The Analysis of Benzodiazepines in Hair Using RRHT LC/MS/MS

Application

Forensics

Authors

Christine Moore, Cynthia Coulter, and Katherine Crompton
Immunalysis Corporation
829 Towne Center Drive
Pomona, CA 91767
USA

Michael Zumwalt
Agilent Technologies, Inc.
9780 S. Meridian Blvd.
Englewood, CO 80112
USA

Abstract

A quantitative analytical procedure for the determination of benzodiazepines and metabolites in hair has been developed and validated. The hair samples were washed, incubated, and any drugs present were quantified using mixed mode solid-phase extraction and liquid chromatography with tandem mass spectrometric detection (LC/MS/MS) in positive electrospray ionization mode. The liquid chromatography is carried out on a ZORBAX Rapid Resolution High Throughput (RRHT) C18 column, which has a 1.8-µm particle size.

For confirmation, two transitions were monitored and one ion ratio was determined, which was within 20% of that of the known calibration standards. The range of concentration analyzed for each compound was 50 to 1,000 pg/mg hair. The intra-day precision of the assays at 100 pg/mg (n = 5) was as low as 1.75% for 7-aminoclonazepam, and as high as 11.8% for a-OH-alprazolam. Inter-day precision (once each day for five days) ranged from as low as 2.55% for diazepam to as high as 13.4% for 7-aminoclonazepam.

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 frequently prescribed sedative, anti-anxiety, hypnotic, and muscle-relaxants. They exert an additive effect when used in conjunction with alcohol or other drugs, and are subject to abuse and show potential for addiction. In particular, health-care professionals have higher rates of abuse with benzodiazepines and opiates than other drugs [1]. Using hair as a biological specimen allows a more historical perspective on the drug use of an individual, depending upon the length of the hair tested, compared to blood or urine, and may be a useful specimen for inclusion in the testing of medical professionals seeking to regain licensing or who are subject to frequent testing.

In 2003, Scott and Nakahara showed the incorporation of eight benzodiazepines into hair [2], while others have reported single drugs for example in cases of drug-facilitated sexual assault [3]. Miller et al recently reported the detection of nine benzodiazepines in hair using immunoassay and LC/MS/MS and their application to authentic spec-

imens. The concentration of drugs found in the hair samples ranged from 30 to well over 200 pg/mg for diazepam [4].

We report the detection of 14 benzodiazepines and 5 metabolites in hair. The procedure includes 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 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. In some cases, monitoring a qualifying transition may inhibit the sensitivity of the assay, but the additional confidence in the result is a critical factor in forensic analysis. The limit of quantitation was 50 pg/mg of hair; the intra-day precision of the assays (n = 5) ranged from 1.75 % for 7-aminoclonazepam to 11.78% for α-hydroxyalprazolam; and the inter-day precision ranged from 2.55% for diazepam to 13.37% for 7-aminoclonazepam (n = 5).

As these compounds have been analyzed in blood and urine in another Agilent application note (5989-7072EN) the reader is referred to that application note for illustrated structures of these compounds.

Experimental

Sample Preparation

Solvents and Reagants

All solvents were of HPLC grade or better; all reagents were ACS grade and purchased from Spectrum Chemical (Gardena, CA).

Standards (purchased from Cerilliant, Round Rock, TX)

Internal standard mix: D7-7-aminoflunitrazepam; D5-alprazolam; D4-clonazepam; D5-temazepam; D5-oxazepam; D5-nordiazepam; D5-diazepam (100 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

For each calibration level used for quantitation, an aliquot of hair (10 mg) was briefly rinsed with methylene chloride (1.5 mL) to remove hair treatments such as mousse, spray, gels, etc., and allowed to dry. The hair was cut into small pieces and both analyte and deuterated internal standard were added as shown below.

Calibration curve:

Negative:	50 µL of deuterated stock solution (100 ng/mL)
50 pg/mg:	50 µL of deuterated stock solution (100 ng/mL) 5 µL of 100 ng/mL stock solution
100 pg/mg:	50 µL of deuterated stock solution (100 ng/mL) 10 µL of 100 ng/mL stock solution
500 pg/mg:	50 µL of deuterated stock solution (100 ng/mL) 50 µL of 100 ng/mL stock solution
1 ng/mg:	50 µL of deuterated stock solution (100 ng/mL) 100 µL of 10 ng/mL stock solution

Deuterated internal standard (50 µL) was also added to proficiency samples used in the validation study.

Add hair extraction buffer (0.025 M phosphate buffer, pH 2.7; 1.5 mL); mix

Sonicate (2 hrs; 75°C); decant liquid

Add 0.1 M sodium phosphate buffer (pH 6.0, 1 mL); vortex

Place extraction tubes (CSDAU020) onto the vacuum manifold

Condition each column:

methanol (3 mL)
deionized water (3 mL)
0.1 M phosphate buffer (pH 6.0, 2 mL)

Important: Do not allow the column bed to go dry.

