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High-performance liquid chromatographic analysis of benzodiazepines using diode array, electrochemical and thermospray mass spectrometric detection

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ABSTRACT

Twenty benzodiazepines were examined via a combination of high-performance liquid chromatography (HPLC)–diode array–dual electrochemical detection and HPLC–thermospray mass spectrometric detection. Although UV detection at 230 nm resulted in extensive overlap of the benzodiazepines, each compound gave unique UV spectra. Twenty percent of the benzodiazepines tested gave response ratios via oxidation detection, and the values obtained were unique. All compounds except chlorazepate gave MH^+ ions as the base peak via thermospray-mass spectrometric detection with filament off; additional ions were detected for many compounds. Using single ion chromatograms all benzodiazepines can be chromatographically resolved.

INTRODUCTION

Benzodiazepines are a commonly prescribed group of drugs well known for their sedative, hypnotic and anticonvulsant effects. Because of their potential for abuse, their analysis is of forensic interest. Typical submissions to forensic laboratories consist of solid-dosage forms and biological specimens.

Gas chromatography (GC) with flame ionization [1–4], electron-capture [4] and mass spectrometric (MS) detection [2–5] have been employed for the analysis of benzodiazepines. Some of these compounds are difficult to analyze via GC because of thermal decomposition [2–4]; in addition, derivatization may be required because of adsorption of benzodiazepines containing hydroxy groups on active sites of the GC column [5].

High-performance liquid chromatography (HPLC) [6–9] has been used for the direct analysis of these compounds. Analysis via retention time alone using single-wavelength detection which lacks specificity is commonly employed. The use of dual-wavelength detection [9,10], diode array detection [9], electrochemical detection in the reduction mode [11,12] and MS detection [13] have all been used to increase specificity of analysis. Huang *et al.* [13] used a heated pneumatic nebulizer interface with atmospheric pressure ionization mass spectrometry for the analysis of 5 benzodiazepines.

In this paper a multi-detection approach for the HPLC analysis of benzodiazepines is reported. These compounds were examined via a combination of HPLC–diode array–dual electrochemical detection in the oxidation mode and HPLC-thermospray MS detection. The structures for the 20 benzodiazepines examined in this study are shown in Fig. 1.

EXPERIMENTAL

Equipment

Two HPLC systems were employed in this study. For diode array and electrochemical detection, a Series 4 liquid chromatograph (Perkin-Elmer, Norwalk, CT, USA) fitted with an ISS 100 autosampler

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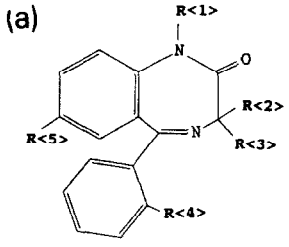
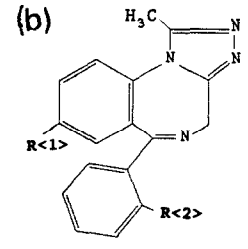


Diazepam	R<5> = Cl	R<1> = CH_3	R<2>,R<3> = H	R<4> = H
Nordiazepam	R<5> = Cl	R<1> = H	R<2>,R<3> = H	R<4> = H
Temazepam	R<5> = Cl	R<1> = CH_3	R<2>,R<3> = H,OH	R<4> = H
Oxazepam	R<5> = Cl	R<1> = H	R<2>,R<3> = H,OH	R<4> = H
Lorazepam	R<5> = Cl	R<1> = H	R<2>,R<3> = H,OH	R<4> = Cl
Prazepam	R<5> = Cl	R<1> = CH_2-▷	R<2>,R<3> = H,H	R<4> = H
Nitrazepam	R<5> = NO_2	R<1> = H	R<2>,R<3> = H,H	R<4> = H
Clonazepam	R<5> = NO_2	R<1> = H	R<2>,R<3> = H,H	R<4>= Cl
Flunitrazepam	R<5> = NO_2	R<1> = CH_3	R<2>,R<3> = H,H	R<4> = F
Halazepam	R<5> = Cl	R<1> = CH_2CF_3	R<2>,R<3> = H,H	R<4> = H
Desalklylflurazepam	R<5> = Cl	R<1> = H	R<2>,R<3> = H,H	R<4> = F



