HPLC–UV [24]. Reubsaet et al. [30] reported limits of detection and quantitation of diazepam using solid-phase extraction and gas chromatography of 2.9–129 and 2.9–138 ng/ml, respectively, and it was 3 ng/ml using GC [28]. Hennis et al. [20] reported 50 ng/ml as a limit of detection of *N*-methyl-3-hydroxypyridinium bromide in dog plasma using ion-exchange liquid chromatography, while Miller and Verma [15] reported 2.5 ng/ml as the detection limit of pyridostigmine bromide in tissues using radioimmunoassay, while using HPLC the limit of detection of pyridostigmine bromide in plasma was 10 ng/ml [16].

A simple chromatographic method was developed for separation and analysis of diazepam, pyridostigmine bromide and their metabolites in rat plasma and urine samples. The method could be used to assess distribution of the parent compounds and metabolites in body tissues and fluids following real-life exposure. The advantage of the method is the ability to analyze simultaneously two parent drugs and their metabolites under similar conditions, saving time and expense for sample preparation analysis.

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