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Use of direct-probe mass spectrometry as a toxicology confirmation method for demoxepam in urine following high-performance liquid chromatography

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Abstract

The identification of the metabolite demoxepam in human urine establishes that chlordiazepoxide, a common benzodiazepine, has been administered. Like N-oxide metabolites of other drugs, demoxepam cannot be detected by gas chromatography–mass spectrometry (GC–MS), due to thermal decomposition, and the product, nordiazepam, is a metabolite common to many benzodiazepines. Demoxepam can be readily screened using a high-performance liquid chromatography (HPLC) system such as REMEDi HS; at 35°C, no thermal decomposition will occur. Currently, there is no confirmation method available for the detection of demoxepam in urine samples. In this study, we demonstrated that following collection of the HPLC fraction, demoxepam can be confirmed using the technique of direct-probe MS. The mass spectra of demoxepam and nordiazepam differ and are easily distinguishable from each other. Ten urine samples that were analyzed by HPLC and determined to contain demoxepam were evaluated; demoxepam was confirmed in each case by direct-probe MS.

Keywords: Demoxepam

1. Introduction

Chlordiazepoxide (Librium) was the first benzodiazepine available for general use and still is widely prescribed for the treatment of mild to severe anxiety. Psychic or physical dependence may develop, especially in addiction-prone individuals. Chlordiazepoxide can undergo metabolism to form norchlordiazepoxide, demoxepam, nordiazepam and oxazepam [1–3]. Fig. 1 illustrates the metabolism of chlordiazepoxide and diazepam. Demoxepam is not produced during the biotransformation of diazepam or any other benzodiazepines.

Gas chromatographic methods are not suitable for the analysis of thermally labile compounds. In several previous studies, the N-oxide metabolites of pethidine, tamoxifen and sulforidazine were shown to be thermally labile [4–7]. Likewise, demoxepam (an N-oxide metabolite) cannot be detected by gas chromatography–mass spectrometry (GC–MS) [8,9], and the in situ thermal decomposition product in GC is mainly nordiazepam. Nordiazepam can be readily analyzed by GC–MS. However, as shown in Fig. 1, nordiazepam is a metabolite produced by many other benzodiazepines, including the commonly prescribed diazepam and the less familiar clorazepate. Nordiazepam itself is sold as a benzodiazepine in some countries [10]. Therefore, the detection of nor-

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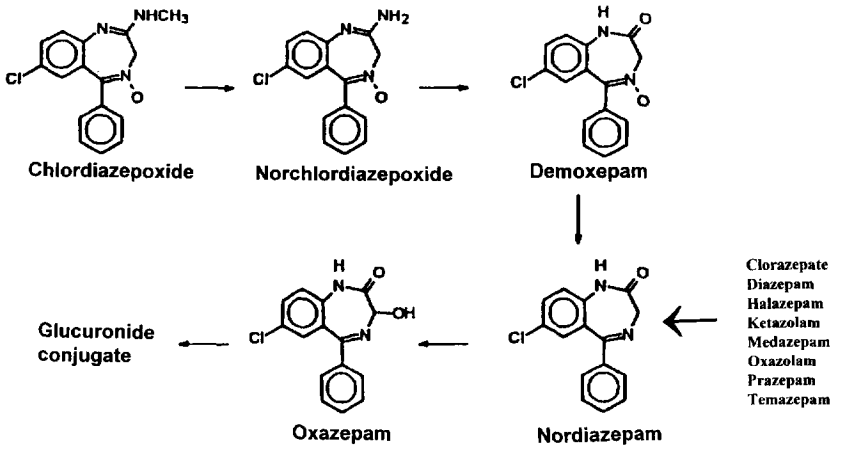

Fig. 1. Biotransformation of chlordiazepoxide, diazepam, clorazepate, halazepam, ketazolam, medazepam, oxazolam, prazepam and temazepam.

diazepam by GC–MS cannot establish the definitive presence of demoxepam in urine samples.

HPLC methods are known to be useful for thermally labile compounds. They can be used for the screening of demoxepam and other benzodiazepines in serum and urine, using a fixed-wavelength UV detection at 254 nm [11], a variable UV detector with a setting at 242 nm [12], a post-column photochemical reactor with fluorescence detection at the emission wavelength of 460 nm [13] and a diode array UV detector for a UV spectrum ranging from 230 nm to 400 nm [14]. In single wavelength detection, the peak assignment is based on retention data only, and the interference from any co-eluting impurities with the UV absorption at the same wavelength cannot be distinguished from the target compound.

