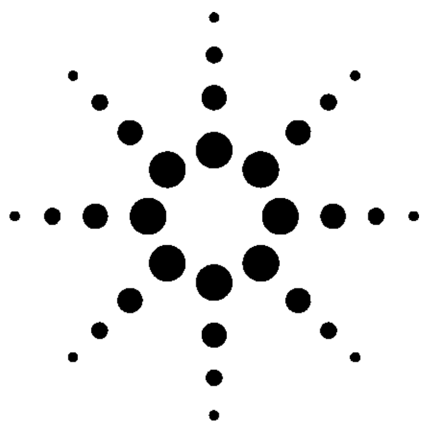

Analysis of Benzodiazepines in Blood by LC/MS/MS

Application Note

Forensic Toxicology

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Abstract

A sensitive and selective method for the simultaneous screening and identification of 13 benzodiazepines and 5 metabolites in human blood using the Agilent LC/MSD Trap is described. The method uses liquid-liquid extraction followed by reverse-phase LC/MS/MS (liquid chromatography/tandem mass spectrometry). The technique is suitable for screening analysis and high-confidence identification of the analytes at their lowest reported dosage concentrations using only 500 µL of blood and the original model ("Classic") of the Agilent LC/MSD Trap. The method has been successfully applied in forensic cases involving low concentrations of benzodiazepines.

Introduction

The analysis of benzodiazepines is of great interest to forensic toxicologists.

Screening of these compounds has been problematic since immunoassays are often not sufficiently specific or sensitive enough for low-dosage benzodiazepines, especially in blood. Benzodiazepines have been analyzed using gas chromatography/nitrogen phosphorus detector (GC/NPD) [1], gas chromatography/electron capture detector (GC/ECD) [1], and gas chromatography/mass spectrometry (GC/MS) [2, 3]. Many benzodiazepines are polar and thermally labile, making them diffi-cult, if not impossible, to analyze with GC or GC/MS without derivatization. Some of the compounds cannot be derivatized for improved chromatographic behavior.

Screening for benzodiazepines can also be carried out using HPLC with UV detection [4], but this technique lacks both the sensitivity and specificity required for forensic applications. Furthermore, some of the newer benzodiazepines, like flunitrazepam, have much lower usage dose ranges and faster clearance, and therefore require identification at lower levels.

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Liquid chromatography/mass spectrometry (LC/MS) is ideally suited for this family of compounds because the technique does not require derivatization, thereby saving time, expense, and experimental difficulty. This class of compounds also ionizes well in either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) modes, and therefore can be easily detected at µg/mL levels and below. A large number of benzodiazepines and related substances have also been analyzed using the Agilent single quadrupole liquid chromatography/mass selective detector (LC/MSD) in APCI mode and selected ion monitoring (SIM) mode [5]. The full scan sensitivity of the Agilent LC/MSD Trap allows for both identification/confirmation and quantitation in a single analysis, and the multiple reaction monitoring (MRM) mode provides for more specific detection in a complex matrix such as blood.

In this work 13 benzodiazepines and 5 metabolites [Table 1] are analyzed in a single run of approximately 20 minutes. This application note is derived from work carried out in the Australian laboratory and previously published in reference 6.

Table 1. Compounds Analyzed

Benzodiazepines	Metabolites
Alprazolam	–
Bromazepam	–
Clobazam	–
Clonazepam	7-aminoclonazepam
Diazepam	Nordiazepam
Flunitrazepam	7-aminoflunitrazepam
Flurazepam	N-desalkylflurazepam
Lorazepam	–
Midazolam	–
Nitrazepam	7-aminonitrazepam
Oxazepam	–
Temazepam	–
Triazolam	–
Prazepam (internal standard)	

Experimental

Sample Preparation

Reference solutions of each analyte were combined, diluted in water and added to drug-free blood, along with the internal standard, to prepare calibrators at low, medium and high dose concentrations of each drug. Typical low and high concentrations are shown in Table 2. The extraction method is the same as used for screening of these drugs by GC/ECD and GC/MS, with any derivatization step omitted and the final residue dissolved in the initial mobile phase rather than in a typical GC solvent.

Table 2. Concentration Ranges for Analytes (mg/L or µg/mL)

Benzodiazepine/ metabolite	Low dosage	High dosage
Alprazolam	0.01	0.1
Bromazepam	0.08	0.2
Clobazam	0.1	1
Clonazepam	0.03	0.08
7-aminoclonazepam	0.03	0.14
Diazepam	0.05	2
Nordiazepam	0.05	2
Flunitrazepam	0.005	0.02
7-aminoflunitrazepam	0.002	0.02
Flurazepam	0.0005	0.028
N-desalkylflurazepam	0.04	0.15
Lorazepam	0.02	0.3
Midazolam	0.08	0.25
Nitrazepam	0.03	0.2
7-aminonitrazepam	0.03	0.2
Oxazepam	0.15	2
Temazepam	0.3	1
Triazolam	0.002	0.02

To 0.5-mL blood in a glass screw-top tube was added 50 µL of freshly prepared internal standard working solution (5 µg/mL in water). To this tube was added 1.75 mL of 4.5% ammonia solution and 10 mL of 1-chlorobutane, and the contents rolled on a mechanical mixer for 10 minutes. After centrifuging, the solvent was drawn off, transferred to a clean glass tube and evaporated to dryness in a Jouan centrifugal evaporator. The residue was dissolved in 100 µL of mobile phase.

LC/MS/MS Instrumentation

The LC/MS/MS system used in this work consisted of an Agilent 1100-series vacuum degasser, binary pump, autosampler, thermostatted column compartment, diode array detector (DAD) with microflow cell, an LC/MSD Trap “Classic” (model

G2445A, equivalent in performance to the current VL model), and a G1947A APCI source. Complete system control and data analysis was provided by the Agilent LC/MS ChemStation.

LC/MS Method Details

LC Conditions

Column:	Agilent ZORBAX Eclipse XDB-C8, 150 × 4.6 mm, 5 µm (p/n 993967-906)
Mobile phase:	A = 20 mM ammonium formate, pH 9 in water
	B = methanol
Flow rate:	0.7 mL/min
Gradient:	60% B until 15 min 100% B at 16 min 100% B to 21 min Post-time (column re-equil): 5 minutes (sufficient for reproducible retention times)
Injection vol:	5 µL

MS Conditions

Source:	Positive APCI
Nebulizer:	60 psig
Vaporizer:	400 °C
Drying gas flow:	5 L/min
Drying gas temp:	350 °C
V_{cap}:	3000 V
Corona:	4 µA
Scan:	*m/z* 150–400
Averages:	2
SPS settings:	Target mass *m/z* 300
	Compound stability 60% (Skim 1: 24 V, Cap exit offset: 69 V)
	Trap drive 100% (resulting value 27)
Precursor isolation width:	2.5 amu
Cutoff:	45% (113–175 *m/z* for these compounds)
MRM:	Eight time segments as shown in Table 3

Results and Discussion

Discussion

Both ESI and APCI were evaluated for the analysis of these compounds. Generally, both gave good sensitivity at the low dosage levels needed for the method. However, both flurazepam and lorazepam showed poor response in ESI with the mobile phase which gave the best chromatographic separation in a reasonable time. Because APCI has also been demonstrated to be less susceptible to matrix suppression effects than ESI, and because both flurazepam and lorazepam showed better sensitivity with APCI, it was chosen as the preferred ionization method.

