TECHNICAL NOTE

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Analysis of Biofluids for Flunitrazepam and Metabolites by Electrospray Liquid Chromatography/Mass Spectrometry*

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ABSTRACT: A rapid and sensitive liquid chromatography/electrospray ionization mass spectrometry (LC/MS) procedure has been developed for the analysis of biofluids containing flunitrazepam and its metabolites. Specimens were spiked with deuterated analogs of the analytes. Urine specimens were enzymatically hydrolyzed and blood specimens were untreated. Extractions were carried out using CleanScreen DAU SPE cartridges. The drugs were separated on a C18 column using a methanol-water-ammonium hydroxide (60:40:0.03 v/v) mobile phase. After determination of base peaks using full scan mass spectrometry, the mass spectrometry method was optimized to operate in selected-ion monitoring (SIM) mode for the base peak of each analyte. Positive findings were confirmed by LC/MS/MS using the same mobile phase and column.

This analytical procedure allows for the detection of low levels of flunitrazepam and metabolites in biofluids. It is useful for ascertaining the role of flunitrazepam in cases of drug-facilitated sexual assault.

KEYWORDS: forensic science, flunitrazepam, Rohypnol®, drug-facilitated, sexual assault, drug rape, date rape, forensic toxicology

Flunitrazepam (Rohypnol®) is a potent benzodiazepine manufactured by Hoffman-LaRoche. While it is not approved for clinical use in the United States, it is prescribed for its sedative and anesthetic properties in more than 80 countries worldwide. The tablets are available in low milligram dosages that have recently been redesigned as gray-green, oval-shaped tablets containing a strong blue dye that releases when dissolved in drinks (1). Older forms of white, circular tablets (without the dye) are still available as street drugs in the United States. It is currently a Schedule IV drug under the Controlled Substances Act of 1974 but is unique in that it carries Schedule I penalties for its possession (2).

Benzodiazepines, including flunitrazepam, are abused worldwide for their strong sedative effects. While flunitrazepam is about seven to 10 times more potent than diazepam, it is four to eight times less potent than triazolam (3). In the United States, the largest occurrence of flunitrazepam abuse has been in Florida and in those states bordering Mexico.

In recent years, flunitrazepam has been used to facilitate sexual assaults and other crimes (4). This has resulted in the media labeling it one of the date rape drugs of the 1990s. Not surprisingly, there have been dramatic increases in requests for flunitrazepam screens in sexual assault cases. Investigators who have kept track of such cases recognize that flunitrazepam has been found in relatively few instances of drug-facilitated sexual assaults (4,5). In fact, there have been more cases of the use of other benzodiazepines to commit this crime than there have been cases of flunitrazepam-related drug-facilitated sexual assault (4). Nonetheless, it should be tested for in cases of suspected drug-facilitated sexual assault, particularly when the investigation fails to provide clues to other likely candidates.

Drug-Facilitated Sexual Assault

There are many difficulties that investigators face in cases of drug-facilitated rape (6). Most of these problems can be directly related to the drug or drugs used to commit the crime. Many of the drugs used, including flunitrazepam, are considered low-dose CNS depressants. Thus, only a small amount of the drug is needed to produce sedation. This sedation is intensified when the drug is consumed with alcoholic beverages, as is often the scenario.

The drugs may cause the victim to lose consciousness for a long period (6). Thus, they may not know they were assaulted or may be uncertain of the events that occurred just hours before. Sometimes they wake up during the assault but cannot remember this later. The victim typically experiences confusion. These factors are important in that they cause a delay in the reporting of the crime and result in a critical loss of time in the collection of biological specimens for toxicological analysis.

Because of this delay, many of the drugs used to facilitate a sexual assault may be eliminated from the blood to undetectable lev-

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els by the time the specimen is collected. Urine specimens provide a longer window for detection compared to blood, but are not routinely collected in rape examinations.

Following oral ingestion, flunitrazepam is rapidly absorbed (7). Blood levels peak one to two hours after ingestion and may never exceed 10 ng/mL (8). The effects may be felt within 20 min of ingestion and may last up to 24 h (9). These effects include profound sedation, dizziness, confusion, anterograde amnesia, and impaired psychomotor function.