Alprazolam	R<1> = Cl	R<2> = H
Triazolam	R<1> = Cl	R<2> = Cl
Midazolam	R<1> = Cl	R<2> = F

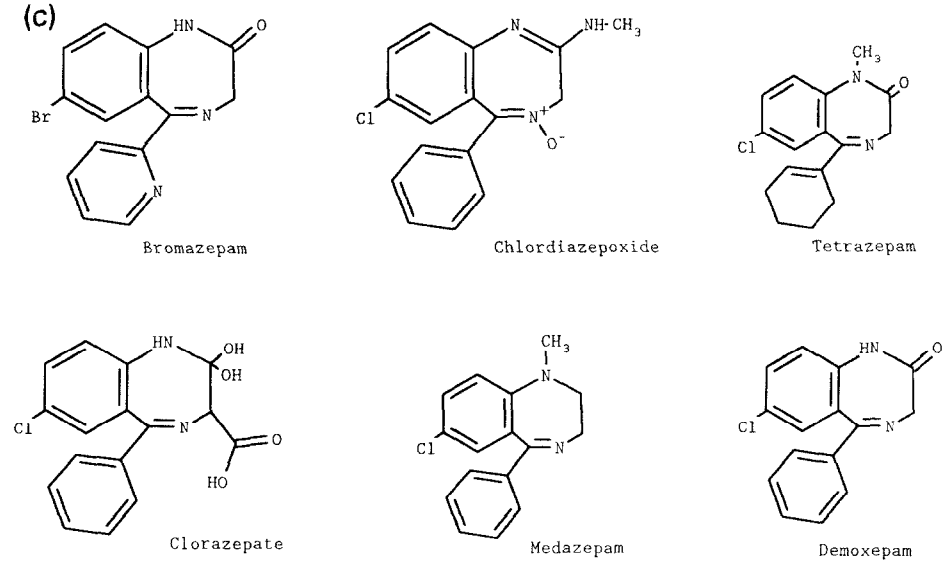


Fig. 1. Structures of benzodiazepines. For explanation of a–c see text.

(Perkin-Elmer); a Partisil ODS-3, 5-μm column (11.0 cm × 4.7 mm I.D.) (Whatman, Clifton, NJ, USA); a 1040 M diode array detection system (Hewlett-Packard, Waldbronn, Germany); and an LC-4B dual amperometric detector fitted with a dual TL-5 glassy carbon electrode cell in the parallel mode, and a Ag/AgCl reference detector with the top half of the cell serving as the auxillary electrode

(Bionalytical Systems, West Lafayette, IN, USA) were used. For UV and thermospray MS detection, a Series 4 liquid chromatograph fitted with a valve injector (Rheodyne, Cotati, CA, USA), a Partisil ODS-3, 5-μm column, an LC85 UV detector (Perkin-Elmer), a switching valve (Rheodyne), an electronically activated switching valve (Valco), a thermospray interface (Vestec Corporation, Houston, TX, USA) and a 4630 quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) were employed. The manual switching valve was used for either flow or column injection while the electronically activated switching valve was used to divert sample either to the thermospray source or to waste.

Materials

Acetonitrile and ammonium acetate were HPLC grade. Water suitable for HPLC analysis and thermospray-mass spectrometric analysis was obtained from a Milli-Q system (Millipore Corporation). All other chemicals were reagent grade. Benzodiazepine drug standards of United States Pharmacopeia/National Formulary quality were employed.

The HPLC mobile phase was internally mixed from solvent reservoirs containing acetonitrile or 0.1 *M* ammonium acetate buffer.