Various mobile phase compositions were evaluated, with the objective being a best compromise among a simple LC method, reasonably short run time, and maximizing chromatographic resolution of the analytes. The choices included isocratic and gradient methods using either 20-mM ammonium formate at pH 3 or pH 9, or 0.1% formic acid. Once APCI was chosen as the preferred ionization method, methanol became the organic component of choice over acetonitrile, as it provides better sensitivity in APCI and does not build up carbon deposits on the APCI corona needle. Acetonitrile has higher gas phase basicity than methanol; since ionization in APCI occurs in the gas phase (rather in the liquid phase as in ESI), acetonitrile can compete with analyte molecules for the available protonation “work”.

The basic aqueous phase with methanol as the organic component was found to give the best separation, chromatographic peak shape, and sensitivity for this analysis. The ZORBAX Eclipse C8 column is stable at the effective pH of this mobile phase for extended periods of time. The C8 stationary phase proved to be sufficiently retentive even for the polar metabolites; C18 would have required longer run times.

Reconstituting the sample extracts in the initial mobile phase, a recommended practice for HPLC, was found to give better peak shapes and therefore better sensitivity than using simply methanol/water. This is especially important for the early-eluting polar analytes.

Prazepam was chosen as a suitable internal standard because of its structural similarity to the other analytes and because it is not prescribed in Australia.

The optimum fragmentation amplitude for each analyte was determined by infusing a 5-µg/mL solution of a single compound into the MS/MS, and increasing the fragmentation amplitude until the precursor ion intensity was reduced to 10%–20% of its major product ion response. The resulting value was used in the data acquisition method as shown in Table 3.

Table 3. Data Acquisition Parameters for MRM

Group number [min]	Benzodiazepine/ metabolite	RT (min)	Precursor ion $[M+H]^+$	Major Product ion (*m/z*)	Fragmentation amplitude (V)	Fragmentation width (*m/z*)
1 [1.00–4.00]	7-aminonitrazepam	2.6	252	224	2.00	10
	7-aminoclonazepam	2.8	286	250	2.50	10
	7-aminoflunitrazepam	3.1	284	264	1.88	10
2 [4.00–5.70]	Bromazepam	5.3	316	288	1.92	10
3 [5.70–6.70]	Clonazepam	6.1	316	270	2.00	10
	Nitrazepam	6.2	282	236	1.86	10
	Flunitrazepam	6.3	314	268	1.90	10
4 [6.70–8.80]	Clobazam	7.3	301	259	3.37	40
	Flurazepam	7.6	388	315	2.60	40
	Triazolam	7.8	343	308	3.57	40
	Alprazolam	8.3	309	281	4.67	40
	Lorazepam	8.3	321	275	2.98	40
	Oxazepam	8.6	287	241	3.32	40
5 [8.80–11.10]	N-desalkylflurazepam	9.2	289	261	4.57	40
	Temazepam	9.6	301	255	3.72	40
6 [11.10–13.00]	Nordiazepam	12.1	271	243	1.88	10
7 [13.00–17.00]	Diazepam	13.9	285	257	1.90	10
	Midazolam	14.9	326	291	2.05	10
8 [17.00–21.00]	Prazepam	19.4	325	271	1.90	10

In spite of the extremely fast scan speed of the Agilent LC/MSD Trap (up to 26,000 amu/s for current models), configuring the MRM method to repetitively step through all of the precursor ions and MS/MS scans for all 18 analytes plus internal standard would result in unacceptably long cycle times and insufficient data points per second to properly define and quantitate each analyte. Therefore the analysis is set up in time-programmed segments in which MRM occurs for a few analytes at a time in a given portion of the chromatogram, as shown in Table 3.

A time delay may be observed during switchover between groups in which a large number of analytes are being monitored if using manual Fragmentation Cut-off values. This delay can be averted by setting the Cut-off selection in all groups (found under Fragmentation in the MS/MS section of the Trap Control window) to an appropriate percentage value of the precursor mass, or to "Default", rather than setting a manual value for each analyte. The original version of this method used a manual cut-off value of 150 *m/z* for each MRM, which resulted in such a delay. Improved sensitivity for these analytes is obtained by setting the Cut-off to 45% rather than the default 27%. This essentially causes the trap to focus on the *m/z* regions where the major product ions of these analytes are found, not trapping lower mass ions less useful for identification and quantitation.

The results of optimization of MS/MS acquisition can be seen by examining the chromatograms in Figure 1 which shows the overlaid principal product ion chromatograms of all the analytes for the analysis described here.

