Determination of Benzodiazepines in Urine and Blood Using Rapid Resolution Liquid Chromatography/ Triple Quadrupole Mass Spectrometry

Application Note

Forensic Toxicology

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Abstract

A rapid, simple, highly sensitive procedure for the simultaneous analysis of 14 benzodiazepines and six metabolites in urine and blood, using the Agilent 6410 Triple Quadrupole Mass Spectrometer in electrospray mode, is described. For the urine samples, preparation included treatment with β-glucuronidase in authentic samples. For the blood samples, preparation included precipitation of the red blood cells with acetonitrile followed by solid phase extraction, evaporation of the final eluent to dryness, and reconstitution in mobile phase for injection into the LC/ MS/ MS system.

To our knowledge, the procedure is the first to include the simultaneous monitoring of a qualifying ion, which is required to be present within a specific ratio to the primary ion for acceptable identification. The unique features of the Agilent software allow the transitions to be monitored and automatically calculated into ratios, which must fall within the range of the calibration standards in order to be considered positive. While monitoring a qualifying ion naturally inhibits the sensitivity of the assay, the additional confidence in the result is a critical factor in forensic analysis

Introduction

Benzodiazepines are the most commonly prescribed class of drugs in the USA. Hegstad et al. published a procedure using LC/MS/MS for the detection of some benzodiazepines in urine, including 7-aminonitrazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, alprazolam, alpha-hydroxyalprazolam, oxazepam, 3-OH-diazepam, and nordiazepam [4]. Following a single dose of lorazepam (2.5 mg), Kintz et al. were able to detect greater than 5 ng/mL of lorazepam in urine for up to 96 hours [5]. After the administration of a single oral dose of bromazepam (6 mg) and clonazepam (2 mg), Cheze et al. reported the bromazepam concentration above 5 ng/mL for 60 hours; 7-aminoclonazepam was detectable for at least 144 hours [6].

Blood is generally collected following traffic safety incidents, and it is also the main biological specimen analyzed following autopsy. However, the detection of benzodiazepines, particularly in blood, is not without difficulty, since the concentrations present, especially following prescrbed medical use, can be low. Several publications have addressed the issue of their analysis in plasma or serum; how-ever, few have attempted the detection in whole blood. Gunnar et al. [7] determined several benzo-diazepines in whole blood using extraction, deriva-tization, and GC/MS analysis.

An excellent publication by Laloup et al. reported the screening of urine, blood, and hair using tandem LC mass spectrometry for 26 benzodiazepines and metabolites [8]. While the authors list a primary transition and a qualifying ion for each drug, the authors noted that a second injection was required for further confirmation of positive samples monitoring two transitions per compound. Using the Agilent system, the monitoring of the qualifying ion and calculation of its ratio to the intensity of the primary transition is an integral part of the software package.

Experimental

Sample Preparation

Standards and Reagents

- Deuterated internal standards: D5-diazepam; D5-temazepam; D5-alprazolam D7-7-aminoflunitrazepam, D4-clonazepam, as well as unlabeled drug standards: 7-aminoflunitrazepam; 7-aminoclonazepam; 7-aminonitrazepam; α-OH-alprazolam; α-OH-triazolam; desalkylflurazepam, bromazepam; clonazepam; nitrazepam; triazolam; alprazolam; flunitrazepam; flurazepam; lorazepam; midazolam; chlordiazepoxide; diazepam, oxazepam, nordiazepam, temazepam were purchased from Cerilliant (Round Rock, TX).
- Mixed-mode solid-phase extraction columns (Clin II) were purchased from SPEWare (San Pedro, CA).
- All solvents were of HPLC grade or better; all reagents were ACS grade and purchased from Spectrum Chemical (Gardena, CA).

Standards (prepared in methanol)

- Internal standard mix: D7-7-aminoflunitrazepam; D5-alprazolam; D4-clonazepam; D5-temazepam; D5-oxazepam; D5-diazepam (1,000 ng/mL)
- Unlabeled drugs: 7-aminoflunitrazepam; 7-aminoclonazepam; 7-aminonitrazepam; α-OH-alprazolam; α-OH-triazolam; desalkylflurazepam; bromazepam; clonazepam; nitrazepam; triazolam; alprazolam; flunitrazepam; flurazepam; lorazepam; midazolam; chlordiazepoxide; diazepam, oxazepam, nordiazepam, temazepam

Extraction Procedure–Urine

Deuterated internal standard (100 µL) was added to urine (1 mL) and mixed.

Calibration Curve:

Negative:	100 µL of deuterated stock solution (1,000 ng/ mL)
10 ng/ mL:	100 µL of deuterated stock solution (1,000 ng/ mL) 10 µL of 1,000 ng/ mL stock solution
25 ng/ mL:	100 µL of deuterated stock solution (1,000 ng/ mL) 25 µL of 1,000 ng/ mL stock solution
50 ng/ mL:	100 µL of deuterated stock solution (1,000 ng/ mL) 50 µL of 1,000 ng/ mL stock solution
100 ng/ mL:	100 µL of deuterated stock solution (1,000 ng/ mL) 100 µL of 1,000 ng/ mL stock solution

A 2 M sodium acetate buffer (pH 5.0; 0.1 mL) was added, and for authentic specimens, α-glucuronidase (50 µL) was also added. The mixture was heated for 3 hours at 45 °C. Following centrifugation (10 min; 2,500 rpm), 0.1 M sodium phosphate buffer (pH 6.0, 1 mL) was added to the decanted upper layer supernatant. Extraction tubes were placed onto the vacuum manifold and conditioned with methanol (3 mL), deionized water (3 mL), and 0.1 M phosphate buffer (pH 6.0, 2 mL). The column bed was not allowed to dry. Each sample was poured through the column and allowed to dry, then rinsed with deionized water (3 mL), 0.1 M phosphate buffer pH 6.0: acetonitrile (80:20; 2 mL) and allowed to dry. Hexane was allowed to flow through the column (1 mL). Finally, the drugs were eluted in ethyl acetate + 2% ammonium hydroxide (2 mL). The eluates were evaporated to dryness under nitrogen (20 psi/37 °C) and reconstituted in methanol[1] (50 µL) for analysis.

[1]Since this work was completed it was found that reconstituting in water worked even more consistently than methanol.

Extraction Procedure–Blood

Acetonitrile (1 mL) was added to whole blood (1 mL). A mix of deuterated internal standards (100 µL; 50 ng/mL) was added and the sample was mixed, then centrifuged (20 min; 2,500 rpm). The supernatant was decanted and 0.025 M sodium phosphate buffer (pH 2.7; 1.5 mL) was added.