A computer-aided HPLC system with a scanning UV detector [15–17] and two-dimensional data analysis capability will provide a more definite identification than retention index alone. The scanning UV detector produces a full UV spectrum that can be used to determine the purity of an analyte peak by comparison with the standard; two-dimensional data analysis uses retention indices and UV spectra fitting.

In toxicology, a confirmation method is defined as the use of an alternate chemical method to verify the result of an initial screening method. In one report, LC–MS was used for the identification of demoxepam, but only pure standards were analyzed [18]. In another report, LC with fast atom bombardment MS was used to identify demoxepam in serum during a pharmacokinetic study [19]. Intact demoxepam was detected in both of these studies, based on the presence of the molecular ion. Because LC–MS is not widely used in most clinical laboratories, we sought an alternate method as a toxicology confirmation method for the demoxepam identified by REMEDi HS.

The technique of direct-probe mass spectrometry (DP-MS) has been studied extensively [20–22]. It is particularly useful for the identification of thermally labile compounds. This study reports the use of DP-MS to confirm demoxepam identified during urine screening by multi-column HPLC with two-dimensional data analysis.

2. Experimental

2.1. Chemicals

HPLC-grade solvents were used for liquid–liquid extraction and preparation of samples. Toluene and hexane (optima grade) were obtained from Pierce (Rockford, IL, USA) and isoamyl alcohol was from Fisher Scientific (Pittsburgh, PA, USA). Analytical grade sodium tetraborate was obtained from Sigma (St. Louis, MO, USA). Demoxepam was purchased from Alltech (Deerfield, IL, USA).

2.2. Direct-probe mass spectrometry

DP-MS was performed on a Vacumetric DCI probe (Ventura, CA, USA) using a Hewlett-Packard (HP) 5989A MS engine (Palo Alto, CA, USA). The probe is equipped with a platinum wire for sample deposition, and it is controlled by a Vacumetric DCI current programmer. A comprehensive discussion of this technique can be found in the literature [23]. Usually, a chemical reagent gas such as methane is used during the analysis. In this study, the probe was used without external chemical reagent gas. The vapor of the analyte functioned as a chemical reagent gas for self chemical ionization. Because of the rapid dissipation of analyte vapor under vacuum, 40 ng of sample on platinum wire was required for a full scan mode and 5 ng on wire in a selected ion monitoring (SIM) mode.

The HP mass spectrometer was operated under the electron impact ionization (EI) mode at 70 eV with source and quadrupole temperatures of 200 and 100°C respectively. Full mass spectrum scan and SIM were both used for the study. The data acquisition time was set at 5 min for both full scan and SIM modes. The probe was inserted into the ionization source through a probe inlet port. The temperature programmer was ramped from 1.0 to 1.5 A in 5 s and held at 1.5 A for another 10 s; the final temperature was about 1200°C.

Limits of detection in full scan and selected ion monitoring modes were defined for the total ion and the 285 a.m.u. ion chromatogram at a signal-to-noise ratio of 10:1. The mass spectrometer was tuned using a MS resolution of 0.5 a.m.u. at half peak height.

2.3. HPLC with two-dimensional data analysis

The HPLC system was a REMEDi HS system (Bio-Rad, Hercules, CA, USA) with a reagent and cartridge set developed for analyzing benzodiazepines in urine (REMEDi UBZ is the abbreviation for this method). It utilizes the technology of on-line sample clean-up, HPLC separation, scanning UV detection and a software algorithm for UV full spectrum matching. The REMEDi HS hardware and chemistry configuration were described in previous studies [17]. The urine samples were hydrolyzed prior to injection for 2 h at 37°C using a glucuronidase–sulfatase preparation that was supplied with the reagent set.

Fig. 2 shows a chromatogram displayed at 235 nm. The identification of demoxepam is made by comparing retention indices and UV spectrum data with reference standard. The matching fitness of two UV spectra is measured throughout the UV range of 220 nm to 265 nm.