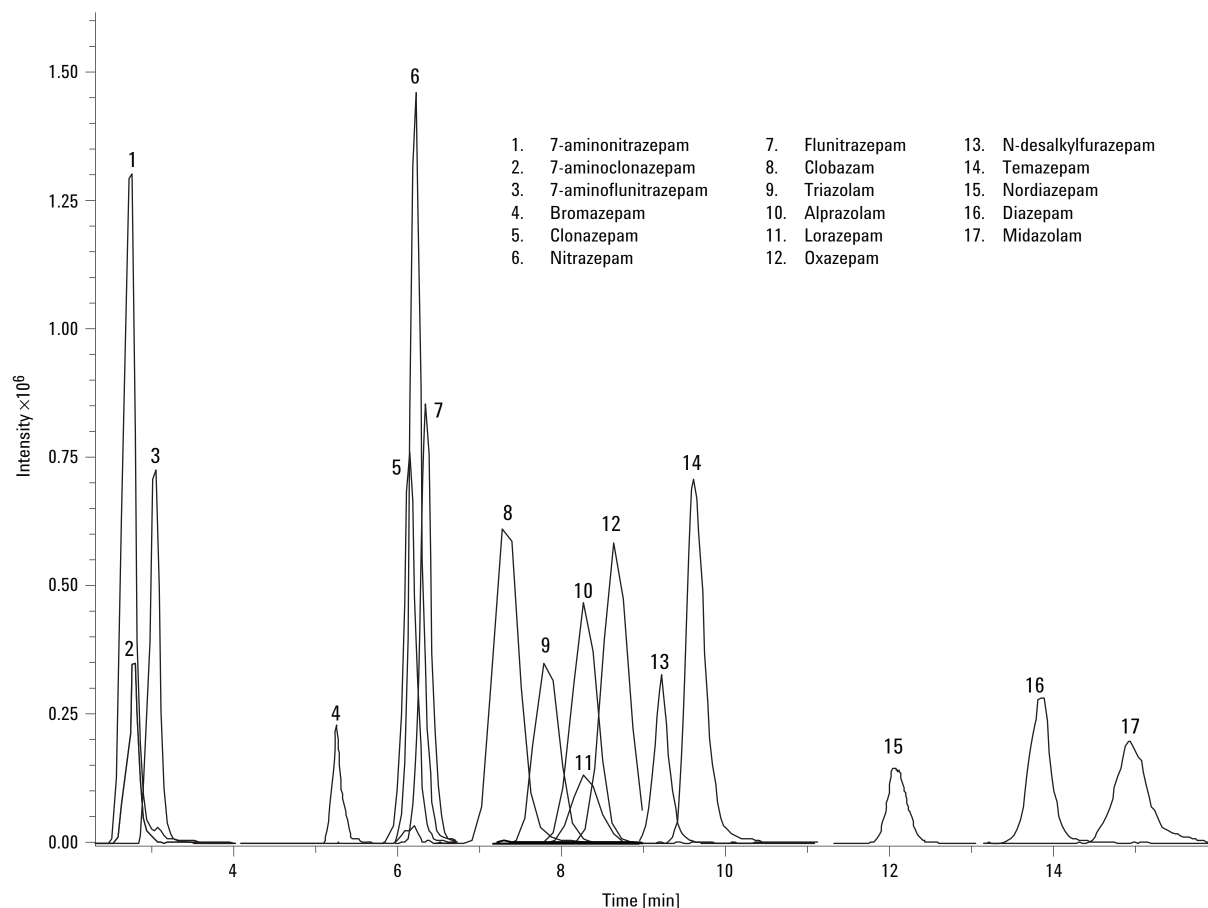

Figure 1. Overlay of principal product ion chromatograms.

The first group, which extends from 1.0 to 4.0 minutes, includes the MRM analysis of three compounds, which practically co-elute. The second group only covers one compound, while the third group covers another three compounds. The fourth group covers six compounds, which reduces the effective duty cycle for analyzing each compound. Whether or not the compounds are eluting, the MRM is cycling through six different precursor ions. However, the chromatographic peak widths are large enough that sufficient data points are produced for each analyte.

The product ion spectra are acquired in full scan mode which allows the MS/MS spectra to be added to a user library as an automated aid to screening and compound identification. Such a library is in use in this laboratory and others in the toxicology field. For screening a larger number of drugs, the AutoMS^n mode of analysis can produce both MS and MS/MS (even MS/MS/MS, or MS^3) spectra which can be searched against a library of spectra created using identical MS^n parameters from authentic standards.

Switching on the SmartFrag option may offer some advantages for qualitative analyses where spectral reproducibility and an abundance of product ions are primary concerns. Switching off the SmartFrag option to maximize the intensity of fewer product ions will assist low level quantitative analyses.

Figures 2–8 show the structures and MS/MS spectra for the analytes under the conditions of the method. The spectra are grouped to illustrate some common losses and the interesting change in fragmentation behavior that can occur with a relatively small change in structure.

Figure 2 shows MS/MS spectra of the chlorine-containing midazolam and triazolam which lose chlorine under these conditions. Triazolam with the five-membered nitrogen-containing ring also shows a major fragment ion corresponding to ring-opening and loss of diatomic nitrogen.

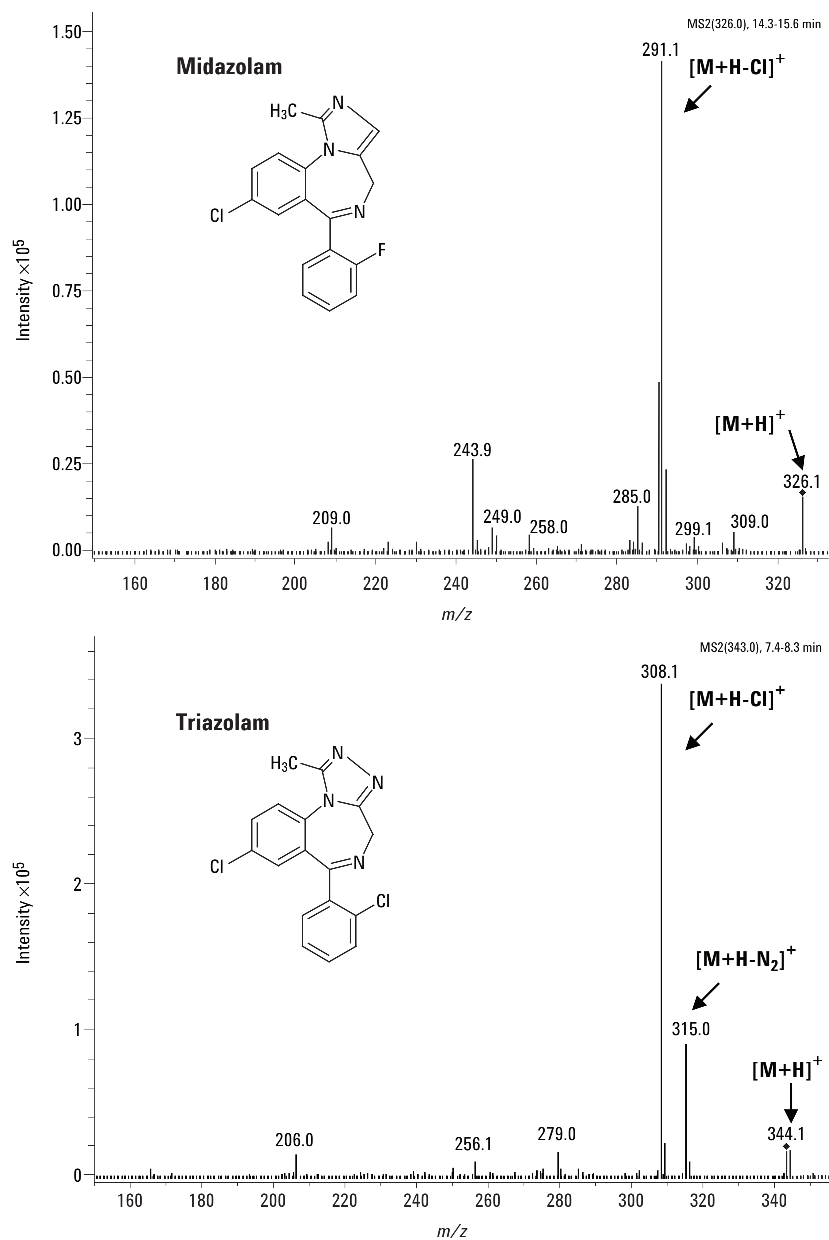

Figure 2. Structures and MS/MS spectra of midazolam and triazolam.