Calibration Curve:

Negative:	50 µL of deuterated stock solution (1,000 ng/ mL)
5 ng/ mL:	50 µL of deuterated stock solution (1,000 ng/ mL) 50 µL of 100 ng/ mL stock solution
10 ng/ mL:	50 µL of deuterated stock solution (1,000 ng/ mL) 10 µL of 1,000 ng/ mL stock solution
25 ng/ mL:	50 µL of deuterated stock solution (1,000 ng/ mL) 25 µL of 1,000 ng/ mL stock solution
50 ng/ mL:	50 µL of deuterated stock solution (1,000 ng/ mL) 50 µL of 1,000 ng/ mL stock solution
100 ng/ mL:	50 µL of deuterated stock solution (1,000 ng/ mL) 100 µL of 100 ng/ mL stock solution

Extraction tubes were placed onto the vacuum manifold and conditioned with methanol (3 mL), deionized water (3 mL), and 0.1 M phosphate buffer (pH 6.0; 2 mL). The column bed was not allowed to dry. Each sample was poured through the column and allowed to dry, then rinsed with deionized water (3 mL), 0.1 M phosphate buffer pH 6.0: acetonitrile (80:20; 2 mL) and allowed to dry. Hexane was allowed to flow through the column (1 mL). Finally, the drugs were eluted in ethyl acetate + 2% ammonium hydroxide (2 mL). The eluates were evaporated to dryness under nitrogen (20 psi / 37 °C) and reconstituted in methanol[2] (50 µL) for analysis.

[2]Since this work was completed it was found that reconstituting in water worked even more consistently than methanol.

Analytical Procedure–Both Urine and Blood

The 7-amino metabolites of flunitrazepam, nitrazepam, and clonazepam eluted from the analytical column rapidly, even though the flow rate was 0.2 mL/min. Optimization of the gradient and flow rate were attempted but did not give acceptable chromatography for the three metabolites. Subsequently, a separate method was implemented, lasting only 3.5 min and monitoring only those three metabolites. The chromatography and sensitivity were greatly improved by separating the two methods.

Both assays employed the Agilent 6410 LC Triple Quadrupole Mass Spectrometer (LC/MS/MS) incorporating an Agilent 1200 Series LC pump; ZORBAX Eclipse XDB C18 4.6 × 50 mm × 1.8-µm analytical column (Agilent PN: 922975-902); and an injection volume of 5 µL. Although the author (CM) obtained good results using the 4.6 mm i.d. column, the 2.1 mm i.d. column with 1.8 um-particle size is normally recommended by Agilent for increased sensitivity at the flow rates used.

The mass spectrometric parameters are shown in Table 1, qualifier ions in parentheses.

Benzodiazepines (except 7-amino metabolites)**:**

Column temperature:	35 °C	
Solvent flow rate:	0.2 mL/ min	
Mobile phase:	A = 20 mM ammonium formate (pH = 8.6) B = acetonitrile	
Isocratic:	50% B	
	Time (minutes)	Flow rate (mL/ min)
	0	0.2
	6.5	0.2
	8	1
	10	0.2
Post time:	4.5 min	

7-Amino Metabolites Only:

Column temperature:	45 °C
Solvent flow rate:	0.6 mL/ min
Mobile phase:	A = 20 mM ammonium formate (pH = 8.6) B = acetonitrile
Isocratic:	35% B
Stop time:	3.5 min

Mass Spectrometer Conditions:

Operation:	Electrospray positive mode	
	7-Amino metabolites	Other benzodiazepines
Gas temperature:	350 °C	300 °C
Gas flow (N_2):	6 L/ min	6 L/ min
Nebulizer pressure:	20 psi	15* psi
Capillary voltage:	4000 V	4500 V

* At LC flow rates of 0.6 mL/ min, nebulizer pressure settings as high as 50 psi are recommended for stable ion spray.

Table 1a. Acquisition Parameters: 7-Amino Metabolites

Compound	Start time (min)	Precursor ion	Product ion	Fragment voltage (V)	CE (V)
Segment 1					
D7-7-Aminoflunitrazepam	0	291	263	120	25
7-Aminoclonazepam	0	286	222 (121)	200	25 (25)
7-Aminonitrazepam	0	252	121 (208)	120	30 (35)
7-Aminoflunitrazepam	0	284	226 (256)	160	30 (25)

Table 1b. Acquisition Parameters: Benzodiazepines

Compound	Start time (min)	Precursor ion	Product ion	Fragment voltage (V)	CE (V)
Segment 1					
Bromazepam	0	316	288 (209)	160	20 (30)
Segment 2					
D4-Clonazepam	4.1	320	274	120	25
Clonazepam	4.1	316	270 (214)	120	25 (35)
α-Hydroxyalprazolam	4.1	325	297 (216)	120	30 (35)
α-Hydroxytriazolam	4.1	359	331 (176)	120	25 (25)
Lorazepam	4.1	321	275 (229)	140	25 (35)
Nitrazepam	4.1	282	236 (180)	160	25 (35)
D5-Alprazolam	4.1	314	286	160	25
Alprazolam	4.1	309	281 (274)	160	25 (30)
Chlordiazepoxide	4.1	300	283 (227)	120	15 (30)
D5-Oxazepam	4.1	292	246	120	20
Oxazepam	4.1	287	241 (269)	120	20 (20)
Triazolam	4.1	343	308 (239)	120	35 (35)
Segment 3					
Flunitrazepam	5.4	314	268 (239)	160	30 (35)
Midazolam	5.4	326	291 (249)	200	30 (40)
D5-Temazepam	5.4	306	260	120	25
Temazepam	5.4	301	255 (177)	120	35 (40)
Desalkylflurazepam	5.4	289	226 (261)	160	30 (25)
Nordiazepam	5.4	271	140 (165)	160	30 (30)
Segment 4					
5-Diazepam	7.2	290	262	160	25
Diazepam	7.2	285	257 (222)	160	25 (25)
Flurazepam	7.2	388	315 (288)	160	25 (25)

* ( ) qualifier ions; qualifier ratios must be within 20% of calibration point

LC/MS/MS Method Verification

The reproducibility of the analytical method was verified according to standard protocols, whereby the limit of quantita-tion, linearity range, correlation, and intra- and inter-day precision were determined via multiple replicates (n = 5) over a period of 5 days. The slope of the calibration curve was not forced through the origin. The equation of the calibration curves and correlation coefficients (R^2) are shown in Tables 2a (urine) and 2b (blood); the inter-day precision and accuracy of the assay are shown in Tables 3a and 3b, respectively. In addition, the intra-day precision and accuracy of the assay are shown in Tables 4a and 4b, respectively. The assay was robust, precise, and accurate at the selected level of 25 ng/mL and was linear over the range 5 to 100 ng/mL. The precision for all drugs was less than 20% both intra-day and inter-day, with most benzodiazepines showing a variation of less than 10%. One exception was 7-amnionitrazepam in urine, which showed a 24.4% variation over five

replicates. The limit of quantitation for all drugs was 5 ng/mL. Commonly encountered drugs were extracted and analyzed at high concentrations and found not to interfere with the assays.