Flunitrazepam is rapidly biotransformed into norflunitrazepam and 7-aminoflunitrazepam. Phase II conjugates of these metabolites are also formed resulting in long half-lives (10). These long half-lives allow for detection of its ingestion up to two days in blood and up to four days in urine when sufficiently sensitive techniques are employed (6).

This procedure was developed to provide a means of screening, confirming, and/or quantitating biological specimens for low levels of flunitrazepam and its common metabolites. The procedure has proven useful for ascertaining the role of flunitrazepam in cases of drug-facilitated sexual assaults.

Materials and Methods

Materials

Reference standards of flunitrazepam, norflunitrazepam, and 7-aminoflunitrazepam were purchased from Radian International LLC (Austin, TX) as 1 mg/mL methanolic solutions. From these, a 100 μg/mL working standard of flunitrazepam and metabolites was prepared in deionized water. Additionally, d_7-flunitrazepam, d_4-norflunitrazepam, and d_7-7-aminoflunitrazepam were obtained from Radian International LLC as 100 μg/mL methanolic solutions, and a 1 μg/mL working standard of the three compounds was prepared in deionized water.

All reagents were analytical grade, and solvents were of HPLC grade or better. Reagents and solvents were obtained from commercial sources. β-glucuronidase (*Helix promatia* —Type H1) was purchased from Sigma Chemical Company (St. Louis, MO). CleanScreen® DAU solid phase extraction cartridges (200 mg) were obtained from United Chemical Technologies, Inc. (Bristol, PA).

Extraction

Five milliliters of urine and 2 mL of blood were spiked with the working standard of deuterated analogs to give final concentrations of 5 ng/mL of each analog. Urine specimens were adjusted to a pH of 5.2 with 1 mL of 1.1 M sodium acetate buffer and enzymatically hydrolyzed with β-glucuronidase for four hours at 37°C prior to extraction. Following hydrolysis, the urine specimens were diluted with 3 mL of 100 mM phosphate buffer (pH 6) prior to extraction.

Blood specimens were not hydrolyzed but were diluted with 7 mL of the phosphate buffer and centrifuged for 10 min at 3000 RPM before extraction.

All specimens were extracted by a modified solid-phase extraction procedure (12) on a Zymark (Hopkinton, MA) RapidTrace robotic system. CleanScreen DAU cartridges were rinsed with elution solvent (1 mL), methanol (3 mL), deionized water (3 mL), and buffer (2 mL) before application of the specimen at 1 mL/min. The cartridges were then washed with deionized water (2 mL) followed by a 20% acetonitrile:80% 100mM phosphate buffer (pH 6) solution (2 mL) and dried with nitrogen (40 psi) for 1 min. This was followed by a hexane rinse (2 mL) and another minute of drying of the cartridge. Additional water rinse (2 mL) and drying steps were then performed to remove all traces of phosphate buffer from the cartridge. The analytes were eluted with 2.5 mL of 98% ethyl acetate/2% ammonium hydroxide and taken to dryness at 40°C. The residue was reconstituted in 20 μL of mobile phase.

Instrumental Analysis

Analyses of the extracts were carried out using a Finnigan MAT LCQ mass spectrometer (San Jose, CA) equipped with a Finnigan MAT P4000 HPLC pump and an AS3000 autosampler. The mobile phase was 60:40 methanol/water with 0.03% ammonium hydroxide at a flow rate of 0.3 mL/min through an Alltech Altima C18 column (15 cm × 2.1 mm × 5 μm). Injections (10 μL) were performed using the autosampler. Atmospheric pressure ionization took place in an electrospray interface with the following parameters: capillary temperature (200°C), source voltage (3.50 kV), source current (approximately 100 μA), sheath gas flow (75 psi), capillary voltage (3.0 V), tube lens offset (45 V), and octapole RF amp (490).

After analysis of single drug solutions by full-scan electrospray ionization mass spectrometry, it was apparent that the protonated molecular ion (M+H) of each analyte was the base peak—often with minimal additional fragmentation. This finding lead to the development of a LC/MS procedure using SIM of the protonated molecular ion (M+H) of each analyte of interest (7-aminoflunitrazepam = m/z 284, d_7–7-aminoflunitrazepam = m/z 291, norflunitrazepam = m/z 300, d_4-norflunitrazepam = m/z 304, flunitrazepam = m/z 314, d_7-flunitrazepam = m/z 321). Figures 1 and 2 are examples of results obtained from specimens of urine and blood, respectively, spiked to 5 ng/mL of each of the analytes.