Chromatographic procedure

For HPLC analysis using the diode array and electrochemical detectors 1 mg of benzodiazepine standard was dissolved in 20.0 ml mobile phase prior to a 10-μL injection onto the liquid chromatograph. When analyzing benzodiazepines using UV and thermospray MS detection, 1 mg of standard or 1 mg each of several standards were dissolved in 20.0 ml mobile phase prior to 50–200-μL injections onto the liquid chromatograph.

HPLC mobile phase consisted of 0.1 *M* ammonium acetate–acetonitrile (60:40, v/v) at a flow-rate of 1.3 ml/min.

Mass spectrometric procedure

The column effluent was introduced into the mass spectrometer via a thermospray LC–MS interface. The vaporizer was operated at 220°C while a source block temperature of 250°C was used. The repeller was set at 45 V. The mass spectrometer was operated under full scan acquisition (*m/z* 104–500) with a 3-s scan.

RESULTS AND DISCUSSION

As shown in Table I there is extensive overlap in retention times between the various benzodiazepines examined via HPLC and UV detection at 230 nm. However, all compounds gave unique UV spectra via diode array detection, *i.e.*, no two spectra directly overlapped. Overall a large number of the spectra can be divided into 3 broad categories. Category 1, as shown in Fig. 2a, contains the generalized 1,4-benzodiazepine structure shown in Fig. 1a. For this category, R<5> = Cl, R<4> = H, Cl or F, R<1> = H,CH_3 or cyclopropylmethyl and R<2>, R<3> = H,H or H,OH. All compounds except lorazepam have UV maxima at 228.5 nm (lorazepam 232.5 nm). In addition, all compounds have UV maxima at 318 nm. Category 2 spectra, as shown in Fig. 2b, also contain the generalized 1,4-benzodiazepine structure shown in Fig. 1a. For this category R<5> = NO_2, R<4> = H,Cl or F and R<1> = H or CH_3. Flunitrazepam and clonazepam have UV maxima at 252.5 nm while nitrazepam has a UV maximum at 258.5 nm. In addition, all 3 compounds have UV maxima at 310.5 nm.

TABLE I

RETENTION DATA AND DUAL ELECTROCHEMICAL RESPONSE RATIOS FOR BENZODIAZEPINES

Compound	Retention time (min)	Area 1.1 V/ Area 1.0 V
Clorazepate	1.2, 1.3	
Bromazepam	2.6	
Demoxepam	2.7	
Oxazepam	3.4	10.5
Lorazepam	3.8	13.2
Chlordiazepoxide	4.0	63.8
Nitrazepam	4.1	
Clonazepam	4.5	
Alprazolam	4.6	
Triazolam	4.8	
Desalkylflurazepam	4.9	
Nordiazepam	5.4	
Temazepam	5.5	
Flunitrazepam	5.8	
Diazepam	8.2	
Midazolam	11.5	
Tetrazepam	13.5	
Prazepam	17.2	
Halazepam	17.5	
Medazepam	23.1	1.38

Category 3, as shown in Fig. 2c, contain the generalized benzodiazepine structure shown in Fig. 1b. For this category R<1> = Cl and R<2> = H,Cl or F. The spectra of the remaining benzodiazepines are shown in Fig. 2d. Clorazepate injected via the autosampler gives 2 peaks with identical UV spectra near the solvent front, which suggests that peak splitting may be occurring. The cause of this phenomena is not clear.

Using dual electrochemical detection in the parallel mode at oxidation potentials of 1.0 and 1.1 V, certain benzodiazepines were found to be electrochemically active. As shown in Table I, the values obtained were unique. The electrooxidation mechanism of chlordiazepoxide, lorazepam, medazepam and oxazepam have been previously examined [14,15] by means other than HPLC. The use of dual electrochemical detection in series with diode array detection results in a significantly higher degree of specificity of detection.