2.4. Sample preparation for DP-MS

A collected fraction of approximately 1 ml, containing the suspected demoxepam peak, was combined with 0.5 ml of saturated sodium borate and 2 ml of toluene–hexane–isoamyl alcohol (78:20:2, v/v). The mixture was mixed vigorously for 4 min and then centrifuged at 3000 *g* for 10 min. The organic layer was transferred to a clean 5-ml reaction-vial, and evaporated to dryness under a gentle stream of nitrogen. The dry extracts were reconstituted in 100 μl of methanol. A 10-μl volume of this methanol solution was applied in droplets to the platinum filament wire of the probe tip, and dried. The extraction efficiency was determined to be 95%, at a concentration of 1 μg/ml.

2.5. GC–MS

GC–MS analysis was performed on an HP 5989A GC–MS MS engine (Hewlett-Packard, Avondale, PA, USA). The mass spectrometer was operated in the EI mode with an ionization voltage of 70 eV. The temperatures of the source and quadrupoles were 200 and 100°C, respectively. A J & W Scientific (Fisons, Folsom, CA, USA) DB5 column (15 m×0.25 mm I.D., 0.25 μm film thickness) was used.

The GC carrier gas was helium with a column head pressure of 10 p.s.i. (70 kPa) and the flow-rate was maintained at 1 ml/min. The injector was at 265°C, the oven temperature was programmed from 160°C to 280°C at 20°C/min and stayed at 280°C for 9 min. The total analysis time was 15 min. The sample was prepared in an ethyl acetate solution (50 ng/μl) and the injection volume was 2 μl.

The standards chlordiazepoxide, norchlordiazepoxide and demoxepam were analyzed. In addition, the TMS-derivatized demoxepam standard

was also analyzed using the same GC–MS conditions.

2.6. Immunoassays

The urine samples that showed positive benzodiazepine-like immunoreactivity were analyzed by the EMIT dau (Syva, Palo Alto, CA, USA) immunoassay at a commercial laboratory.

3. Results and discussion

3.1. HPLC identification of demoxepam using two-dimensional data analysis

Fig. 2 shows a chromatogram of a urine sample from the REMEDi HS, which yields a positive screen by EMIT. A large peak was identified as demoxepam using two-dimensional data analysis. Table 1 lists the benzodiazepines and metabolites identified by this system for ten different urines which contained demoxepam. When compared to immunoassay, the advantage of an HPLC system such as REMEDi HS for urines that contain benzodiazepines is that parent compounds and metabolites can be individually separated and identified. The total analysis time for a hydrolyzed urine sample is less than 15 min in this automatic HPLC system, which requires minimal sample pre-treatment compared with the GC–MS method. In addition, thermally labile compounds, such as demoxepam and norchlordiazepoxide, possess unique UV spectra and can be routinely identified by the HPLC method with two-dimensional data analysis.

Demoxepam is the only metabolite that can be used to indicate the ingestion of chlordiazepoxide or norchlordiazepoxide, because the latter two were not found in these ten urine samples. Furthermore, two urines appeared to contain only demoxepam. In these two cases, the ability to confirm the presence of demoxepam is important because there were no other benzodiazepine parent compounds or metabolites present. The detection limit is about 250 ng on-column.

In a separate study of REMEDi [24], among the 588 urine samples that were found to contain one or more benzodiazepines or their metabolites, 318 were found to contain demoxepam or other benzodiazepines; among these 318 urine samples, 74 contained only demoxepam, 5 contained trace amounts of chlordiazepoxide and norchlordiazepoxide in addition to demoxepam and other benzodiazepines, 4 contained trace amounts of chlordiazepoxide in