When desired, quantitative estimations were achieved by comparison of the integrated area(s) of the detected analyte(s) to the respective deuterated analog. Alternatively, calibration curves of each analyte have been constructed.

Any indication of flunitrazepam or one of its metabolites was confirmed with extraction of a separate aliquot and analysis by LC/MS/MS(ESI). This was carried out by monitoring the full scan (m/z 100–325) daughter ions of the M+H parent ion using the same parameters listed above and a relative collision energy of 14% (Figs 3 to 5).

Validation

Validation of this analytical procedure included studies on recovery, linearity, accuracy, precision, and sensitivity (Table 1). Blood and urine specimens spiked at 5 and 30 ng/mL were analyzed for the recovery study (four of each at both levels). Recoveries were determined by comparison of areas of the extracted analytes to areas of equal amounts of deuterated analogs of the analytes added after the extraction. While the recovery of 7-aminoflunitrazepam was less than 65% for both blood and urine, the sensitivity of the instrumental assay compensates for this lower recovery. For comparison purposes, extracted samples were also analyzed by LC/UV. The procedure allowed for detection of flunitrazepam and its metabolites by LC/UV, but interferences from biological specimens resulted in detection limits about 100 times higher than those observed using the LC/MS method.

Blood and urine specimens (four of each) spiked at 5 ng/mL were analyzed for the precision and accuracy studies. This was done three different days to determine day-to-day variances.

Linearity was very good for each of the analytes in both blood and urine. This was determined by constructing multi-level (5–7 levels) calibration curves within the ranges listed in Table 1.