Figure 1a shows a typical calibration curve for lorazepam in urine ($R^2 > 0.998$). Figure 1b shows a typical calibration curve for midazolam, with a correlation coefficient greater than 0.999.

Table 2a. Linearity, Correlation Coefficient, and Acceptable Qualifier Ratio for Benzodiazepines in Urine

Analyte	Equation	Correlation (R^2)	Qualifying ratio (20% range)
7-Aminoflunitrazepam	Y = 0.0210x – 0.0481	0.9985	69.4 (55.4–83.2)
7-Aminonitrazepam	Y = 0.5293x – 0.2512	0.9990	8.6 (6.9–10.3)
7-Aminoclonazepam	Y = 0.0523x – 0.1647	0.9959	84.5 (67.6–101.4)
α-Hydroxyalprazolam	Y = 0.0019x – 0.0053	0.9997	40.4 (32.3–48.5)
α-Hydroxytriazolam	Y = 0.000971x – 0.0024	0.9996	92 (73.6–110.45)
Alprazolam	Y = 0.0117x + 0.00063	0.9998	15.8 (12.6–18.9)
Bromazepam	Y = 0.0035x + 0.0095	0.9948	59.4 (47.5–71.25)
Chlordiazepoxide	Y = 0.0064x + 0.0284	0.9982	80.2 (64.1–96.2)
Clonazepam	Y = 0.0121x – 0.0342	0.9997	24.5 (19.5–29.3)
Desalkylflurazepam	Y = 0.0027x + 0.023	0.9986	26.7 (21.3–32)
Diazepam	Y = 0.0116x +0.0166	0.9996	82.5 (66–99)
Flunitrazepam	Y = 0.0025x – 0.000311	0.9994	49.4 (39.5–59.2)
Flurazepam	Y = 0.1291x + 0.2849	0.9993	13.6 (10.8–16.3)
Lorazepam	Y = 0.0104x – 0.0457	0.9981	34.2 (27.3–41)
Midazolam	Y = 0.0117x + 0.0149	0.9997	31.4 (25–37.6)
Nitrazepam	Y = 0.015x + 0.0176	0.9948	20 (34.9–52.3)
Nordiazepam	Y = 0.0032x + 0.0139	0.9998	65.8 (52.6–78.9)
Oxazepam	Y = 0.0079x – 0.0123	0.9999	24.3 (19.4–29.1)
Temazepam	Y = 0.0062x + 0.0011	0.9998	31 (24.8–37.2)
Triazolam	Y = 0.0076x + 0.0522	0.9983	92.1 (73.7–110.5)

Table 2b. Linearity, Correlation Coefficient, and Acceptable Qualifier Ratio for Benzodiazepines in Blood

Analyte	Equation	Correlation (R^2)	Qualifying ratio (20% range)
7-Aminoflunitrazepam	Y = 0.0199x – 0.0196	0.9997	73.3 (58.6–88)
7-Aminonitrazepam	Y = 0.525x – 0.2845	0.9985	7.3 (5.8–8.7)
7-Aminoclonazepam	Y = 0.0403x – 0.0429	0.9996	97.8 (78.2–117.3)
α-Hydroxyalprazolam	Y = 0.001x – 0.0016	0.9989	41.0 (32.8–49.2)
α-Hydroxytriazolam	Y = 0.00033x + 0.00065	0.9985	90.4 (72.3–108.5)
Alprazolam	Y = 0.0124x – 0.0092	0.9999	15.0 (12–18)
Bromazepam	Y = 0.0029x – 0.0128	0.9940	59.2 (47.4–71.1)
Chlordiazepoxide	Y = 0.0136x + 0.0708	0.9833	78.9 (63.1–94.7)
Clonazepam	Y = 0.0113x – 0.0332	0.9980	25.2 (20.2–30.3)
Desalkylflurazepam	Y = 0.0029x + 0.0006	0.9996	26.6 (21.3–31.9)
Diazepam	Y = 0.0105x – 0.0197	0.9992	83.3 (66.6–100)
Flunitrazepam	Y = 0.00083x + 0.00084	0.9989	49.7 (39.8–59.7)
Flurazepam	Y = 0.1303x + 0.1446	0.9994	13.8 (11.0–16.6)
Lorazepam	Y = 0.0153x – 0.0538	0.9971	35.1 (28.1–42.2)
Midazolam	Y = 0.0142x – 0.0088	0.9986	31.8 (25.4–38.2)
Nitrazepam	Y = 0.0273x + 0.0974	0.9951	42.7 (34.2–51.3)
Nordiazepam	Y = 0.0048x + 0.0058	0.9980	65.5 (52.4–78.6)
Oxazepam	Y = 0.009x – 0.0136	0.9997	23.6 (18.9–28.4)
Temazepam	Y = 0.0063x – 0.0041	0.9999	30.6 (24.5–36.7)
Triazolam	Y = 0.0032x + 0.00091	0.9966	92.7 (74.2–111.3)

Table 3a. Inter-Day Precision and Accuracy (25 ng/ mL Control Specimens; n = 5) for Benzodiazepines in Urine

Drug	Mean recovery (ng/ mL)	SD	Precision (%)	Accuracy (%)
7-Aminoclonazepam	25.18	3.15	12.5	99.29
7-Aminoflunitrazepam	23.92	1.55	6.47	104.52
7-Aminonitrazepam	23.52	2.14	9.09	106.29
α-Hydroxyalprazolam	24.8	1.74	7.02	100.81
α-Hydroxytriazolam	24.94	2.21	8.85	100.24
Alprazolam	25.5	0.81	3.16	98.04
Bromazepam	27.1	1.63	6.02	92.25
Chlordiazepoxide	25.3	1.35	5.32	98.81
Clonazepam	24.86	0.84	3.37	100.56
Desalkylflurazepam	26.16	0.3	1.13	95.57
Diazepam	25.02	1.01	4.04	99.92
Flunitrazepam	25.2	0.31	1.22	99.21
Flurazepam	25.64	1.4	5.46	97.5
Lorazepam	23.8	1.85	7.76	105.04
Midazolam	25.58	0.98	3.83	97.73
Nitrazepam	26.84	1.11	4.15	93.14
Nordiazepam	26.26	0.65	2.46	95.2
Oxazepam	24.94	0.55	2.19	100.24
Temazepam	25.4	0.34	1.34	98.43
Triazolam	27.16	1.96	7.23	92.05