Figure 3 shows three benzodiazepines whose base peak in the MS/MS spectrum corresponds to loss of NO_2. The figure also shows the spectra of the metabolite of each parent drug in which the nitro group has been reduced to an amino group. In each case, the structural change gives rise to a different fragmentation: 7-aminoclonazepam loses HCl; 7-aminonitrazepam loses CO, apparently via opening of the 7-membered ring; and 7-amino-flunitrazepam loses HF. Note the similarity in structures for the analytes with HCl and HF losses.

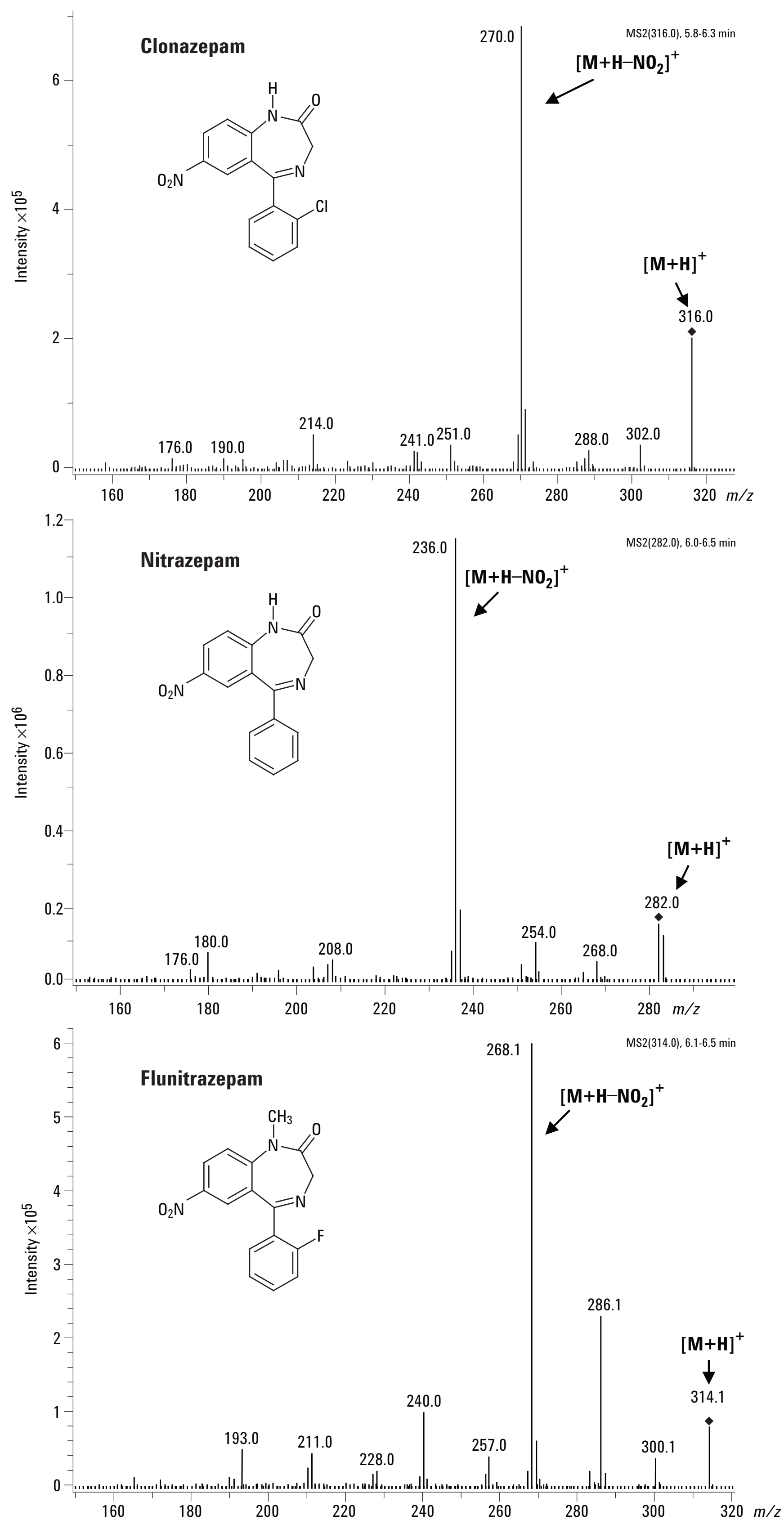

Figure 3. Structures and MS/MS spectra for clonazepam, nitrazepam, flunitrazepam, 7-aminoclonazepam, 7-aminonitrazepam, and 7-aminoflunitrazepam.