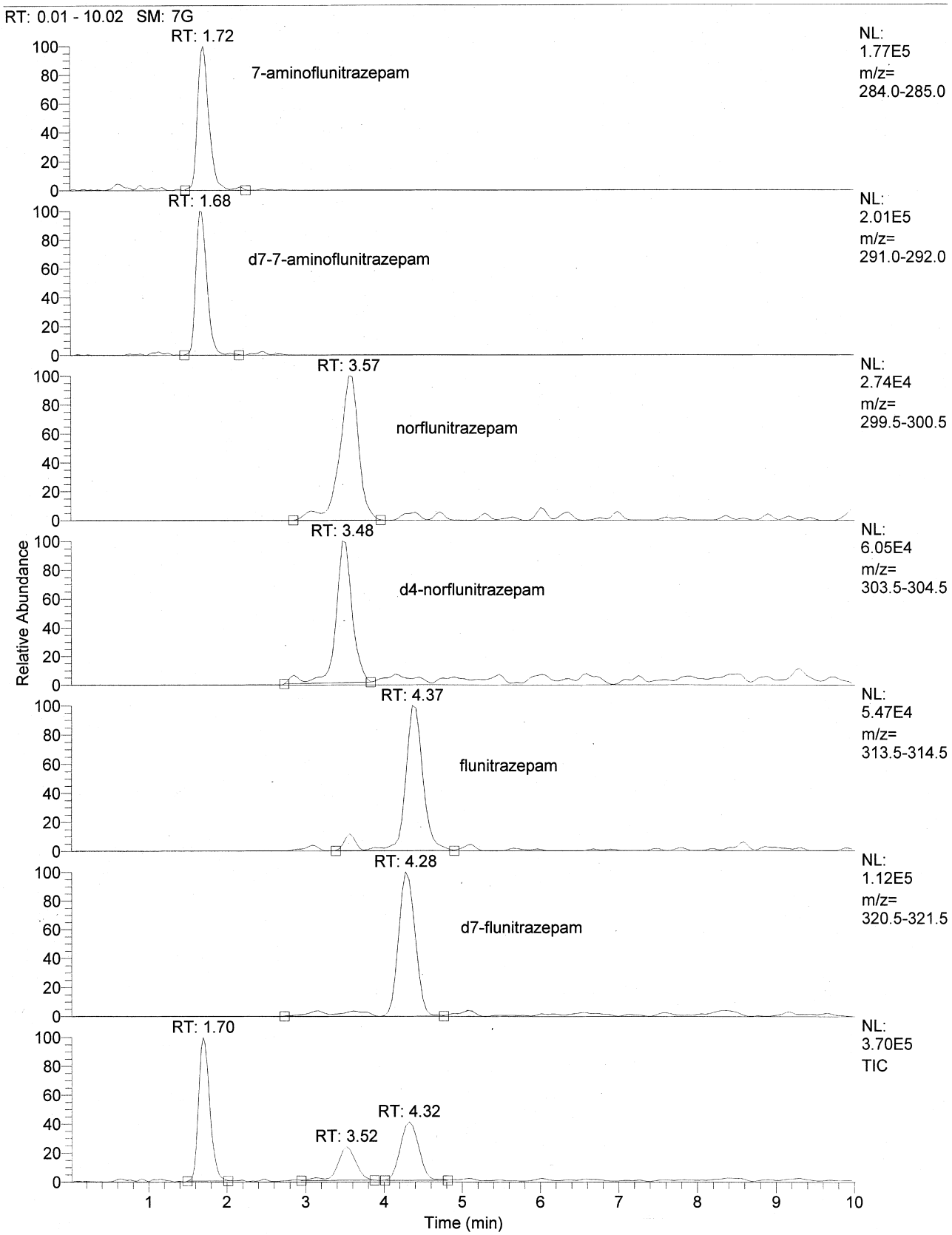

FIG. 1—*5 ng/mL SIM chromatogram from spiked urine.*

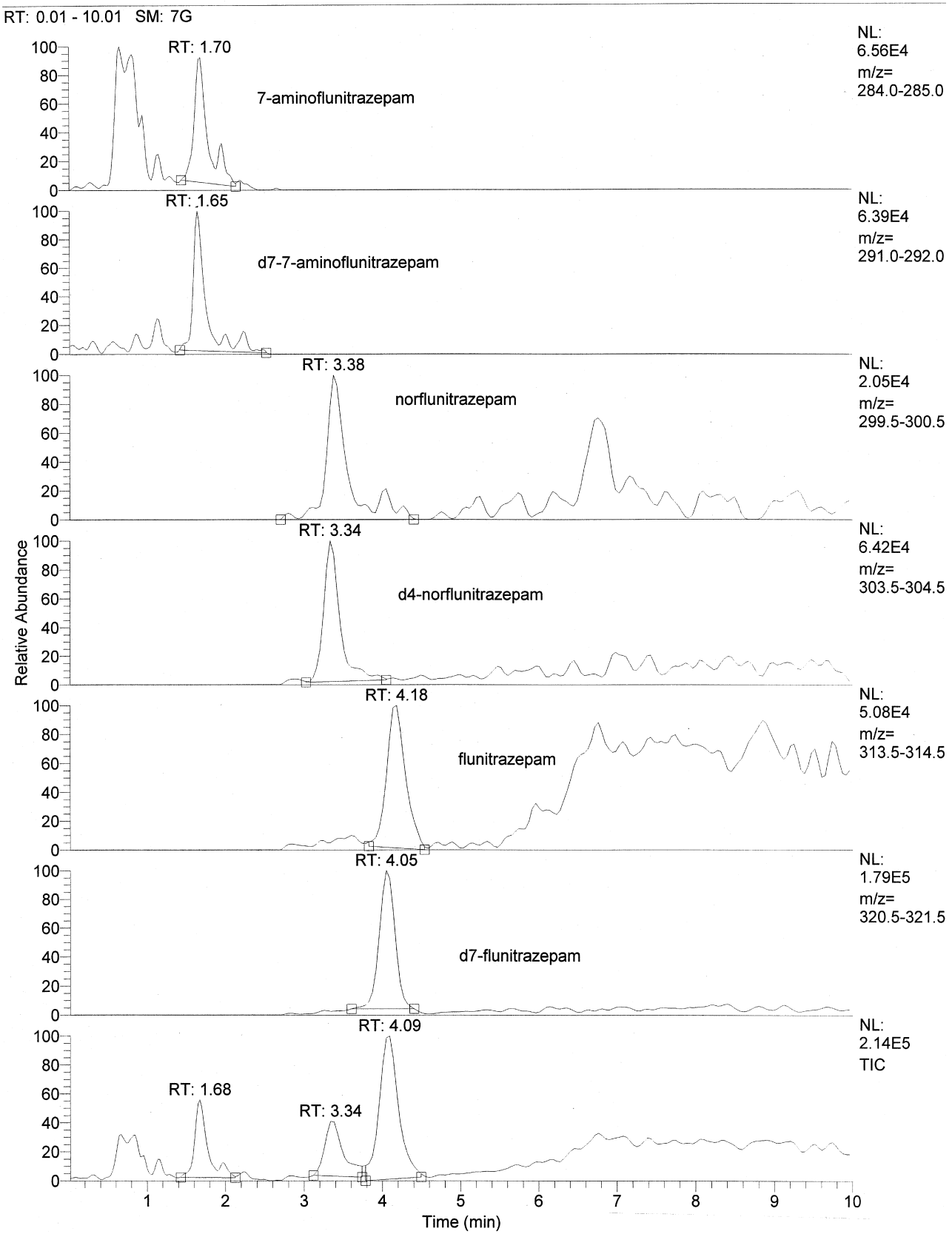

FIG. 2—*5 ng/mL SIM chromatogram from spiked blood.*

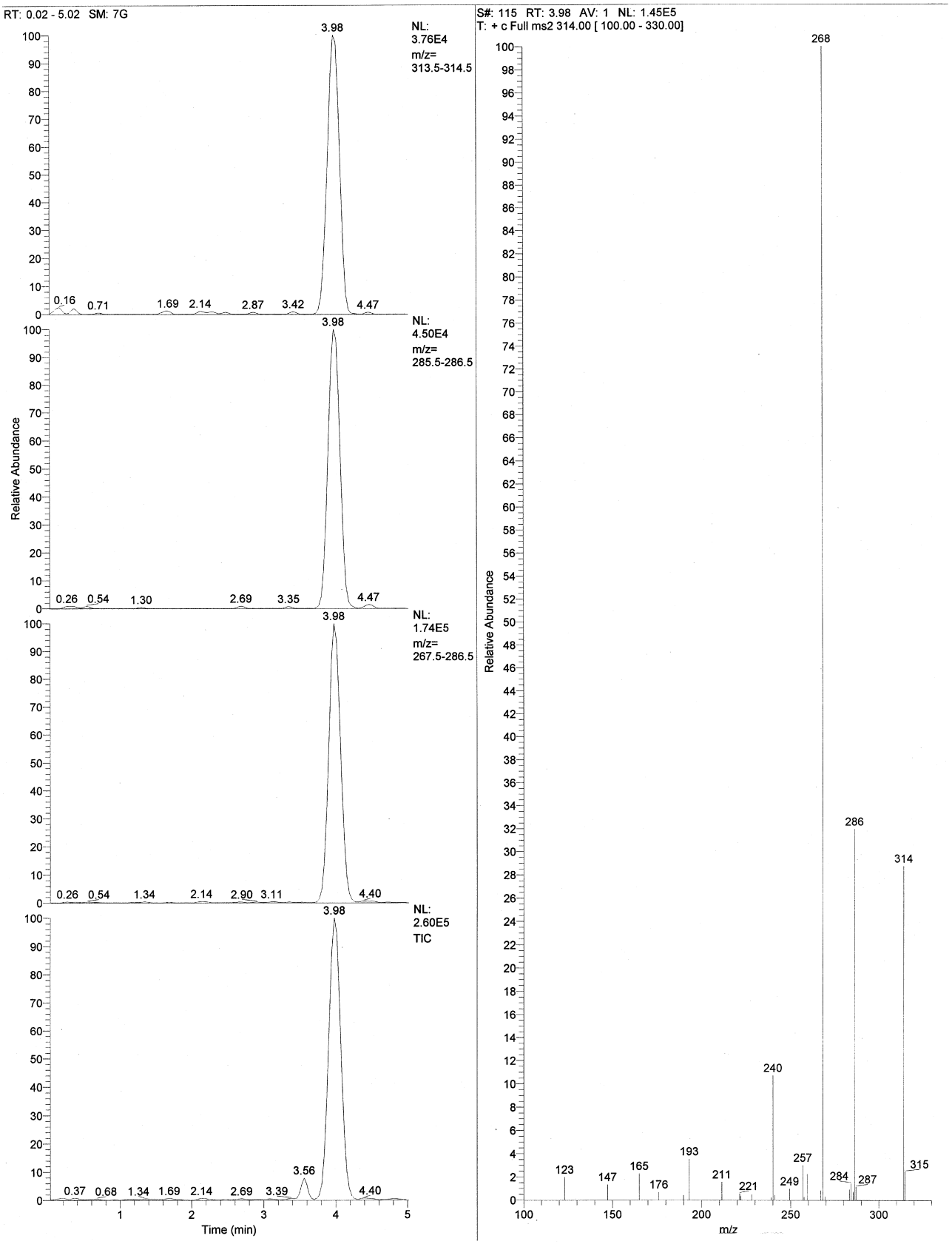

FIG. 3—*MS/MS spectrum of flunitrazepam.*

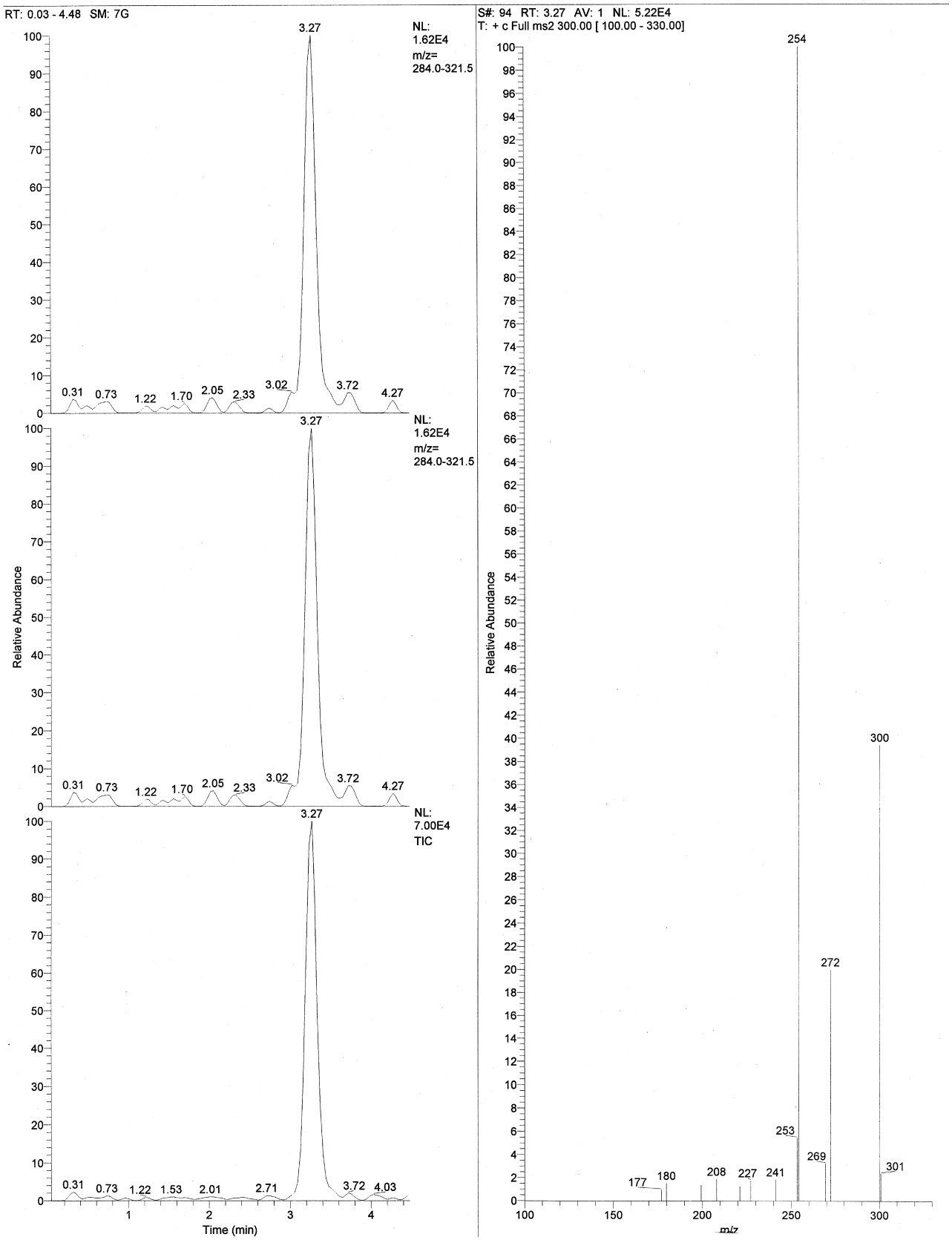

FIG. 4—*MS/MS spectrum of norflunitrazepam.*

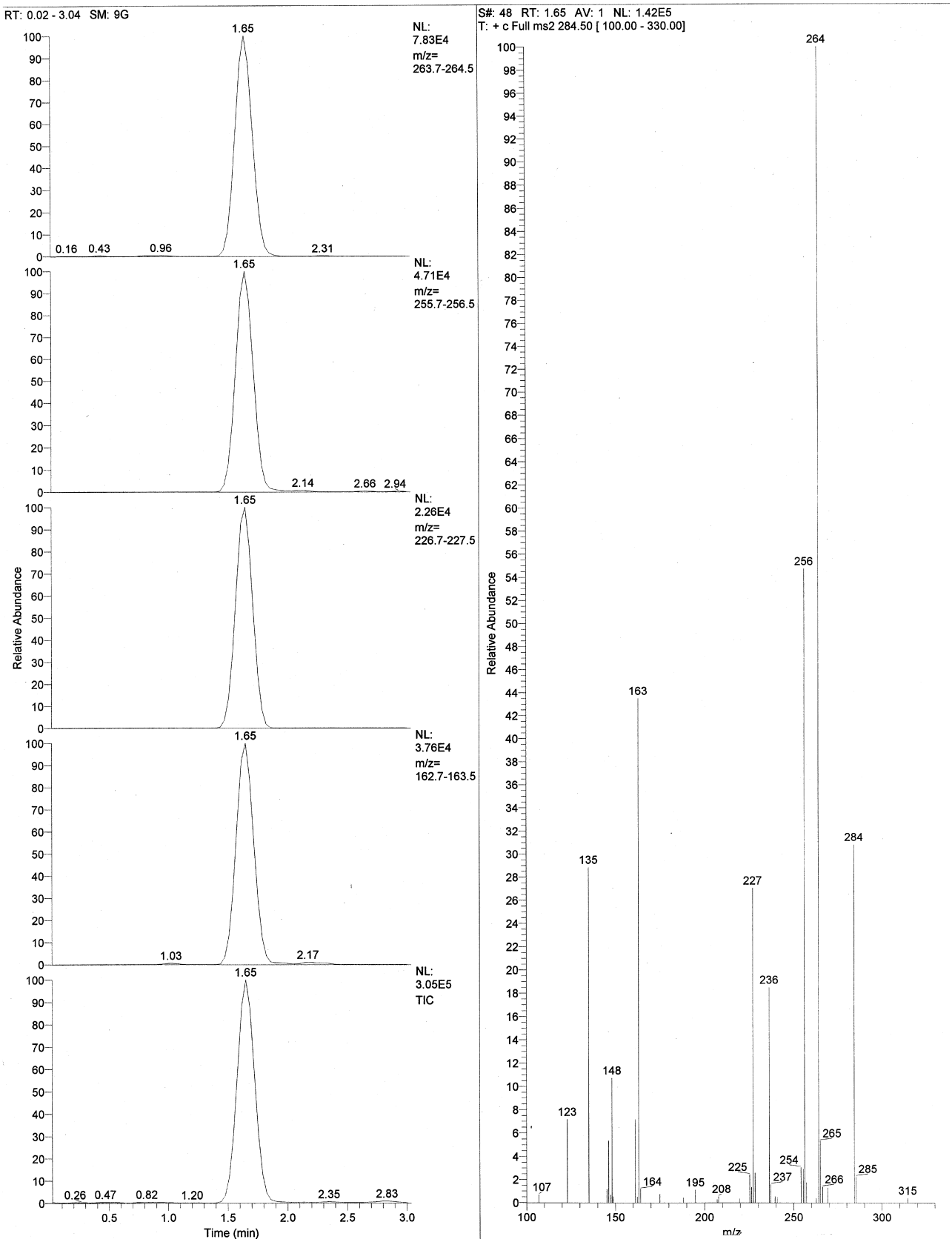

FIG. 5—*MS/MS spectrum of 7-aminoflunitrazepam.*

TABLE 1—*Validation results.*

	Flunitrazepam		Norflunitrazepam		7-Aminoflunitrazepam	
	Blood	Urine	Blood	Urine	Blood	Urine
Limit of Detection	0.5 ng/mL	0.1 ng/mL	1.0 ng/mL	0.1 ng/mL	0.5 ng/mL	0.2 ng/mL
Linearity (ng/mL)	2–200 ($r^2 = 0.99$)	1–200 ($r^2 = 0.99$)	5–200 ($r^2 = 0.99$)	1–200 ($r^2 = 0.99$)	2–200 ($r^2 = 0.99$)	1–200 ($r^2 = 0.99$)
Recovery						
5 ng/mL	57%	95%	61%	124%	24%	62%
30 ng/mL	74%	96%	65%	101%	26%	57%
Accuracy (5 ng/mL)	3.7 ± 0.4	4.9 ± 0.4	4.2 ± 0.6	5.0 ± 0.2	3.8 ± 0.1	5.2 ± 0.5