Caution must be excercised in using electrochemical ratios because of potentially poor day-to-day reproducibility [16]. Changes in ratios can result from drifts in the reference-electrode redox potential, from over-potential effects or from variations in temperature. The injection-to-injection reproducibility is much better. The dual electrochemical ratio for medazepam on a given day did not change by more than 5% over a 7-h period. Therefore, suspected compounds should be confirmed by standard injection on the same day of analysis.

In addition, all the benzodiazepines analyzed in

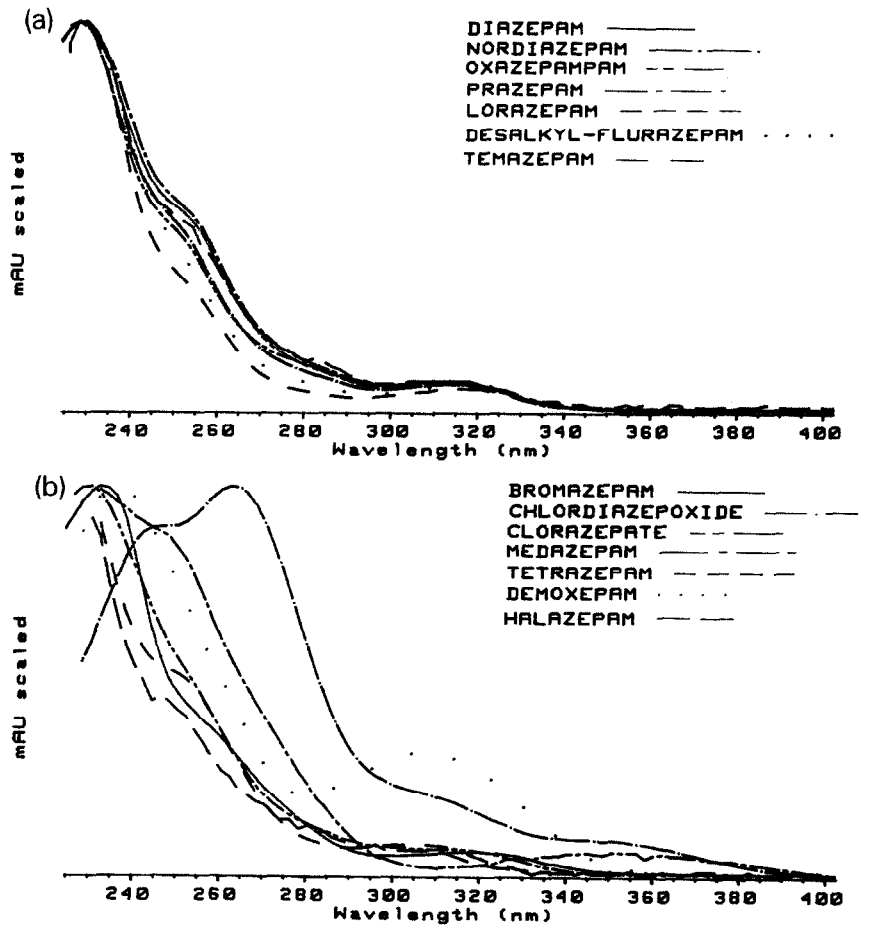


Fig. 2.