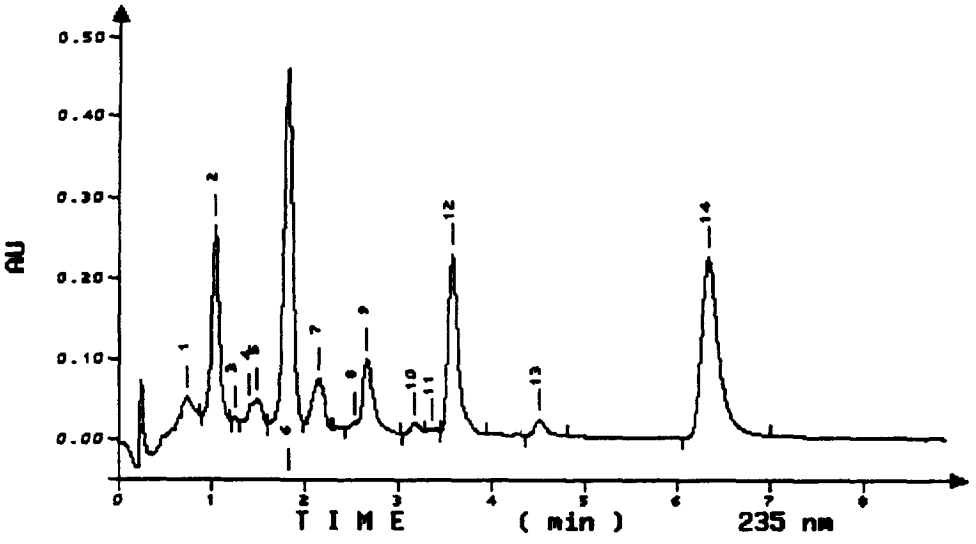

Fig. 2. HPLC chromatogram of an enzymatically hydrolyzed urine sample. Peak 6, demoxepam; peak 9, oxazepam; peak 12, IS1 (triazolam); peak 13, desmethyl diazepam; peak 14, IS2 (ethyl-oxazepam).

Table 1
The DP-MS and REMEDi UBz results for ten urine samples that contained benzodiazepine-like immunoreactivity

Sample ID	DP-MS result	REMEDi UBZ result	Estimated concentration of demoxepam (ng/ml) by REMEDi HS
940 700	Demoxepam	Demoxepam	>1800
940 731	Demoxepam	Demoxepam, oxazepam, nordiazepam	1006
940 743	Demoxepam	Demoxepam, oxazepam	>1800
940 746	Demoxepam	Demoxepam, oxazepam, lorazepam, Temazepam, nordiazepam	>1800
940 749	Demoxepam	Demoxepam	>1800
940 768	Demoxepam	Demoxepam, oxazepam	338
940 775	Demoxepam	Demoxepam, oxazepam, nordiazepam	547
940 800	Demoxepam	Demoxepam, oxazepam	>1800
940 802	Demoxepam	Demoxepam, oxazepam	>1800
940 806	Demoxepam	Demoxepam, oxazepam, temazepam Nordiazepam	508

addition to demoxepam and other benzodiazepines. Again, this demonstrates that demoxepam (97%; 309 out of 318) is more frequently detected in urine than either chlordiazepoxide or norchlordiazepoxide, using the HPLC method.

Demoxepam was more abundant than chlordiazepoxide and norchlordiazepoxide in urine. This is consistent with previous studies [25] which showed that less than 1% of parent chlordiazepoxide was excreted unchanged in urine, about 6% was excreted as demoxepam. The parent compound can be N-desmethylated to form norchlordiazepoxide and subsequently it will be transformed to demoxepam.

3.2. GC–MS analysis of chlordiazepoxide and demoxepam

As shown in Fig. 3A,B, in GC–MS analysis, chlordiazepoxide and norchlordiazepoxide (100 ng on-column) both underwent thermal decomposition and yielded an earlier elution by-product in addition to the intact compounds of chlordiazepoxide or norchlordiazepoxide. The by-products were formed through the loss of the oxygen atom at the N-oxide group of chlordiazepoxide or norchlordiazepoxide, as depicted in Fig. 4 and Fig. 5. Fig. 3C shows that demoxepam standard (100 ng on-column) also follows the same thermal decomposition pattern in GC–MS, to yield nordiazepam, and there was no detectable peak of demoxepam. In addition, a separate analysis of TMS-derivatized demoxepam standard only produced nordiazepam. Therefore, GC–MS analysis cannot be used as a confirmation method for demoxepam.

3.3. DP-MS analysis

Fig. 6A,B are the DP mass spectra of demoxepam and nordiazepam, respectively. These two mass spectra are different and distinguishable from each other. The molecular ions and some fragmentation ions were both observed. The molecular ions for demoxepam and nordiazepam were 285 and 269, respectively. Fig. 6C is a mass spectrum of a demoxepam-containing HPLC peak collected during urine screening, and it matches well with the standard. Because of the rapid dissipation of analyte vapor under vacuum, a minimum of 40 ng of demoxepam was required on the platinum wire for a full scan mode.

The ions at m/z 285 and 242 were used in the SIM mode for the detection of demoxepam, and the detection limit was 5 ng on platinum wire at a signal-to-noise ratio of 10:1.