Table 3b. Inter-Day Precision and Accuracy (25 ng/ mL Control Specimens; n = 5) for Benzodiazepines in Blood

Drug	Mean recovery (ng/ mL)	SD	Precision (%)	Accuracy (%)
7-Aminoclonazepam	26.3	1.46	5.54	105.2
7-Aminoflunitrazepam	24.84	1.05	4.24	99.36
7-Aminonitrazepam	25.1	1.57	6.27	100.4
α-Hydroxyalprazolam	24.62	0.88	3.56	98.48
α-Hydroxytriazolam	25.7	1.39	5.41	102.8
Alprazolam	24.56	0.42	1.72	98.24
Bromazepam	26.14	2.9	11.1	104.56
Chlordiazepoxide	25.26	4.03	15.94	101.04
Clonazepam	24.32	0.85	3.51	97.28
Desalkylflurazepam	25.54	0.53	2.06	102.16
Diazepam	24.84	0.59	2.39	99.36
Flunitrazepam	24.82	1.49	5.99	99.28
Flurazepam	26	1.05	4.04	104
Lorazepam	24.82	0.53	2.12	99.28
Midazolam	24.72	1.41	5.7	98.88
Nitrazepam	28.32	2.73	9.65	113.28
Nordiazepam	25.86	0.62	2.41	103.44
Oxazepam	24.32	0.89	3.67	97.28
Temazepam	24.72	0.41	1.65	98.88
Triazolam	25.8	3.41	13.22	103.2

Table 4a. Intra-Day Precision (n = 5) for Benzodiazepines in Urine

Drug	Mean recovery (ng/mL)	SD	Precision (%)
7-Aminoclonazepam	27.36	2.84	10.4
7-Aminoflunitrazepam	24.74	0.57	2.31
7-Aminonitrazepam	28.26	6.9	24.4
α-Hydroxyalprazolam	23.9	2.74	11.47
α-Hydroxytriazolam	23.6	3.16	13.4
Alprazolam	26.26	0.74	2.83
Bromazepam	23.5	3.93	16.7
Chlordiazepoxide	23.2	1.49	6.42
Clonazepam	25.9	0.29	1.13
Desalkylflurazepam	26.2	1.06	4.03
Diazepam	25.78	0.82	3.18
Flunitrazepam	25.42	0.79	3.13
Flurazepam	26.88	1.09	4.05
Lorazepam	24.78	0.47	1.9
Midazolam	25.8	0.74	2.86
Nitrazepam	27.62	1.76	6.37
Nordiazepam	25.28	0.47	1.77
Oxazepam	25.28	0.92	3.64
Temazepam	25.42	0.36	1.43
Triazolam	27.24	2.2	8.09

Table 4b. Intra-Day Precision (n = 5) for Benzodiazepines in Blood

Drug	Mean recovery (ng/mL)	SD	Precision (%)
7-Aminoclonazepam	24.02	1.57	6.52
7-Aminoflunitrazepam	23.82	1.35	5.67
7-Aminonitrazepam	28.64	1.04	3.86
α-Hydroxyalprazolam	24.36	1.77	7.28
α-Hydroxytriazolam	24.66	3.35	13.57
Alprazolam	24.6	0.33	1.35
Bromazepam	27.38	4.24	15.5
Chlordiazepoxide	25.52	2.69	10.54
Clonazepam	23.84	0.34	1.41
Desalkylflurazepam	26.96	2.32	8.61
Diazepam	24.96	1.82	7.29
Flunitrazepam	24.54	4.37	17.8
Flurazepam	25.74	0.55	2.12
Lorazepam	17.66	2.38	13.48
Midazolam	23.74	1.53	6.43
Nitrazepam	30.52	2.88	9.45
Nordiazepam	27.28	2.76	10.1
Oxazepam	23.84	0.6	2.51
Temazepam	25.04	0.53	2.12
Triazolam	26.02	4.17	16.02

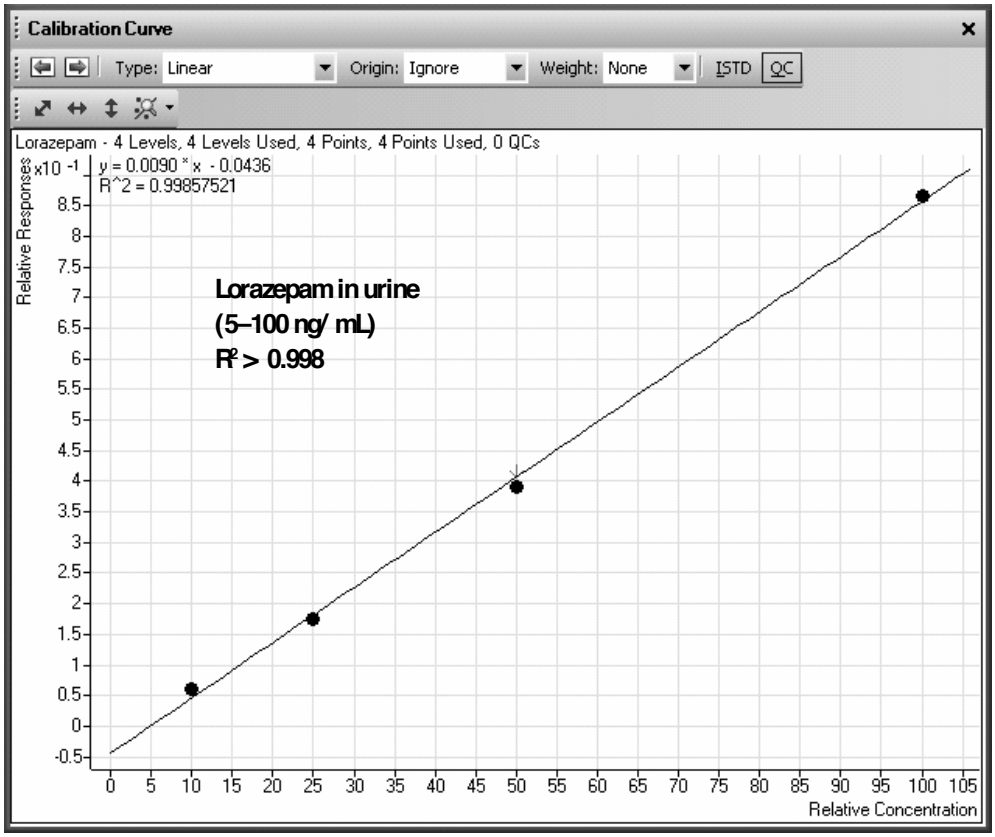

Figure 1a. Calibration curve for lorazepam in urine (5, 10, 25, 50, and 100 ng/mL).