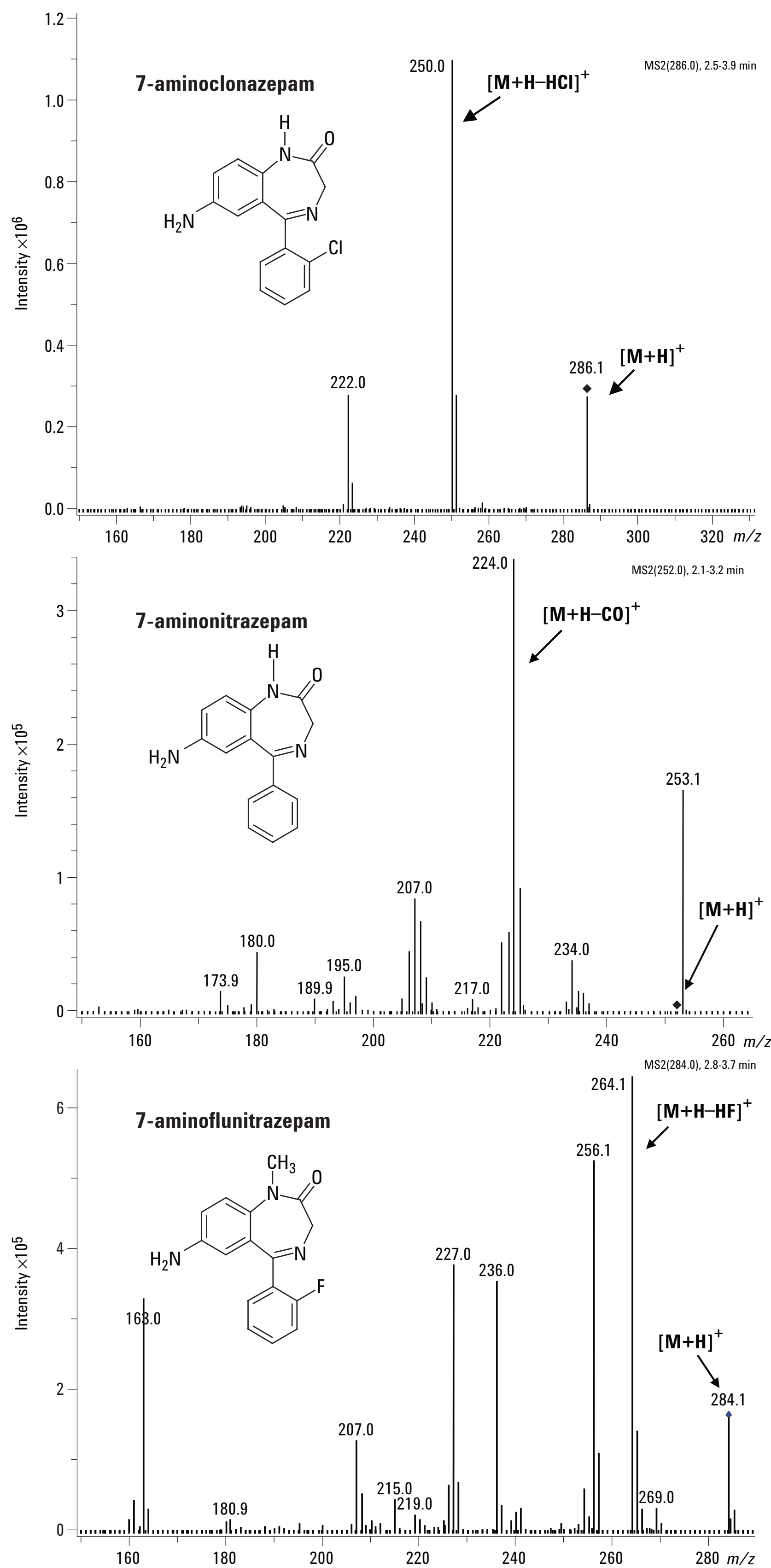

Figure 3 (continued). **Structures and MS/MS spectra for clonazepam, nitrazepam, flunitrazepam, 7-aminoclonazepam, 7-aminonitrazepam, and 7-aminoflunitrazepam.**

Flurazepam and prazepam lose alkyl groups as shown in Figure 4, and the desalkylflurazepam metabolite which has lost the entire alkylamino substituent develops a different fragmentation behavior with the major ion corresponding to loss of CO.

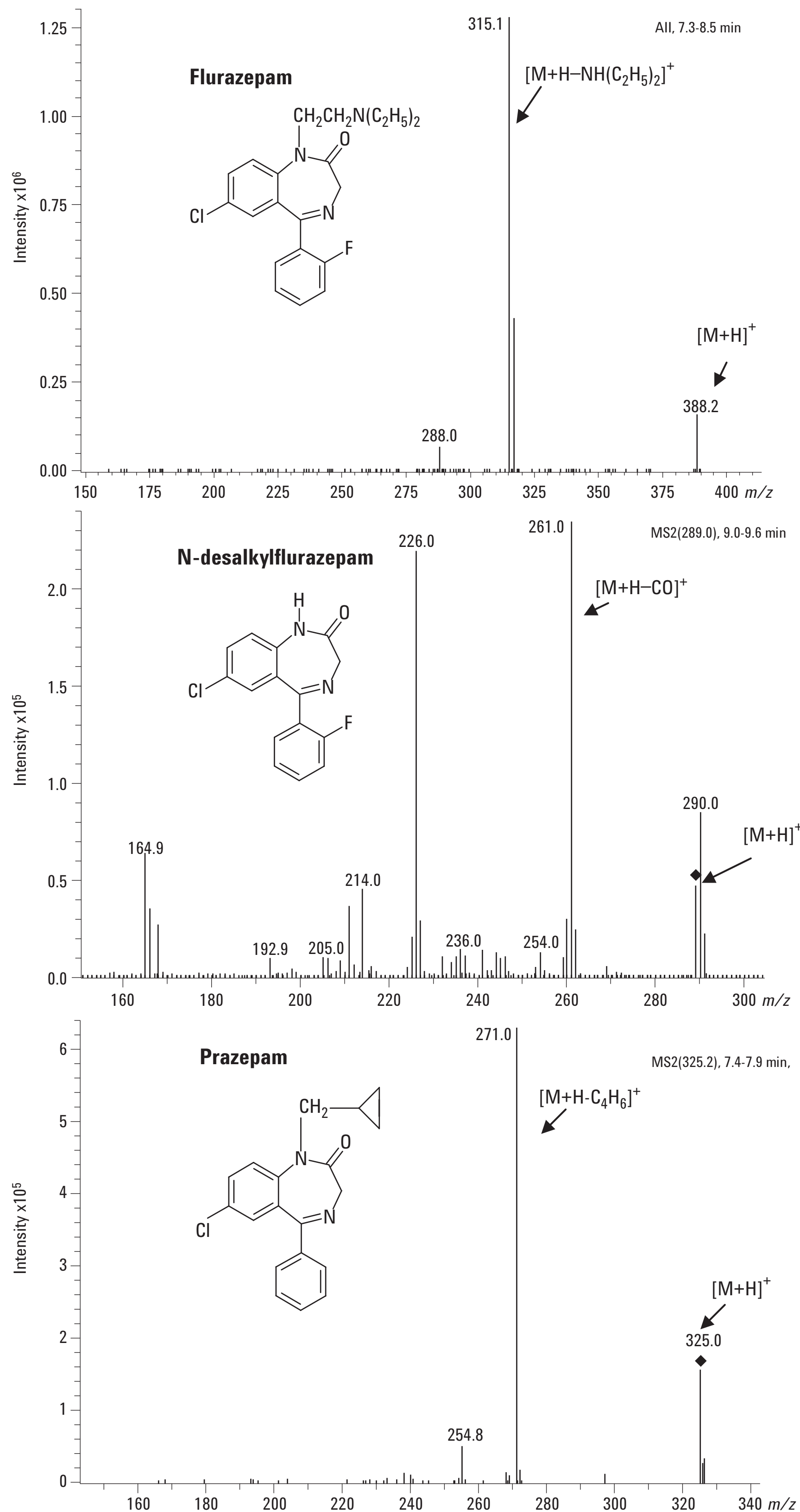

Figure 4. Structures and MS/MS spectra for flurazepam, N-desalkylflurazepam, and prazepam.

Figure 5 shows the loss of N_2 from alprazolam under these conditions. Its structure is very similar to that of triazolam (Figure 2) which also loses N_2, presumably also from the five-membered ring.