4. Conclusion

This study reported the use of DP-MS as a confirmation method of the demoxepam peak iden-

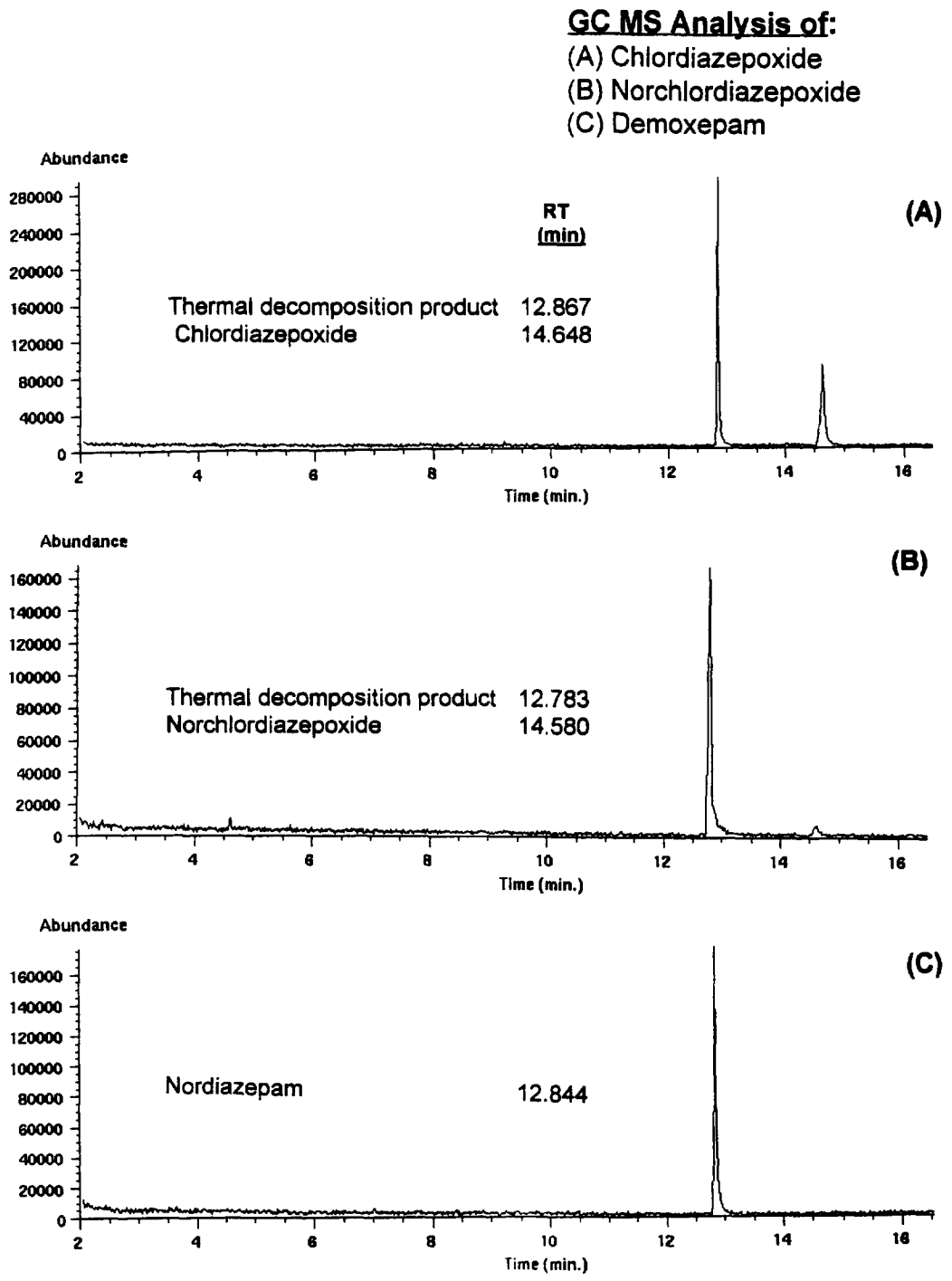

Fig. 3. GC–MS total ion chromatograms: (A) chlordiazepoxide standard that had undergone in situ thermal decomposition to form by-product in addition to intact chlordiazepoxide compound; (B) norchlordiazepoxide standard that had undergone in situ thermal decomposition to form by-product in addition to intact norchlordiazepoxide compound; (C) demoxepam standard that had undergone in situ thermal decomposition to form nordiazepam only.