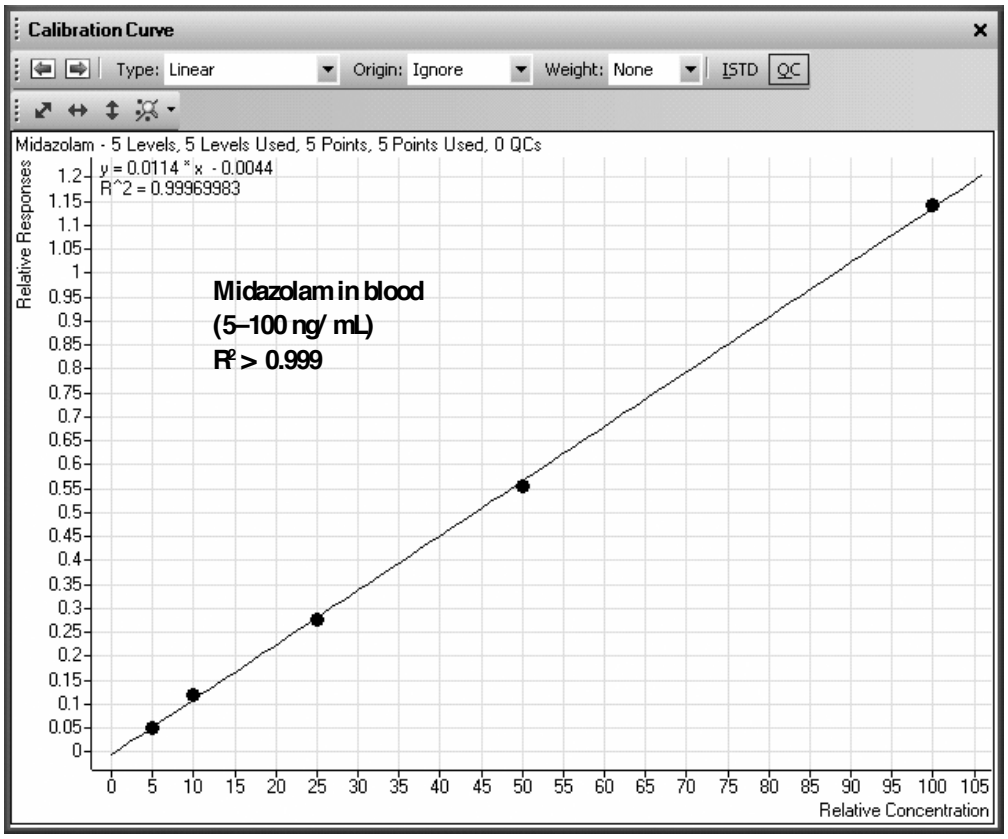

Figure 1b. Calibration curve for midazolam in blood (5, 10, 25, 50, and 100 ng/ mL).

Discussion

The Agilent instrumentation allowed the rapid determination of 14 benzodiazepines and six metabolites in urine and blood. The chromatographic separation produced by the small-particle analytical column allowed separation of the peaks in each group segment (Figures 2a and 2b, respectively). The metabolites 7-aminonitrazepam, flunitrazepam, and clonazepam showed poor chromatography when analyzed on this LC program, so they were analyzed separately in a fast run (3.5 min).

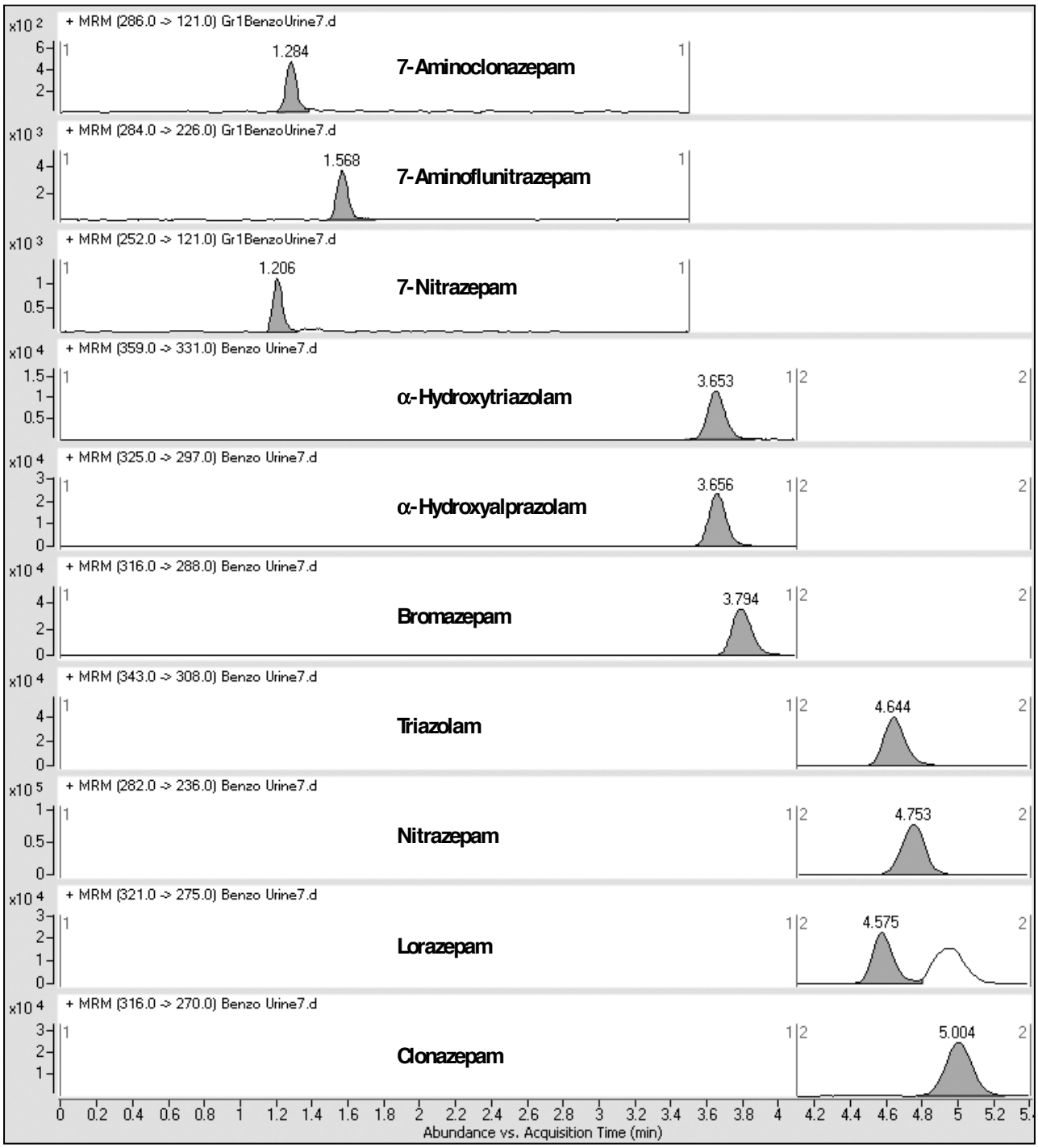

Figure 2a. Benzodiazepines extracted from urine (25 ng/ mL) : primary transitions, for clarity, internal standards not shown.

