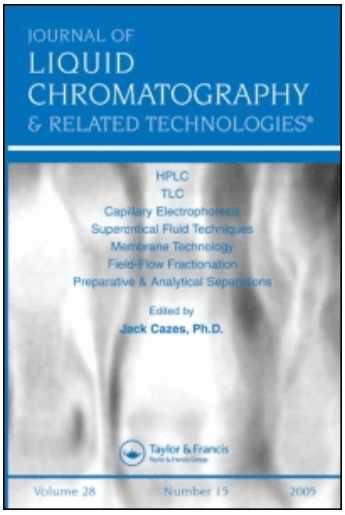

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F. T. Noggle Jr.[a]; C. Randall Clark[b]; Jack De Ruiter[b]
[a] Alabama Department of Forensic Sciences Wire, Alabama [b] Department of Pharmacal Sciences, School of Pharmacy Auburn University Auburn, Alabama

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LIQUID CHROMATOGRAPHIC SEPARATION OF SOME COMMON BENZODIAZEPINES AND THEIR METABOLITES

F. T. NOGGLE, JR.[1], C. RANDALL CLARK[2], AND JACK DE RUITER[2]

[1]*Alabama Department of Forensic Sciences*
Wire Road
Auburn, Alabama 36830
[2]*Department of Pharmacal Sciences*
School of Pharmacy
Auburn University
Auburn, Alabama 36849

ABSTRACT

A series of benzodiazepines commonly encountered in forensic samples were separated using isocratic reversed-phase liquid chromatography. These compounds display a wide range of capacity factors on a C_{18} stationary phase in a pH 8 phosphate buffer and methanol mobile phase. Clorazepate must be analyzed under basic mobile phase conditions to prevent its decomposition to N-desmethyldiazepam. The separation of common parent benzodiazepines such as chlordiazepoxide, diazepam and flurazepam from their corresponding metabolites was achieved under a variety of reversed-phase conditions.

INTRODUCTION

The discovery of the unique CNS depressant activity of the benzodiazepines several decades ago led to development and eventual marketing of more than 20 compounds of this structural class as anxiolytics, sedative/hypnotics, muscle relaxants and anticonvulsants. As a result of therapeutic efficacy, the benzodiazepines have become the most widely prescribed of all psychotropic drugs [1]. However, the antianxiety and sedative effects produced by the benzodiazepines has also contributed to their emergence as substances of abuse in recent years [2,3]. Abusers of the benzodiazepines typically obtain these drugs by diversion from legitimate sources or inappropriate prescribing [4]. Therefore rapid and reliable analytical methods are required to analyze forensic benzodiazepine samples diverted from legal medical use to potential abusers.

Structurally most of the benzodiazepines are characterized by a 1,3-dihydro-5-aryl-2H-1,4-benzodiazepin-2-one ring system as represented by structure **1** (Chart 1). The structural features required for CNS depressant activity include: 1) an electron withdrawing moiety (Cl or NO_2) at the 7-position, 2) a hydrogen atom or relatively small alkyl group at position 1, and 3) a 5-aryl substituent, frequently containing a halogen atom at the ortho (2′) position [5]. A number of modifications of the benzodiazepine ring structure have resulted in compounds with comparable depressant activities. For example, fusion of a heterocyclic ring at positions 1 and 2 such as the triazole of triazolam, estazolam and alprazolam results in enhanced activity (structure **2**) [5].

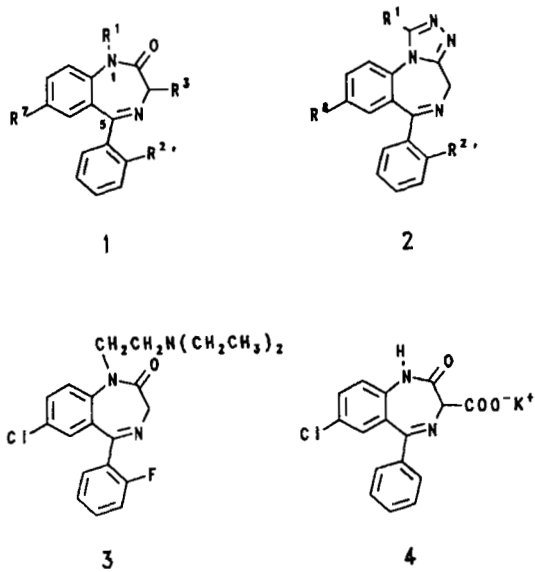


Chart 1. Benzodiazepine Structures.