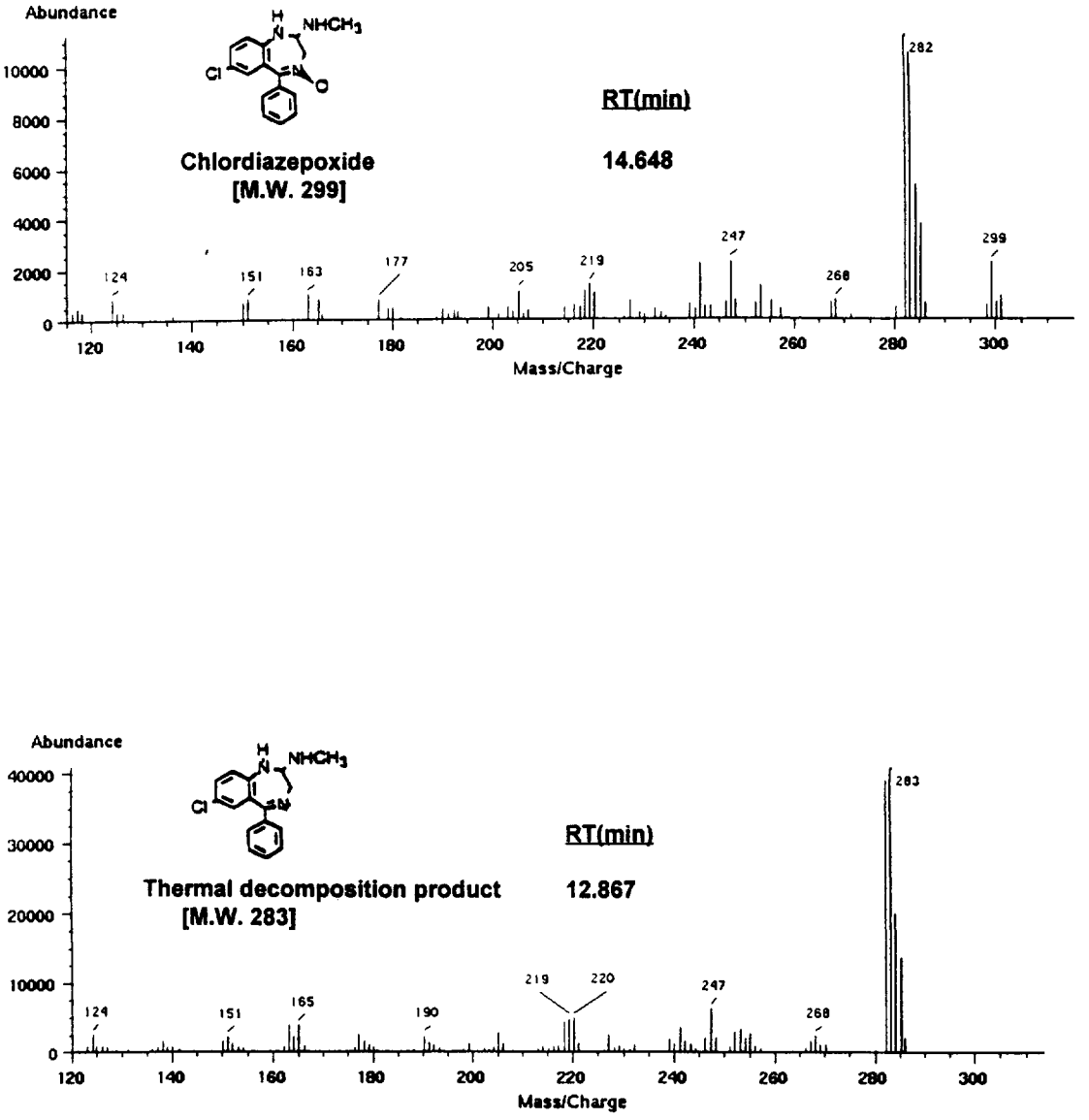

Fig. 4. Mass spectra and molecular structures of thermal decomposition by-products of chlordiazepoxide.

tified in HPLC elution of the REMEDi HS system, using two-dimensional data analysis. The mass spectra of demoxepam and nordiazepam obtained under this condition showed both molecular ions and some fragmentation ions. Their fragmentation patterns were different and distinguishable from each other. The HPLC demoxepam peak fractions were collected for DP-MS analysis, and all of the ten samples from patients were shown to contain demoxepam, by DP-MS confirmation.