Pour sample through column. Dry.

Rinse each column with:

Deionized water (3 mL),
0.1 M phosphate buffer pH 6.0: acetonitrile (80:20; 2 mL)
Dry column; wash column with hexane (1 mL)

Elute drugs: ethyl acetate + 2% ammonium hydroxide (2 mL)
Evaporate to dryness under nitrogen (20 psi/ 37 °C)
Reconstitute in water (50 µL); transfer to autosampler vials; cap

Analytical Procedure

Instrument: Agilent 1200 Series RRLC; 6410 LC Triple Quadrupole Mass Spectrometer

LC Conditions:

Column:	ZORBAX RRHT Eclipse XDB C18, 4.6 mm x 50 mm x 1.8 µm (PN: 922975-902)

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 was 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. Although the author (CM) obtained good results using the 4.6-mm id column, the 2.1-mm id column with 1.8-µm particle size is normally recommended by Agilent for increased sensitivity at the flow rates used.

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
Post time:	Off

Benzodiazepines (except 7-amino metabolites):

Column temperature:	35 °C
Solvent flow rate:	0.2 mL/min (initial)
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

Stop time = 10 min; Post time = 5 min

MS Conditions:

Operation:	Electrospray ESI 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	50 psi
Capillary voltage:	4000 V	4500 V

The multiple reaction monitoring (MRM) transitions are shown in Table 1. For all compounds, the first quadrupole, for the precursor ion, is operated at low resolution, or full width half maximum (FWHM) equal to 2.5 amu. The last quadrupole, for the product ion, is operated at unit resolution, or FWHM = 0.7 amu.

Retention times are given as used in the quantitation method. The two parameters requiring optimization for each compound include the fragmentor (Frag) voltage and the collision energy (CE), expressed in units of voltage. The fragmentor is part of the ion transfer optics located between the ion source and the mass analyzer, responsible for transferring the precursor ion mass of the specified compound. This parameter is optimized for each compound using flow injection analysis (FIA) of the corresponding standard in which the fragmentor voltage is varied with each injection and the voltage for the optimal response is determined.

Once the fragmentor voltage is optimized, the collision energy voltages are determined for which an optimal response of both the quantifier and the qualifier ions are obtained. The quantifier ion corresponds to the product ion that has the best signal response overall. The qualifier ion corresponds to the second most-intense product ion and is used for confirmation based on its peak area ratio versus that of the quantifier ion.

Table 1. Multiple Reaction Monitoring (MRM) Ttransitions for the Benzodiazepines Analyzed in the Work

Compound	RT (min)	MRM transition	Frag (V)	CE (V)
7-amino metabolites only:				
D7-7-aminoflunitrazepam	1.102	291 > 263	120	25
7-aminoclonazepam	0.94	286 > 222 (121)	200	25 (25)
7-aminonitrazepam	0.95	252 > 121 (208)	120	30 (35)
7-aminoflunitrazepam	1.104	284 > 226 (256)	160	30 (25)
Remaining benzodiazepines: **Segment 1 (0.0 min)**				
α-OH-triazolam	3.71	359 > 331 (176)	120	25 (25)
α-OH alprazolam	3.72	325 > 297 (216)	120	30 (35)
Bromazepam	3.85	316 > 288 (209)	160	20 (30)
Segment 2 (4.1 min)				
D5-oxazepam	4.40	292 > 246	120	20
Oxazepam	4.44	287 > 241 (269)	120	20 (20)
D5-alprazolam	4.57	314 > 286	160	25
Alprazolam	4.63	309 > 281 (274)	160	25 (30)
Lorazepam	4.67	321 > 275 (229)	140	25 (35)
Triazolam	4.79	343 > 308 (239)	120	35 (35)
Nitrazepam	4.85	282 > 236 (180)	160	25 (35)
Chlordiazepoxide	5.07	300 > 283 (227)	120	15 (30)
D4-clonazepam	5.07	320 > 274	120	25
Clonazepam	5.12	316 > 270 (214)	120	25 (35)
Segment 3 (5.6 min)				
D5-temazepam	6.34	306 > 260	120	25
Temazepam	6.43	301 > 255 (177)	120	35 (40)
Flunitrazepam	6.44	314 > 268 (239)	160	30 (35)
Nordiazepam	6.46	271 > 140 (165)	160	30 (30)
Midazolam	7.05	326 > 291 (249)	200	30 (40)
Segment 4 (7.4 min)				
D5-diazepam	7.78	290 > 262	160	25
Diazepam	7.83	285 > 257 (222)	160	25 (25)
Flurazepam	8.08	388 > 315 (288)	160	25 (25)

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

LC/MS/MS Method Validation

The analytical method was validated according to standard protocols, whereby the 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 forced through the origin. The typical equations of the calibration curves and correlation coefficients (R^2) are shown in Table 2; the inter-day precision and accuracy of the assay are shown in Table 3. In addition, the intra-day precision and accuracy of the assay are shown in Table 4. The assay was robust, precise, and accurate at the selected level of 100 pg/mg and was linear over the range 50 to 1,000 pg/mg. The precision for all drugs was less than 20% both within day and between days, with most benzodiazepines showing a variation of less than 10%.