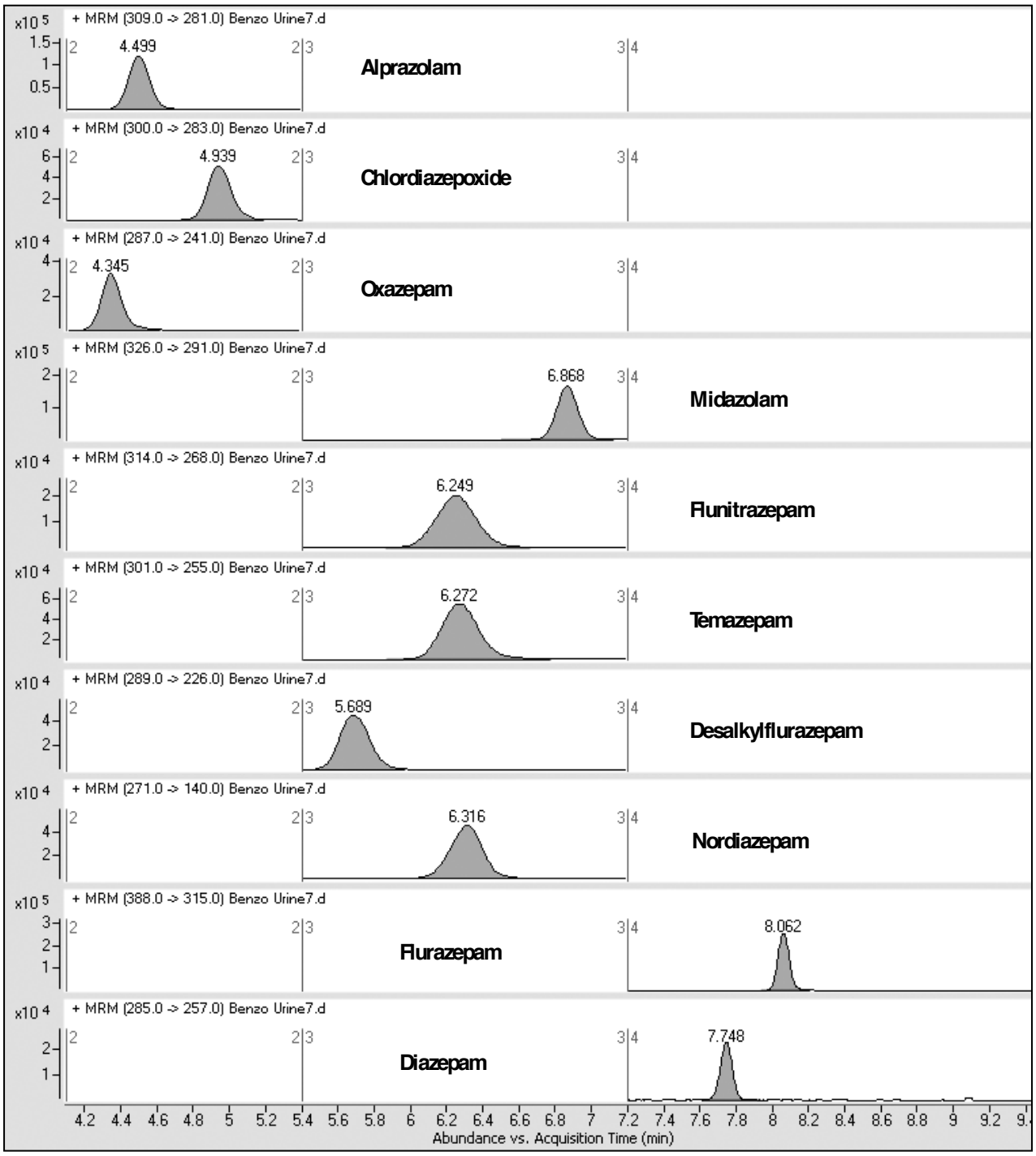

Figure 2a. Benzodiazepines extracted from urine (25 ng/ mL): primary transitions, for clarity, internal standards not shown. (continued)