Very sensitive LC–MS methods have been developed by fine-tuning experimental conditions for a particular drug. For example, the detection limits of individual benzodiazepines in LC–FAB-MS were

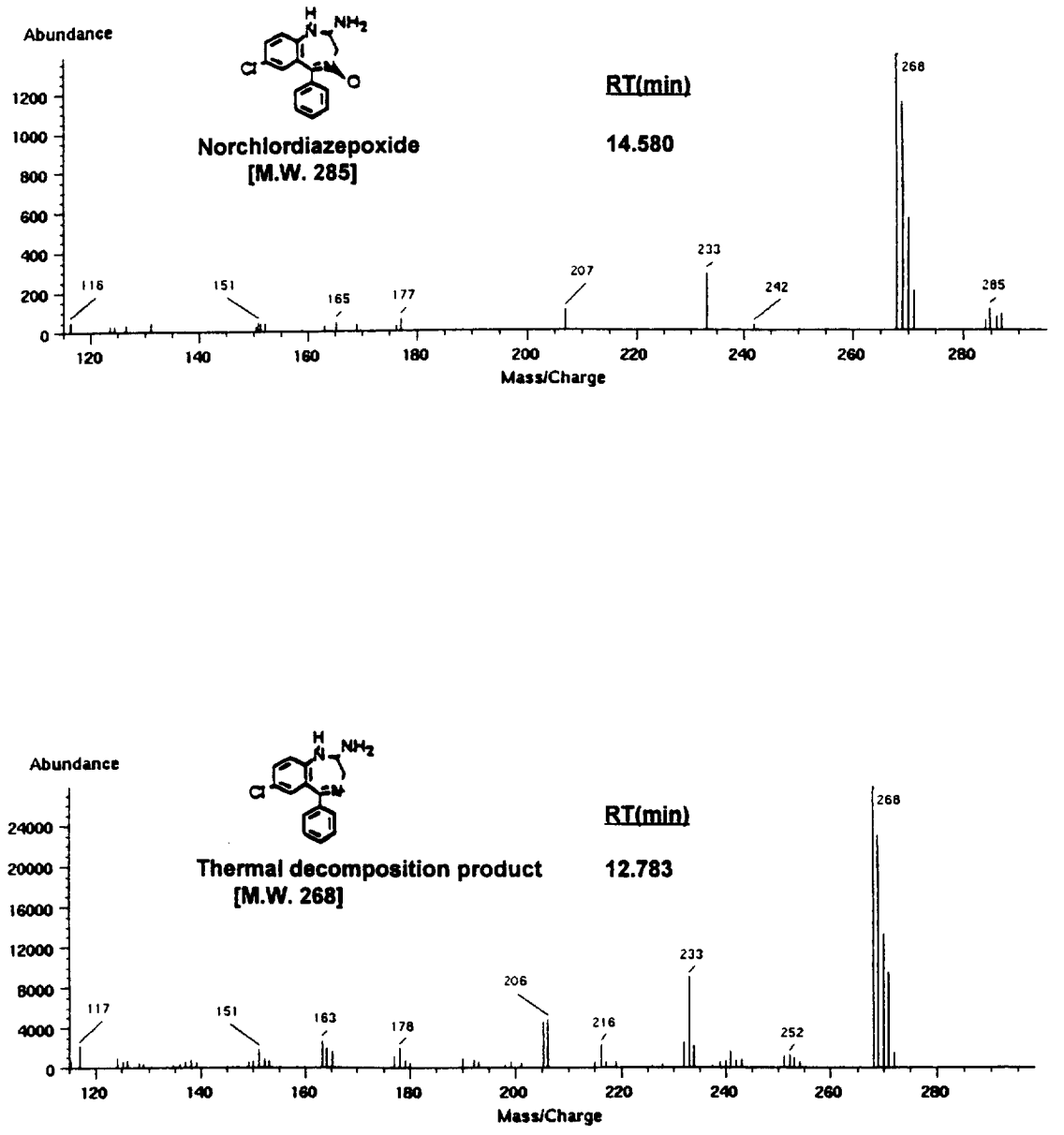

Fig. 5. Mass spectra and molecular structures of thermal decomposition by-products of norchlordiazepoxide.

reported to be 0.5 ng to 5.0 ng on-column [19]. Screening conditions may yield significantly different results; an amount as high as 25–100 μg on-column was required for broad-spectrum thermospray LC–MS analysis of benzodiazepine standards [18]. The use of DP-MS following HPLC is a rapid and straightforward method, and it provides a confirmation method when LC–MS is not available.