The differences in lipophilicity, basicity and chemical reactivity between various benzodiazepines are important determinants of therapeutic utility. Most of the benzodiazepines contain only a weakly basic imine moiety (the N_4 atom) and hence have pKas ranging from 1.5 to 3.5 [5]. However, benzodiazepines which contain an N_1-alkylamino moiety such as flurazepam (structure 3) are considerably more basic; flurazepam's side chain nitrogen has a pKa of 8.2. In addition to basicity, the chemical reactivity differences between benzodiazepines are often significant. For example, benzodiazepines such as clorazepate (structure 4) contain a 3-carboxylate moiety which spontaneously decarboxylates in acid. Also, the 1,2-heterocyclic fused benzodiazepines (structure 2) undergo ring opening by imine hydrolysis in the presence of aqueous acid. The primary amine group of the

ring opened species is a relatively strong base having a pKa in the 6.5 range compared to 1.5 for the cyclized parent benzodiazepine [6]. Furthermore, all of the benzodiazepines contain functional groups susceptible to metabolic transformations in mammalian systems [5].

In addition to determining therapeutic profiles, the differences in lipophilicity, basicity and chemical reactivity between various benzodiazepines are also significant factors in the development of chromatographic methods for the analysis of these compounds. The isocratic reversed-phase HPLC methods described in this paper allow for separations based on differences in lipophilicity and control of benzodiazepine reactivity. These methods also allow for separation of parent benzodiazepines from their primary metabolites and therefore may be used for samples excracted from biological samples.

EXPERIMENTAL

Instrumentation. The liquid chromatograph consisted of a Waters Associates Model 6000A pump, U6K injector, Model 440 UV detector with a dual wavelength accessory operated at 254 nm and 280 nm, and a Houston Instruments OmniScribe dual pen recorder. Infrared spectra were recorded on a Perkin-Elmer Model 1710 Fourier transform infrared (FTIR) spectrophotometer. The GC-MS studies were performed using a Hewlett-Packard 5970B mass selective detector. The ionization voltage was 70 eV and the source temperature was 220°C. The samples were dissolved in methanol (1 mg/mL) and 0.5 uL introduced into the mass spectrometer via a gas chromatograph equipped with a 12 m X 0.31 mm i.d. fused silica column with a 0.52 um thickness of OV-1. The column temperature

was programmed from 70°C to 150°C at a rate of 15°C/min and from 150°C to 250°C at a rate of 25°C/min and the split ratio for the GC was 10:1. Solid probe EI and FAB mass spectra were obtained using a VG 70-70 EHF mass spectrometer.

Liquid Chromatographic Procedures. The analytical column was a 30 cm X 3.9 mm i.d. packed with u-Bondapak C_{18} (Waters Associates). The analytical column was preceded by a 7 cm X 2.1 mm i.d. guard column packed with CO:Pell ODS (Whatman). The benzodiazepines, with the exception of clorazepate, were dissolved in HPLC grade methanol and chromatographed using a mobile phase of pH 8 phosphate buffer and methanol (100:75, 500:425 or 500:425) or methanol, water and acetic acid (50:49:1). Clorazepate was dissolved in pH 8 buffer. The pH 8 phosphate buffer was prepared by mixing 5.0 mL of pH 7 stock solution (9.2 g of sodium biphosphate, NaH_2PO_4 in 1 L of double distilled water) and 95.0 mL of stock solution prepared by dissolving 17.86 g of disodium phosphate (Na_2HPO_4) in 1 L of double distilled water. The mobile phase flow rate was 1.5 mL/min and the detector was operated at 0.2 AUFS. A 5 uL aliquot of each benzodiazepine solution was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

The isocratic reversed-phase separation of a series of benzodiazepines is shown in Figure 1. Under the conditions of the separation, these compounds display a wide range of capacity factors. The mobile phase conditions for this separation consisted of pH 8 phosphate buffer and methanol (100:75) and the stationary phase was a covalently-bonded octadecylsilane. The pH of the mobile phase was buffered at 8 in order to prevent the