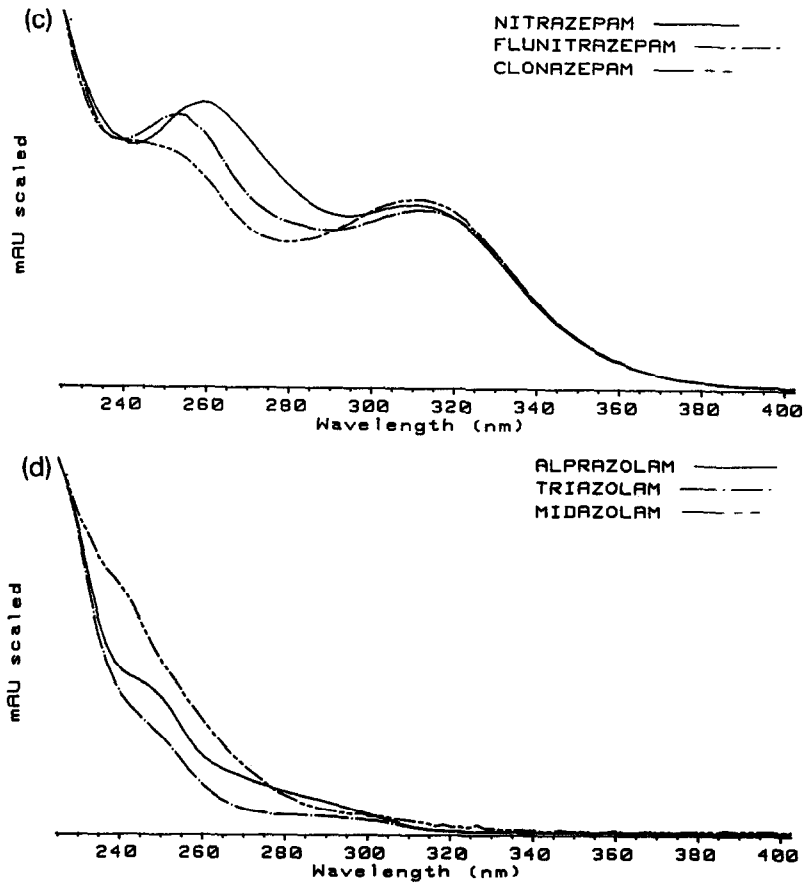


Fig. 2. UV spectra of benzodiazepines. For explanation of a–d see text.

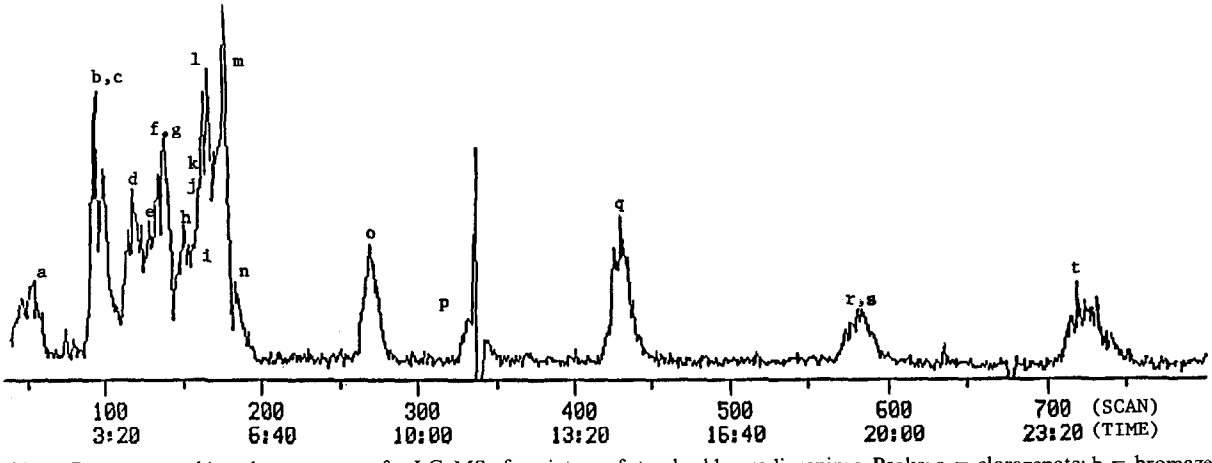


Fig. 3. Reconstructed ion chromatogram for LC–MS of a mixture of standard benzodiazepines. Peaks: a = clorazepate; b = bromazepam; c = demoxepam; d = oxazepam; e = lorazepam; f = chlordiazepoxide; g = nitrazepam; h = clonazepam; i = alprazolam; j = triazolam; k = desalkylflurazepam; l = nordiazepam; m = temazepam; n = flunitrazepam; o = diazepam; p = midazolam; q = tetrazepam; r = prazepam; s = halazepam; t = medazepam. Times scale in min:s.