The REMEDi HS system used in this study has a detection limit of 250 ng on-column for demoxepam; 80 ng for other benzodiazepines. In this study, the patients' urine samples were from routine toxicology

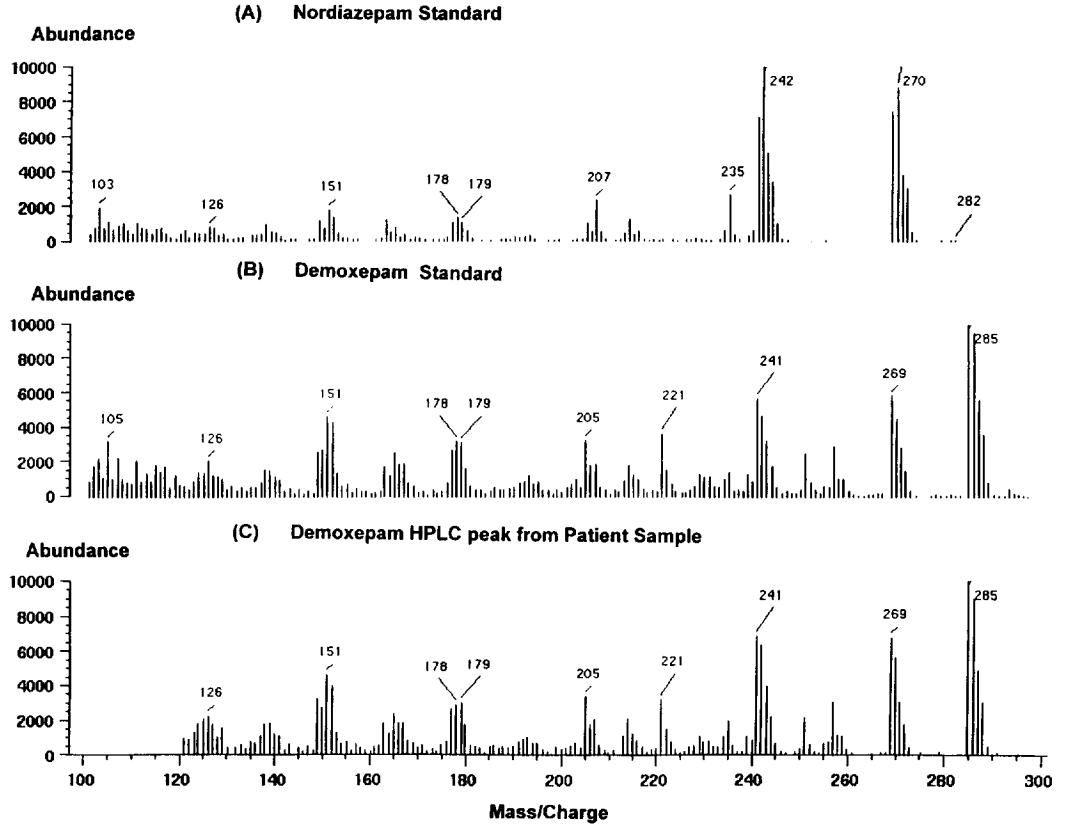

Fig. 6. DP mass spectra of (A) nordiazepam standard, (B) demoxepam standard and (C) a demoxepam-containing HPLC peak from a patient's urine sample.

screening, and the detectable demoxepam levels in the ten urine samples from patients were from 338 ng/ml to over 1800 ng/ml. In addition to demoxepam, the HPLC method with two-dimensional data analysis identified other benzodiazepines and metabolites, including nordiazepam, oxazepam, lorazepam and temazepam. This study demonstrates the usefulness of identifying demoxepam in routine toxicology screening of urine samples by using this HPLC system; it establishes that chlordiazepoxide has been administered.

The method of DP-MS has a detection limit of 40 ng on wire for demoxepam, and it provides a way to confirm the LC peak of demoxepam directly. The collection of LC eluates can be automated by using a fraction collector. All other benzodiazepines and metabolites can be confirmed by GC–MS.

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