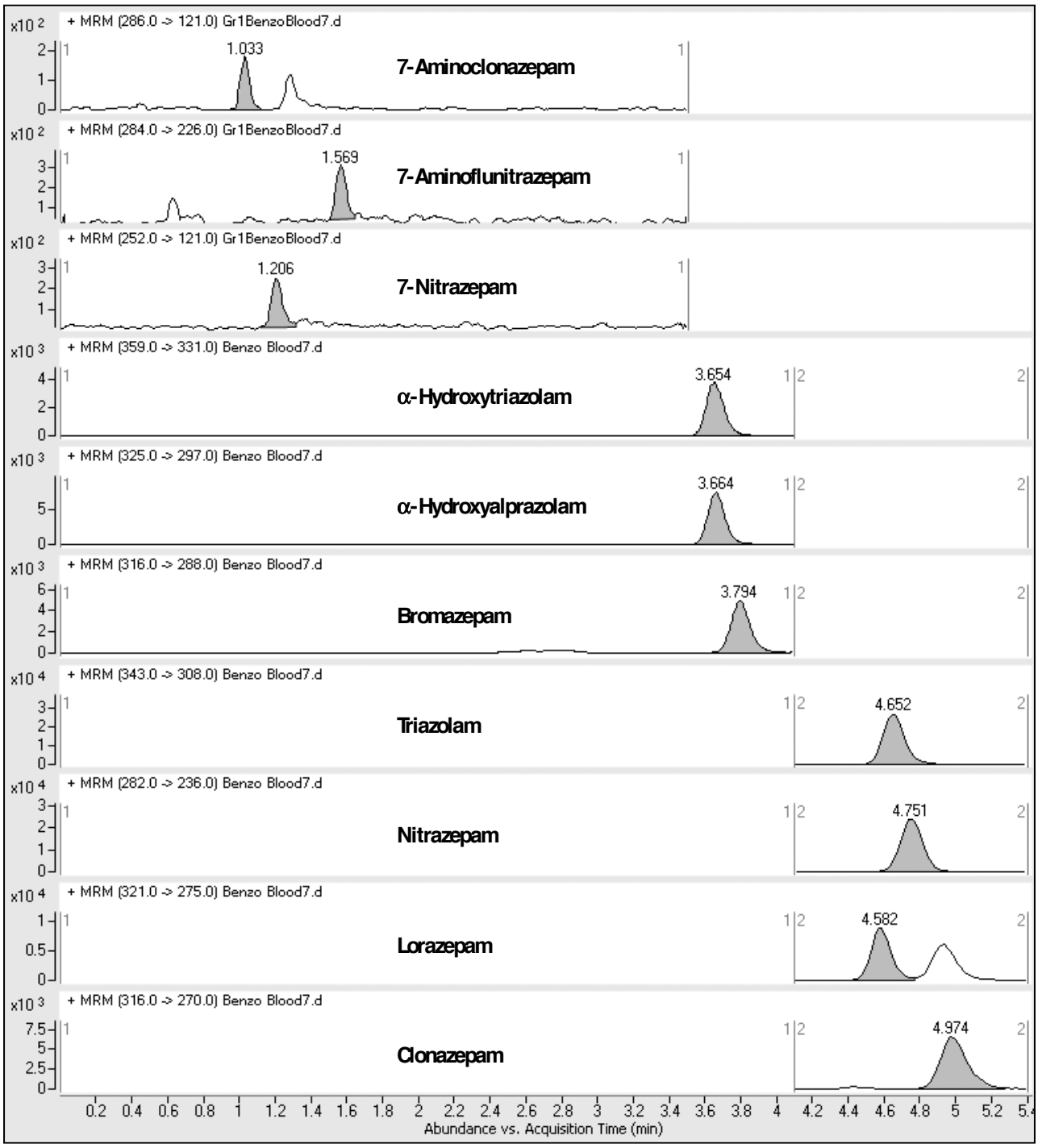

Figure 2b. Benzodiazepines extracted from blood (25 ng/ mL): primary transitions, for clarity, internal standards not shown.

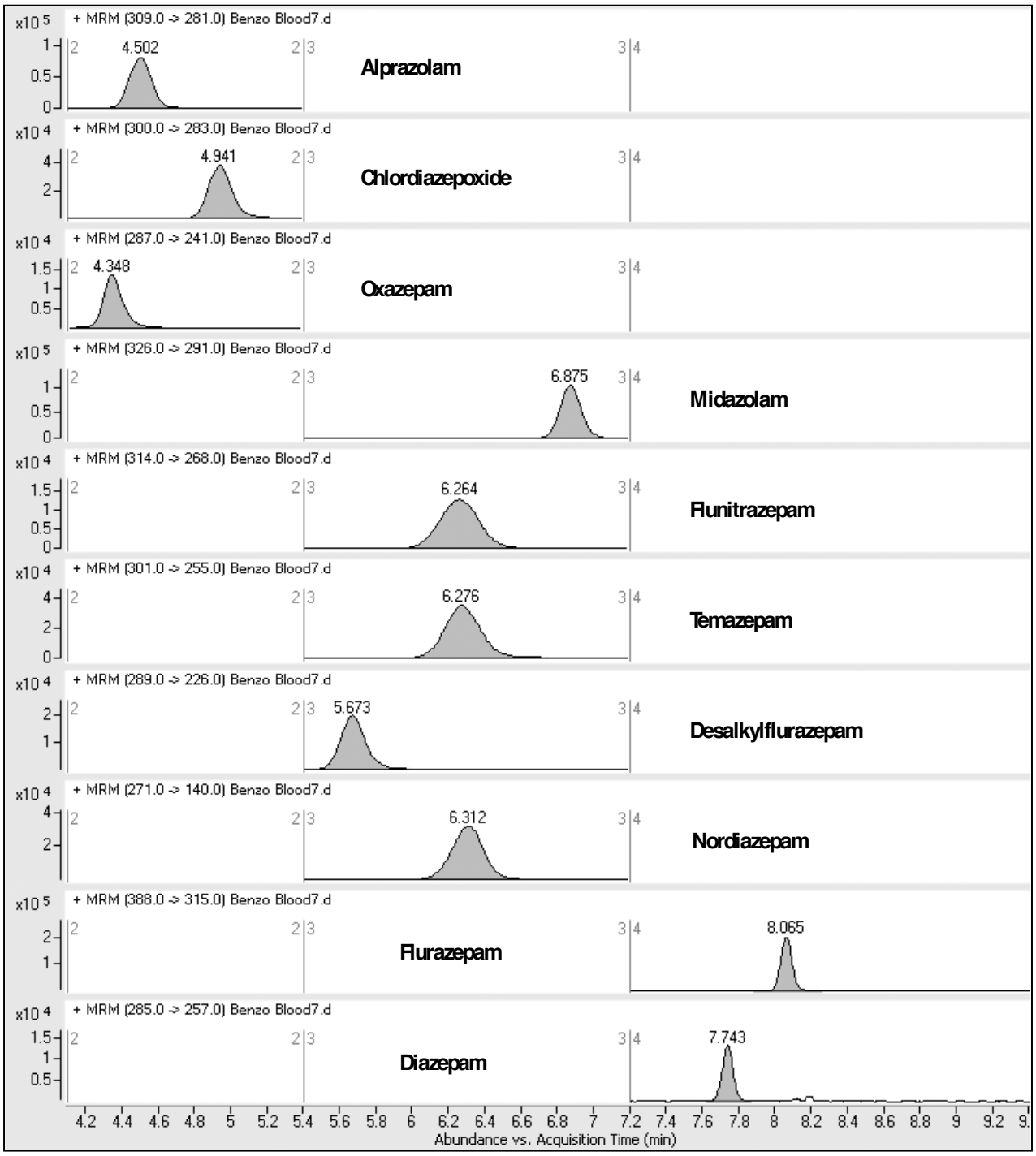

Figure 2b. Benzodiazepines extracted from blood (25 ng/ mL): primary transitions, for clarity, internal standards not shown. (continued)

The software provided with the instrument is unique in its ability to monitor a secondary transition from the precursor ion and automatically calculate the ratio to the primary ion. If the ratio is not within 20% of a calibration standard, the identification is rejected. This is an additional feature of the triple quadrupole mass spectrometer, which is extremely important in forensic analysis, where court challenges to laboratory data are frequent.