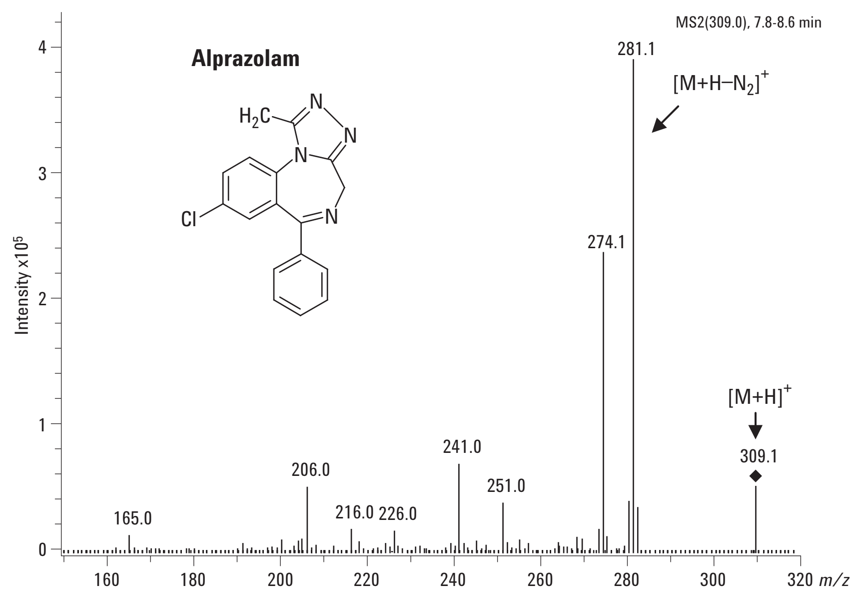

Figure 5. Structure and MS/MS spectrum of alprazolam.

Figure 6 shows several more benzodiazepines which lose the elements of CO, like N-desalkylflurazepam in Figure 4. It is interesting that all three lose CO from the 7-ring even though they all have a halogen in the 7-position of the fused benzene ring. Notice the HX loss from 7-aminoclonazepam and 7-aminoflunitrazepam (Figure 3) where a halogen is on the 2'-position of the non-fused benzene ring.

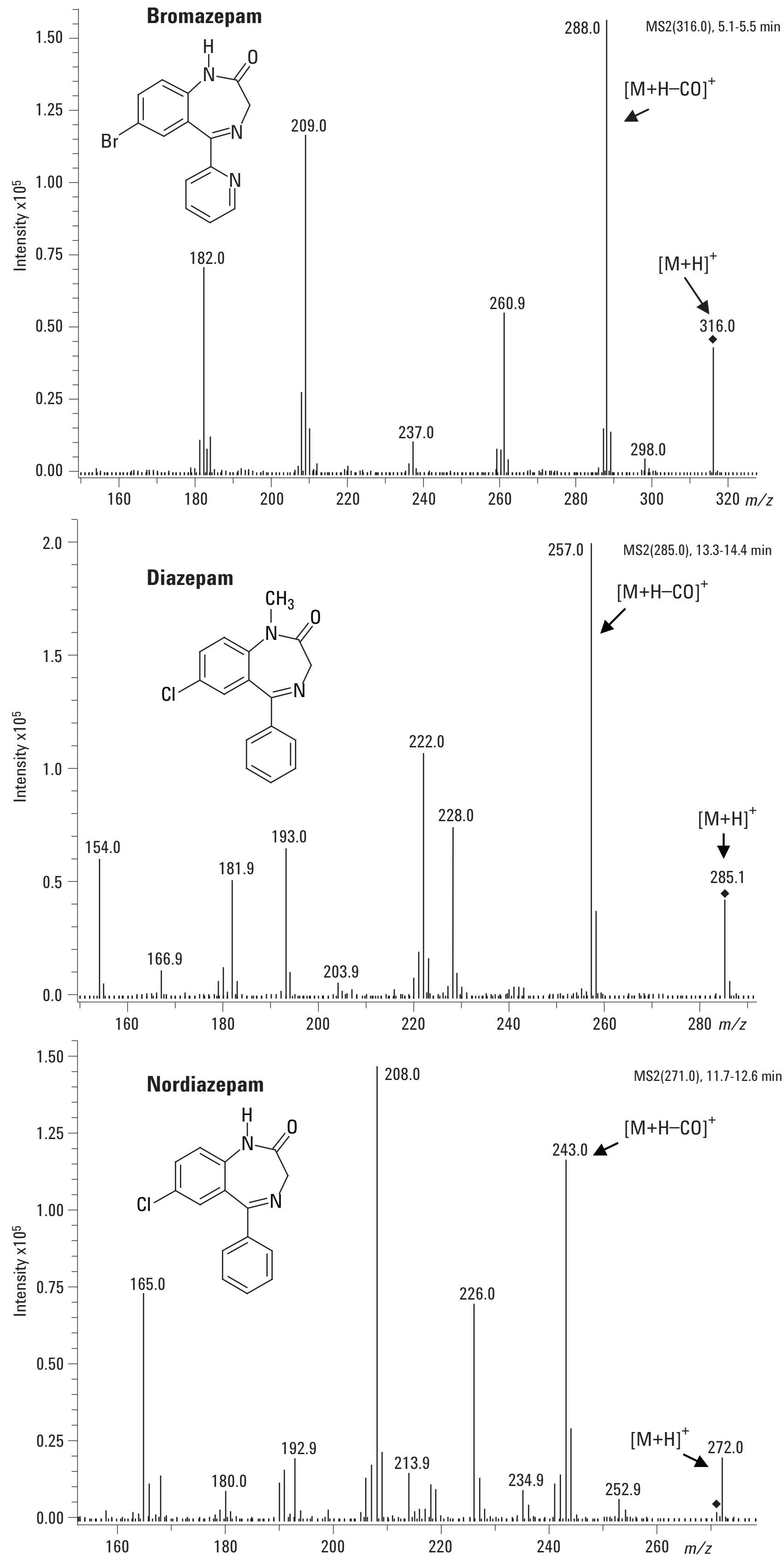

Figure 6. Structures and MS/MS spectra for bromazepam, diazepam, and nordiazepam.

Clobazam shows an extremely simple MS/MS spectrum and a unique loss of CH_2CO in Figure 7.

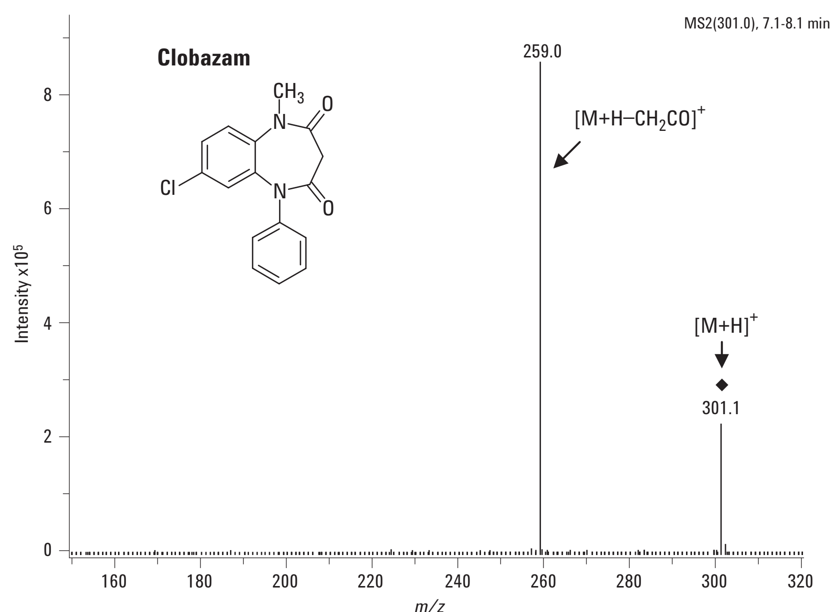

Figure 7. Structure and MS/MS spectrum for clobazam.