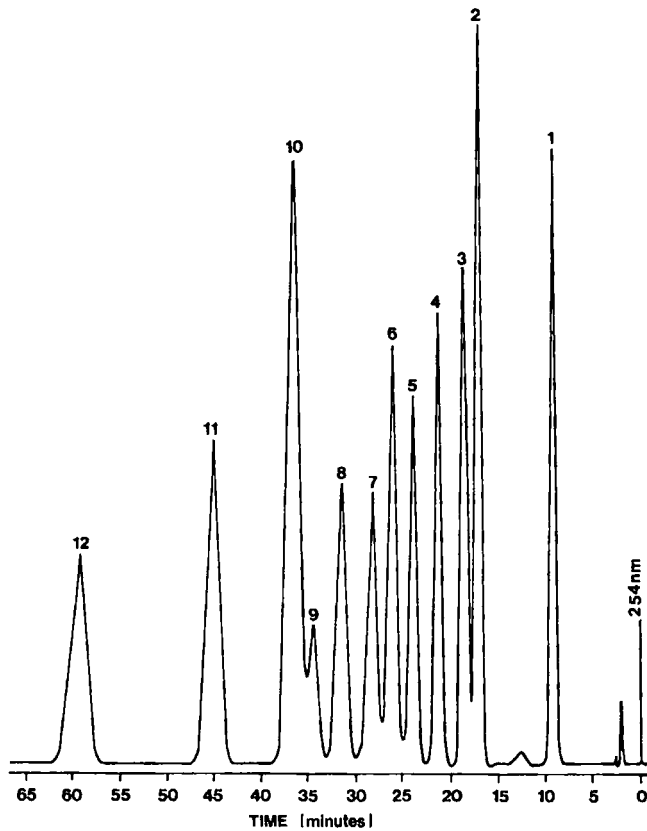


Figure 1. Reversed-phase liquid chromatographic separation of benzodiazepines. Peaks: 1=chlorazepate, 2=nitrazepam, 3=clonazepam, 4=flunitrazepam, 5=estazolam, 6=oxazepam, 7=lorazepam, 8=alprazolam, 9=triazolam, 10=chlordiazepoxide, 11=nordiazepam and 12=diazepam. Solvent system: pH 8 phosphate buffer: methanol (100:75).

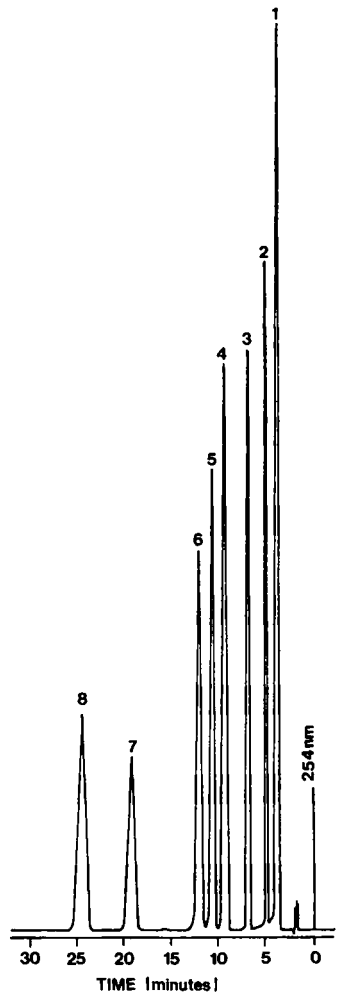


Figure 2. Reversed-phase liquid chromatographic separation of benzodiazepines. Peaks: 1=midazolam, 2=bromazepam, 3=clobazam, 4=nordiazepam, 5=fludiazepam, 6=diazepam, 7=halazepam and 8=prazepam. Solvent system: methanol:water:acetic acid (55:44:1).

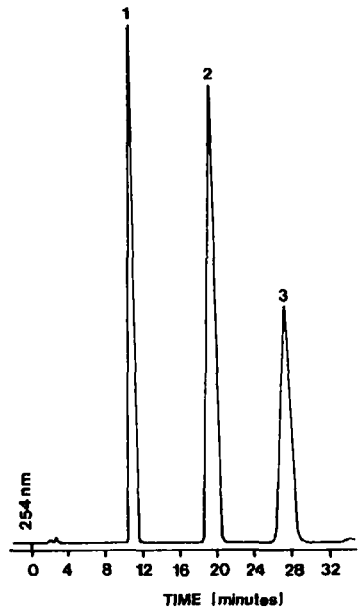
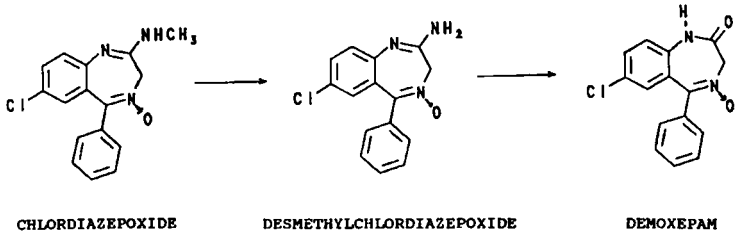