Monitoring a second transition gives additional confidence in the result; applying a ratio to that second transition compared to the primary product ion is a further enhancement to the identification of drugs in urine. The software plots the ratio in the chromatographic window, so the operator is able to assess positiveness visually using the "uncertainty" band imposed by the software (Figure 3a: urine; Figure 3b: blood).

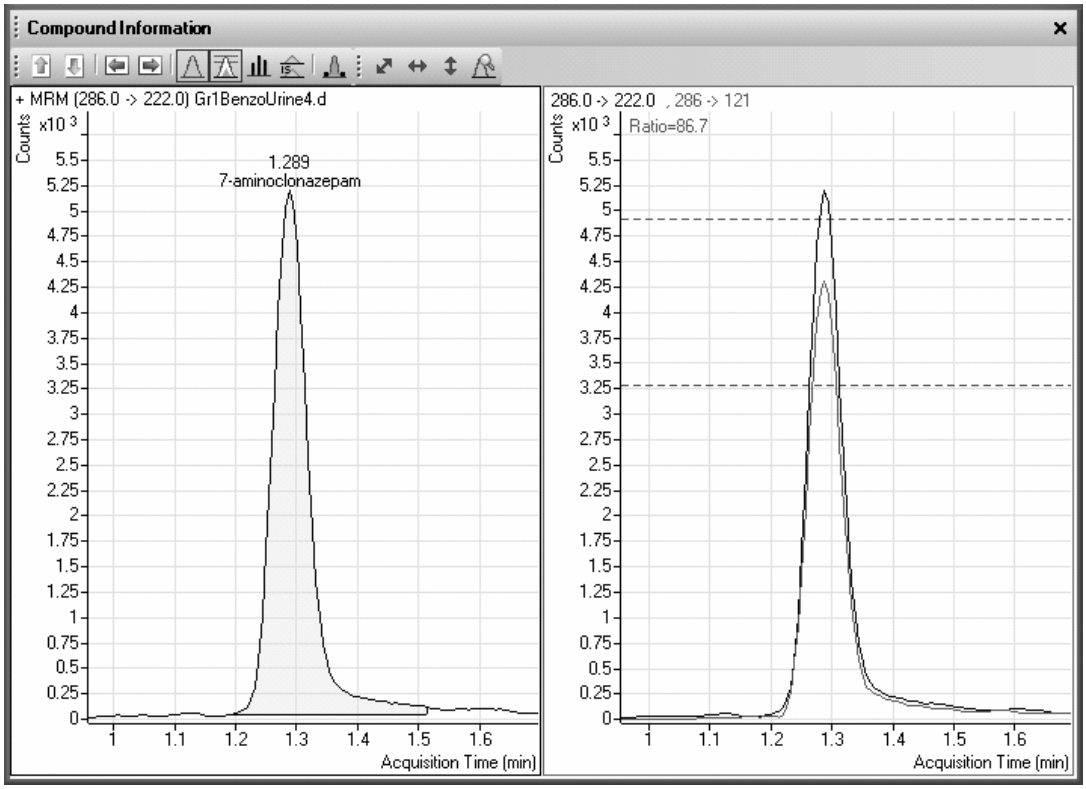

Figure 3a. 7-Aminoclonazepam extracted from urine (50 ng/ mL) showing qualifying ion (normalized by area) and acceptable ratio 86.7 with ± 20% tolerance (range: 69.4–104.0).

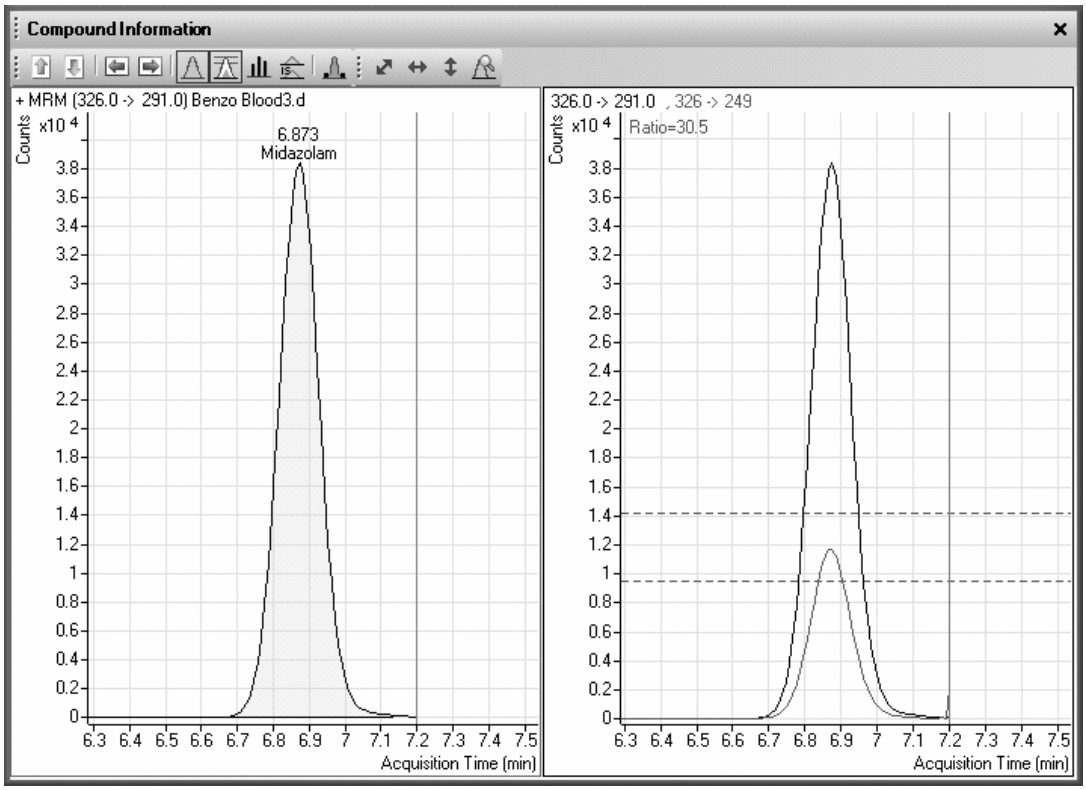

Figure 3b. Midazolam extracted from blood (10 ng/ mL) showing qualifying ion and acceptable ratio 30.5 with ± 20% tolerance (range: 24.4–36.6).

Conclusions

The procedure described is suitable for the detection of benzodiazepines in urine using an Agilent Technologies triple quadrupole LC/MS/MS system. To our knowledge, this is the first method where the intensity of qualifying transitions are required to be within a specific ratio compared to the primary transition.

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