Figure 8 shows the MS/MS spectra of the remaining benzodiazepines which are obtained using an important feature of the Agilent LC/MSD Trap. On initial examination of MS/MS spectra during method optimization, lorazepam, oxazepam, and temazepam were found to have the major product ion to be the result of loss of the elements of water. As this is not the most specific loss one might prefer for identification purposes, a more information-rich MS/MS spectrum was obtained for each using the following technique. By increasing the fragmentation window from 10 amu to 40 amu (±20 amu centered on the precursor ion mass), fragmentation energy is applied to both the $[M+H]^+$ and the $[M+H–H_2O]^+$ ions, and the resulting MS/MS spectra are much more specific for identification.

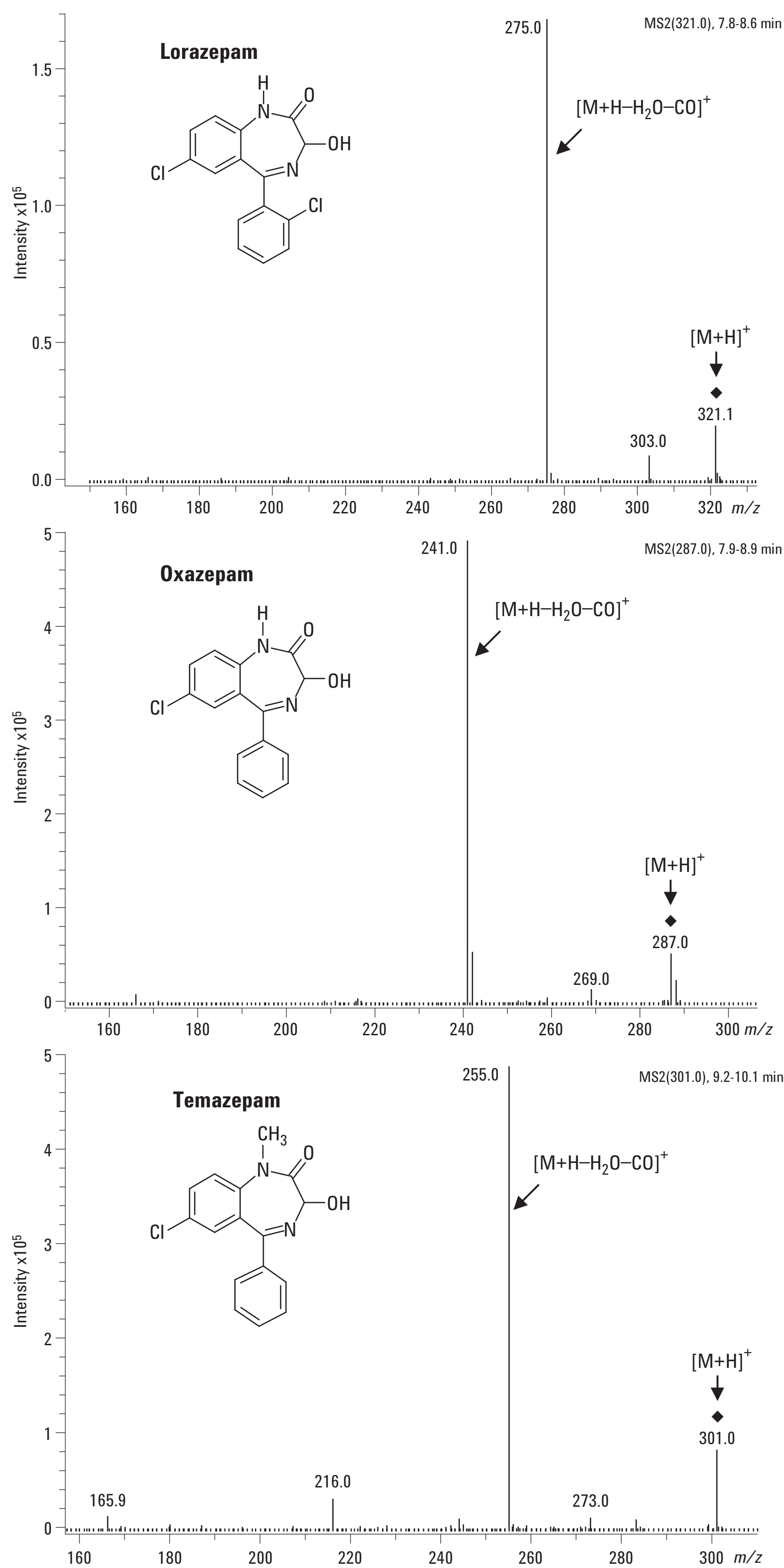

Figure 8. Structures and MS/MS spectra for lorazepam, oxazepam, and temazepam.

For example, with temazepam, fragmentation can result in the water-loss ion at *m/z* 283.0 and the water-loss plus CO loss ion at *m/z* 255.0. If the fragmentation energy is applied not only to the pseudomolecular ion at *m/z* 301.0, but also to the water-loss ion by using a fragmentation window of 40 amu, the intensity of the *m/z* 255.0 is improved, resulting in better detection and identification.

Application to Forensic Cases

Blood extracts from a wide variety of case types including multiple drug overdose, sexual assault victims and motor vehicle accident victims have been analyzed by this LC/MS/MS procedure. A number of benzodiazepines have been identified using this screening method, and are subsequently quantified by GC/ECD or HPLC-UV. A selection of the cases and their blood drug concentrations are shown in Table 4.

The ion chromatograms from the blood sample in Case 1, which provide the detection of nitrazepam, 7-aminonitrazepam, diazepam, nordiazepam and prazepam, are shown in Figure 9.