Figure 3. Reversed-phase liquid chromatographic separation of chlordiazepoxides and its metabolites. Peaks: 1=demoxepam, 2=desmethylchlordiazepoxide and 3=chlordiazepoxide. Solvent system: pH 8 phosphate buffer:methanol (500:425).

decomposition of clorazepate by decarboxylation to nordiazepam [7]. The decarboxylation of clorazepate occurs upon protonation of the carboxylate anion and therefore is pH dependent [8]. The ionic clorazepate shows the lowest capacity factor, followed by the other more polar nonionic compounds such as nitrazepam and clonazepam. Clonazepam differs from nitrazepam by the additional 2'-chloro substituent on the 5-phenyl ring and therefore is more lipophilic yielding the higher capacity factor. The very lipo-



Scheme 1. Metabolism of chlordiazepoxide.

philic compounds such as chlordiazepoxide, nordiazepam and diazepam display very high capacity factors eluting at up to 60 minutes in this chromatographic system.

The chromatogram shown in Figure 2 was obtained using a slightly more polar mobile phase consisting of methanol and water made acidic with acetic acid. While the more polar benzodiazepines from Figure 1 were omitted from the chromatogram in Figure 2, the relative solvent strength of the two systems can be compared based on the elution of nordiazepam: approximately 45 minutes in Figure 1 compared to about 9 minutes in Figure 2. A similar comparison can be made based upon the relative elution of diazepam in the two systems. It should be pointed out that acetic acid plays a role in the strength of the mobile phase and is not just a method for controlling the pH of the solution. Both halazepam and prazepam are considerably more lipophilic than diazepam due to the large nonpolar groups attached at the N-1 position of the benzodiazepine nucleus.

The chromatogram in Figure 3 shows the separation of demoxepam, desmethylchlordiazepoxide and chlordiazepoxide (Librium). These three compounds represent a likely combination from biolog-

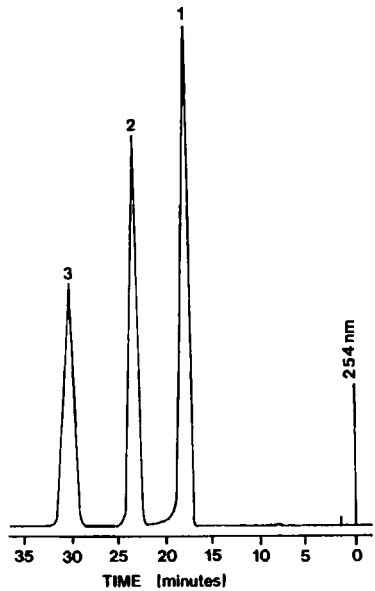
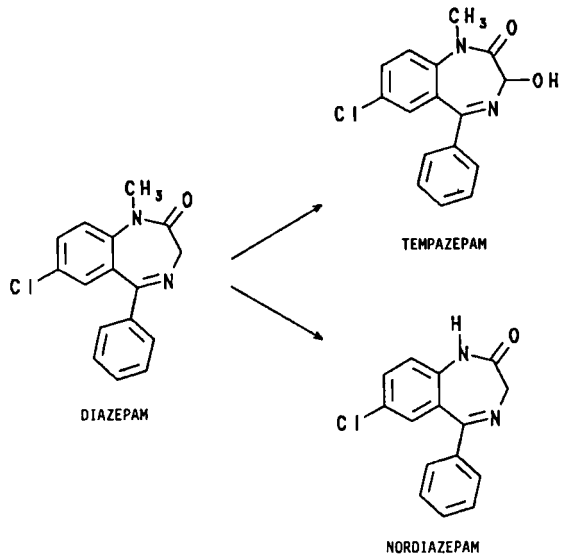


Figure 4. Reversed-phase liquid chromatographic separation of diazepam and its metabolites. Peaks: 1=temazepam, 2=nordiazepam and 3=diazepam. Solvent system: pH 8 phosphate buffer:methanol (500:425).