Precision (% CV)						
Within-Day	11.2%	5.2%	12.2%	0.6%	7.0%	8.8%
Day-to-Day	11.5%	7.7%	15.9%	4.6%	9.6%	11.1%

Discussion

While it is common to rely on commercially available immunoassays for benzodiazepine screening, most tend to lack the sensitivity needed to detect flunitrazepam or its metabolites in specimens following single-dose administration. This is particularly true when there is a delay in specimen collection (11). Relying on such methodologies to determine if an individual was drugged by flunitrazepam may lead to false-negative results (7).

Other commonly used methods for flunitrazepam and its metabolites also have limitations. Gas chromatography (GC) with an electron-capture detector (ECD) is quite sensitive for flunitrazepam and norflunitrazepam, but not for 7-aminoflunitrazepam. GC-MS procedures are also sensitive, but require derivatization of 7-aminoflunitrazepam for detection. HPLC with UV detection lacks sufficient sensitivity to be of use in most investigations of drug-facilitated sexual assault.

LC/MS coupled with solid phase extraction allows for a sensitive and selective analysis of biological specimens for flunitrazepam and its major metabolites without derivatization. The sensitivity of the procedure allows for an extended window of detection of flunitrazepam exposure, an issue particularly important to cases of drug-facilitated rape. The use of deuterated internal standards allow for rapid quantitative estimates of the amount of each analyte present.

The procedure's large sample volume (5 mL for urine) is higher than what others have reported (7,8,11), however, this volume is to further enhance the sensitivity of the procedure. This is usually not a concern when the analyst is provided 100 mL of urine.