this study would be expected to give electrochemical response ratios in the reduction mode [12] due to the presence of a diphenylimine moiety. This is in contrast to the higher specificity of detection in the oxidation mode, where only 20% of the compounds were electrochemically active. However, analysis in the reduction mode requires a slow reflux of the eluent to deoxygenate the mobile phase and the presence of coulometric detector prior to a mercury electrode for high-sensitivity work [12].

HPLC–MS, as shown in Fig. 3, was also investigated for the analysis of benzodiazepines. A summary of the spectral data for the thermospray-mass spectrometric detection of the benzodiazepines obtained with filament off is shown in Table II. In all instances except clorazepate, the $[M+H]^+$ ion is the base peak. For clorazepate, the base peak is the $[M+H-CO_2-H_2O]^+$ ion. This ion is formally identical to the MH^+ ion derived from nordiazepam. It should be noted that although nordiazepam and clorazepate give very similar mass spectra, their retention times as shown in Table I are vastly different and thus these compounds are chromatographically distinct. It has been shown that at a slightly basic pH (mobile phase pH for this study 6.8), the decarboxylation of clorazepate to nordiazepam is delayed [6]. For most of the benzodiazepines the only ion of any consequence is $[M+H]^+$. Demoxepam and chlordiazepoxide, both containing N-oxides, exhibit a small relative abundance of $[M+H-O]^+$ ions. In addition, oxazepam and lorazepam, both containing H and OH groups at the <2> and <3> positions, exhibit a relatively high abundance of $[M+H-H_2O]^+$ ions. It is unclear at this point why temazepam, which also contains H and OH groups at the <2> and <3> positions, does not also show this loss. It is of interest to note that most of the benzodiazepines contain either a single Cl, Br or two Cl atoms. Because of natural isotopic abundances of these halogens, additional characteristic mass fragment ions are observed which increase the specificity of the mass spectral data.

Besides providing characteristic mass fragment

TABLE II

MASS SPECTRA (THERMOSPRAY IONIZATION) OF BENZODIAZEPINES

Relative abundance in parentheses.

Benzodiazepine	*m/z*		
	$[M+H]^+$	$[M+H-O]^+$	$[M+H-H_2O]^+$
Clorazepate	271(100)[a]		
Bromazepam	316(100)		
Demoxepam	287(100)	271(1)	
Oxazepam	287(100)		269(56)
Lorazepam	321(100)		303(55)
Chlordiazepoxide	300(100)	284(1)	
Nitrazepam	282(100)		
Clonazepam	316(100)		
Alprazolam	309(100)		
Triazolam	343(100)		
Desalkylflurazepam	289(100)		
Nordiazepam	271(100)		
Temazepam	301(100)		
Flunitrazepam	314(100)		
Diazepam	285(100)		
Midazolam	326(100)		
Tetrazepam	289(100)		
Prazepam	325(100)		
Halazepam	353(100)		
Medazepam	271(100)		