ical samples since desmethylchlordiazepoxide and demoxepam are metabolites of chlordiazepoxide. This metabolic sequence is illustrated in Scheme 1 and the chromatographic elution of these compounds parallels the additional polarity resulting from metabolism. Chlordiazepoxide is the most lipophilic of the group (peak 3) and demethylation to yield the desmethyl metabolite (peak 2) enhances polarity. Deamination of the 3-amino group to yield demoxepam (peak 1) produces a further increase in polarity. A specific method for the analysis of these compounds should be



Scheme 2. Metabolism of diazepam.

possible in a shorter time using a stronger solvent system based on the high resolution factors illustrated in Figure 3.

A similar situation is shown in Figure 4 where diazepam and two of its major metabolites (Scheme 2) are separated under the same chromatographic conditions as in Figure 3. Diazepam (peak 3) is similar in polarity to chlordiazepoxide and displays a high capacity factor in this chromatographic system. Hydroxylation at the 3-position of the benzodiazepine ring system yields the more polar temazepam (peak 2) and N-demethylation of diazepam further increases the polarity to yield nordiazepam (peak 1).

The chromatogram in Figure 5 illustrates the separation of flurazepam and three of its major metabolites (Scheme 3) in

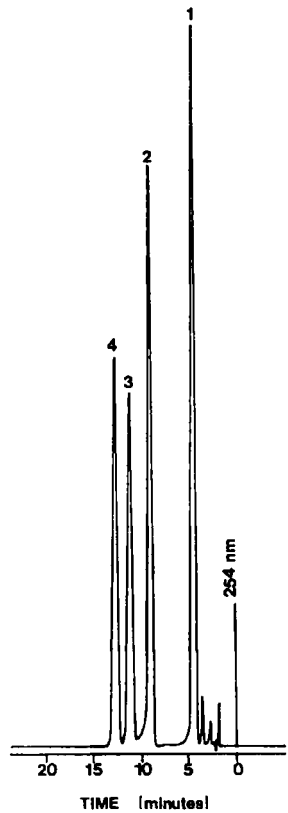
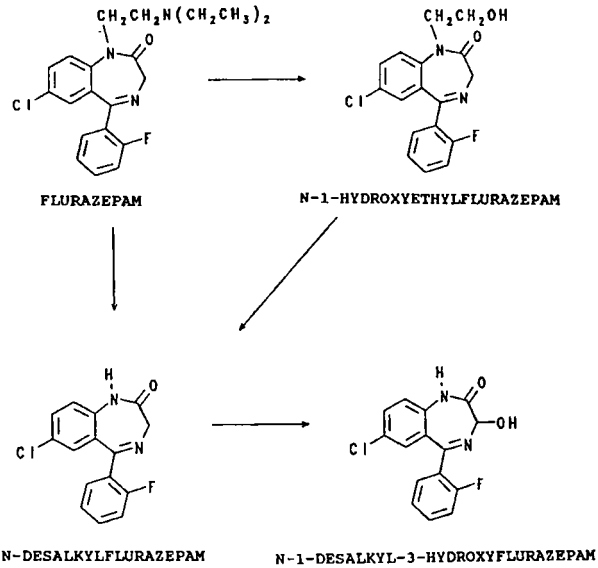


Figure 5. Reversed-phase liquid chromatographic separation of flurazepam and its metabolites. Peaks: 1=flurazepam, 2=N-1-desalkyl-3-hydroxyflurazepam, 3=N-1-hydroxyethylflurazepam and 4=N-1-desalkylflurazepam. Solvent system: methanol:water:acetic acid (50:49:1).



Scheme 3. Metabolism of flurazepam.

humans. In this example, the parent drug elutes first (most polar) since it is likely chromatographed as the protonated amine species. The mobile phase used for this separation is acidic (1% acetic acid) and the side chain diethylaminoethyl moiety is a relatively strong base (pKa approximately 8.2) thus existing in the ionic form under these mobile phase conditions. The benzodiazepine ring nitrogen atoms are significantly less basic (pKa of 3.0 or lower) and upon metabolic deamination of the side chain, the compounds have a higher affinity for the stationary phase hydrocarbons since less protonated species should result. The most polar of the metabolites is N-1-desalkyl-3-hydroxyflurazepam (peak 2), followed by N-1-hydroxyethylflurazepam (peak 3).