Table 4. Case Examples of Drugs and their Concentrations in Blood

Case	Benzodiazepine/ metabolite	Concentration mg/L
1	Nitrazepam	0.01
	7-aminonitrazepam	0.08
	Diazepam	0.33
	Nordiazepam	0.32
2	Diazepam	0.06
	Clonazepam	0.009
	7-aminoclonazepam	0.02
3	Bromazepam	0.40
4	Alprazolam	0.006
	Diazepam	0.05
	Nordiazepam	0.01
5	Diazepam	0.58
	Nordiazepam	0.66
	Oxazepam	0.04
	Temazepam	0.12
6	Clobazam	0.10

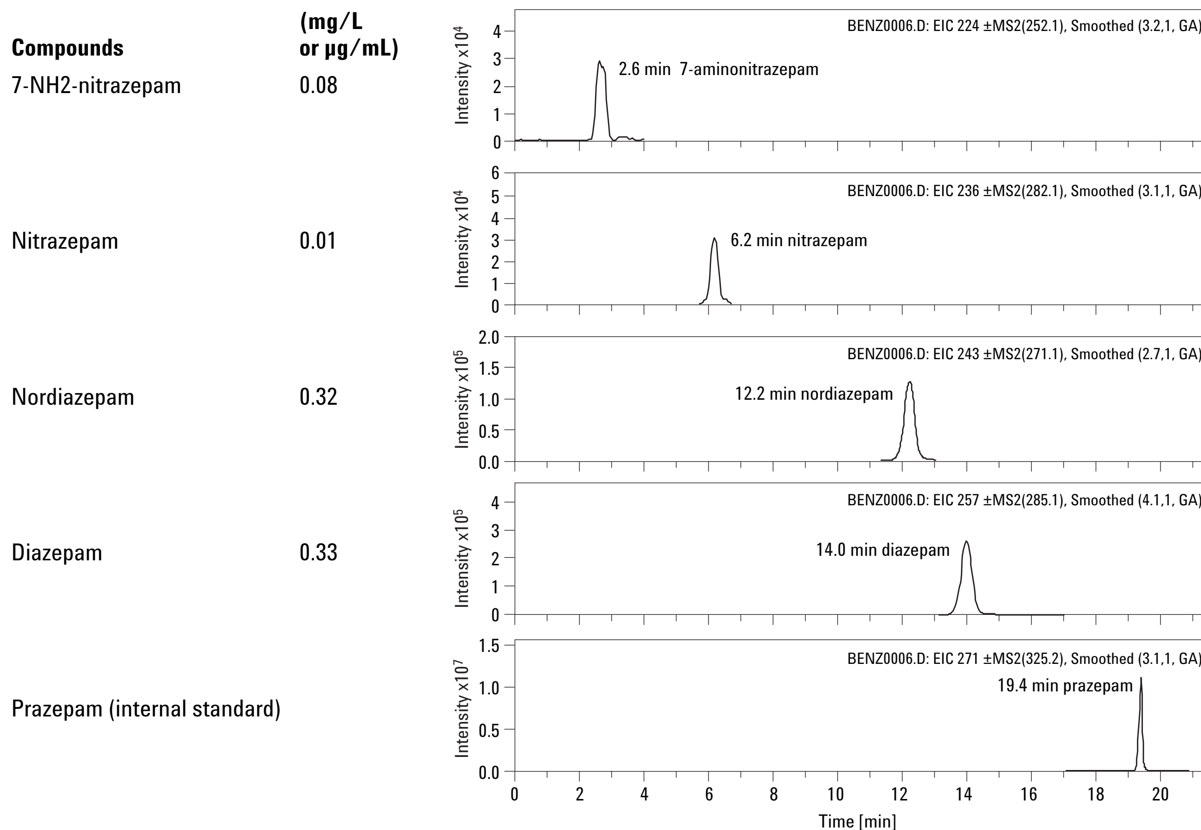

Figure 9. Ion chromatograms from Case 1 example.

Polypharmacy in such cases is not uncommon, and the method can easily detect, confirm and quantify multiple benzodiazepines and metabolites in a single analysis, as illustrated by this case.

Typical ion chromatograms from a blank blood sample are shown in Figure 10. Note that in Case 4 (see Table 4) it was possible to detect 0.006 mg/L (6 ng/mL) of alprazolam while still obtaining a clear identification with a full-scan MS/MS spectrum. Case 4 contains the lowest level of benzodiazepine detected in these cases, that of alprazolam at a level of 6 µg/L (6 ng/mL).

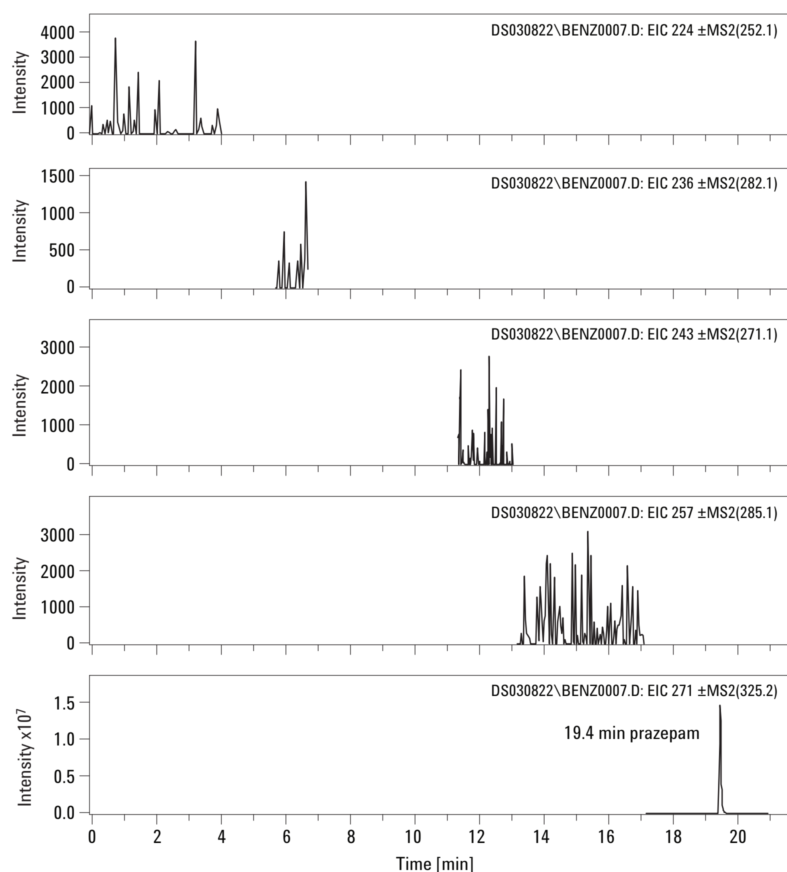

Figure 10. Ion chromatograms from blank blood with internal standard.

Conclusions

The LC/MS/MS method described here provides a single procedure for the identification of a wide range of benzodiazepines available in Australia and their metabolites, with a simple adaptation of an existing GC/MS sample preparation procedure, and without the need for derivatization. The MS/MS spectra provide a high-confidence identification of the drugs. The technique is suitable for screening analyses and confirmation of identity of the benzodiazepines at their lowest reported dosage oncentrations using only 500 µL of blood. The data in Table 4 and Figure 9 illustrate that the procedure is able to identify a range of dose concentrations of benzodiazepines in casework samples. Low concentrations of various benzodiazepines have been rapidly and successfully identified in forensic cases.

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