[a] $[M+H-CO_2-H_2O]^+$

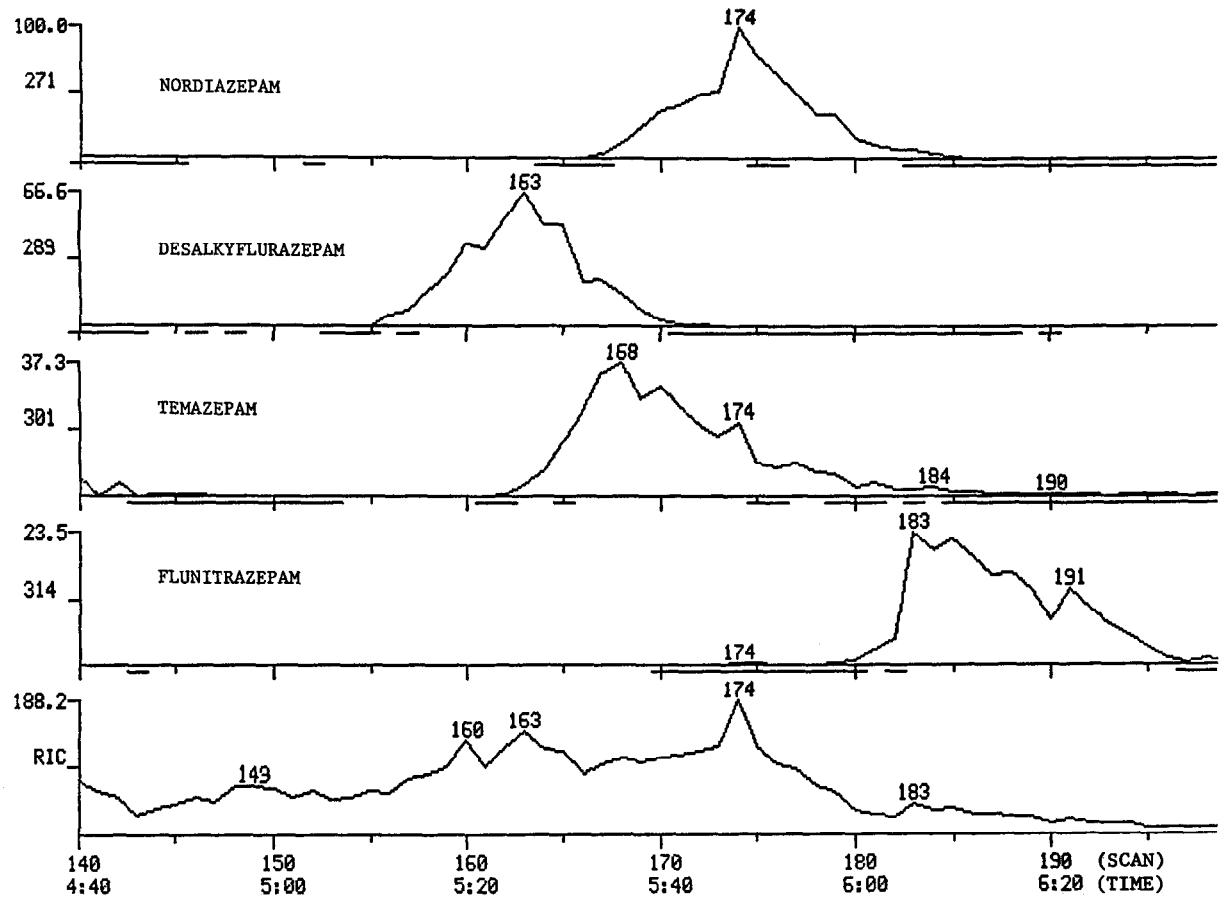


Fig. 4. Ion chromatograms and reconstructed ion chromatogram for LC–MS of a mixture of standard benzodiazepines. Time scale in min:s.

ions, one of the great utilities of HPLC–thermospray MS detection is the ability to perform single-ion monitoring (SIM) to deconvolute complex chromatograms. This capability is illustrated in Fig. 4, where 4 benzodiazepines that overlap in a reconstructed ion chromatogram can be resolved using SIM. In fact, all 20 benzodiazepines can be chromatographically resolved using SIM.

CONCLUSION

The combination of complimentary diode array, dual electrochemical and thermospray MS detection greatly increases the specificity of HPLC analysis of benzodiazepines. This technique is potentially applicable to both solid-dosage forms and biological specimens.

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