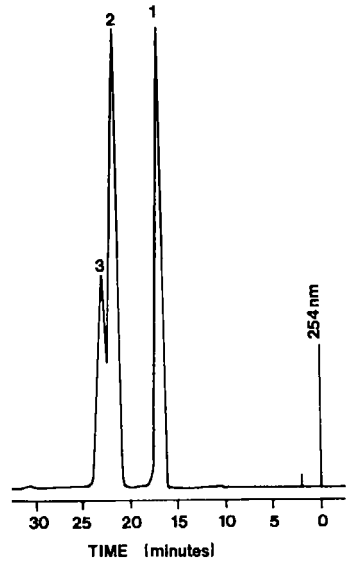
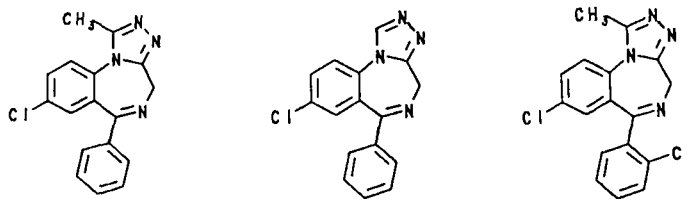


Figure 6. Reversed-phase liquid chromatographic separation of triazolobenzodiazepines. Peaks 1=estazolam, 2=alprazolam and 3=triazolam. Solvent system: pH 8 phosphate buffer:methanol (500:425).

The compound displaying the highest capacity factor in this system is N-desalkylflurazepam (peak 4).

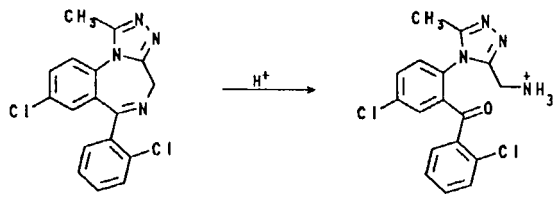
The chromatogram in Figure 6 shows the separation obtained for three of the triazobenzodiazepines using the pH 8 phosphate buffer and methanol (500:425) mobile phase. The structures for these three compounds are shown in Scheme 4. These compounds differ in structure by a methyl group in the triazolo ring and a 2'-chloro substituent on the 5-phenyl ring. These substituents generally enhance the lipophilicity of the parent molecule and the absence of both groups in estazolam (peak 1) yields the



ALPRAZOLAM ESTAZOLAM TRIAZOLAM

Scheme 4. Structures of the triazolobenzodiazepines.

lowest capacity factor. The additional methyl group in the triazolo ring of alprazolam enhances its lipophilic character (peak 2). The partially resolved peak 3 is for triazolam which contains the additional 2'-chloro substituent in the 5-phenyl ring. This chloro substituent should add sufficient lipophilicty to allow for complete resolution from alprazolam. However, triazolam is known to undergo a rapid ring opening reaction to break the 4,5-imine bond in the benzodiazepine ring to yield the triazolobenzophenone (Scheme 5). The exact position of this equilibrium and the contribution of each species to retention are likely factors contributing to the poor resolution of triazolam and alprazolam. The ring opening reaction in triazolam is known to be pH dependent and increases with a decrease in solution pH [6]. The open form of triazolam is stable and easily cyclizes back to the parent compound under various conditions including lower acidity and heat. The thermal cyclization of the open form back to triazolam occurs under GC and solid probe MS conditions. In our studies, GC analysis of either triazolam or its open form gave only one peak whose mass spectrum was that of triazolam. Solid probe MS analysis yielded only the spectrum for triazolam



Scheme 5. Solution hydrolysis of triazolam.

upon probe heating. Only fast atom bombardment (FAB) mass spectrometry techniques allowed for the identification of a molecular ion for the open form of triazolam. This form has a molecular ion 18 mass units higher than triazolam since the hydrolysis essentially adds a molecule of water across the imine bond. Interestingly, the other triazolobenzodiazepines do not appear to hydrolyze at a rate sufficient for participation in the chromatographic separation under these conditions.

In summary, reversed-phase liquid chromatographic methods are very sensitive to the relatively small polarity changes in the various CNS active benzodiazepines and their metabolites. A large group of the more common benzodiazepines of abuse potential can be separated under isocratic conditions. These methods are very useful for the resolution of a parent drug from its various major metabolites.

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