While many benzodiazepines/metabolites exhibit improved sensitivity by the use of negative ion mass spectrometry, this was not the case with flunitrazepam and its metabolites. 7-Aminoflunitrazepam, in particular, was not detected in negative ion mode. These results are in agreement with observations by other authors (13).

The final aqueous wash in the solid phase extraction proved to be crucial to obtaining the observed detection limits of the procedure. Without this step, some adduct formation onto the analytes occurred with phosphate buffer cations ($M+Na^+$) and methanol (from the mobile phase). For instance, the predominant ion for the 7-aminoflunitrazepam was $M+Na^+$ without the extraction's final aqueous wash step. The response of SIM and MS/MS experiments of the instrumental procedure was drastically hampered by these adducts. Additionally, once phosphate was introduced into the system, it was very difficult to remove, often requiring shutdown of the system in order to flush it out.

Conclusions

Although the media has labeled flunitrazepam a commonly used drug to commit rape, there have been few proven cases of its use as such (4). This is consistent with our findings. While some may argue that the small percentage of positive flunitrazepam findings precludes its inclusion in the analytical scheme of drug-facilitated rape cases, the general public's understanding of its effects necessitates its inclusion.

This procedure allows for semi-automated analysis of biological specimens for the presence of flunitrazepam and its metabolites utilizing the power of LC/MS and LC/MS/MS with electrospray ionization. The sensitivity of the procedure allows for the detection of these analytes into the subnanogram per milliliter range. This proves useful for investigations into claims of drug-facilitated sexual assault, even when there is considerable delay in the reporting and collection of specimens.

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