Figure 1 shows a typical calibration curve for oxazepam in urine ($R^2 > 0.9996$).

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

Analyte	Equation	Correlation (R^2)	Qualifying ratio (20% range)
7-aminoflunitrazepam	y = 0.0013x	0.9984	69.4 (55.5–83.3)
7-aminonitrazepam	y = 0.0112x	0.9678	8.6 (6.9–10.3)
7-aminoclonazepam	y = 0.0027x	0.9978	84.5 (67.6–101.4)
α-hydroxyalprazolam	y = 0.0001x	0.9992	51.7 (41.4–62.0)
α-hydroxytriazolam	y = 0.000073x	0.9964	95.5 (76.4–114.6)
Alprazolam	y = 0.001x	0.9999	15.6 (12.5–18.7)
Bromazepam	y = 0.00035x	0.9974	61.3 (49.0–73.6)
Chlordiazepoxide	y = 0.0004x	0.9996	91.3 (73.0–109.6)
Clonazepam	y = 0.0015x	0.9999	30.1 (24.1–36.1)
Diazepam	y = 0.0012x	0.9987	76.0 (60.8–91.2)
Flunitrazepam	y = 0.00038x	0.9946	56.5 (45.2–67.8)
Flurazepam	y = 0.0011x	0.9998	11.9 (9.5–14.3)
Lorazepam	y = 0.00005x	0.9832	34.5 (27.6–41.4)
Midazolam	y = 0.00064x	0.9994	31.2 (25.0–37.4)
Nitrazepam	y = 0.00026x	0.997	47.7 (38.1–57.2)
Nordiazepam	y = 0.00036x	0.9955	59.6 (47.7–71.5)
Oxazepam	y = 0.001x	0.9996	26.0 (20.8–31.2)
Temazepam	y = 0.00045x	0.9987	39.1 (31.3–46.9)
Triazolam	y = 0.00036x	0.9998	75.2 (60.2–90.2)

Table 3. Inter-Day Mean, Standard Deviation (SD), Precision (CV), and Accuracy (100 pg/mg Control Specimens; n = 5) for Benzodiazepines in Hair

Analyte	Mean	SD	CV (%)	Accuracy (%)
7-aminoflunitrazepam	103.38	13.80	13.35	96.73
7-aminonitrazepam	93.72	11.54	12.31	106.70
7-aminoclonazepam	101.50	13.57	13.37	98.52
α-hydroxyalprazolam	105.56	3.23	3.06	94.73
α-hydroxytriazolam	106.38	3.91	3.67	94.00
Alprazolam	97.70	6.77	6.93	102.35
Bromazepam	98.78	5.42	5.49	101.24
Chlordiazepoxide	95.24	9.07	9.52	105.00
Clonazepam	101.66	5.59	5.50	98.37
Diazepam	100.38	2.56	2.55	99.62
Flunitrazepam	100.52	12.24	12.18	99.48
Flurazepam	96.98	11.44	11.80	103.11
Lorazepam	107.72	12.38	11.50	92.83
Midazolam	97.18	6.38	6.57	102.90
Nitrazepam	107.90	7.03	6.51	92.68
Nordiazepam	106.14	5.25	4.95	94.22
Oxazepam	100.28	11.33	11.30	99.72
Temazepam	97.56	4.66	4.78	102.50
Triazolam	103.52	10.82	10.45	96.60

Table 4. Intra-Day Mean, Standard Deviation, Precision, and Accuracy (100 pg/mg Control Specimens; n = 5) for Benzodiazepines in Hair

Analyte	Mean	SD	CV (%)	Accuracy (%)
7-aminoflunitrazepam	99.78	5.43	5.44	100.22
7-aminonitrazepam	107.73	12.28	11.40	92.83
7-aminoclonazepam	110.58	1.94	1.75	90.43
α-hydroxyalprazolam	93.24	10.99	11.78	107.25
α-hydroxytriazolam	97.00	5.13	5.29	103.09
Alprazolam	97.72	4.28	4.38	102.33
Bromazepam	93.00	7.13	7.66	107.53
Chlordiazepoxide	91.36	7.00	7.66	109.46
Clonazepam	92.98	5.32	5.72	107.55
Diazepam	102.32	3.70	3.62	97.73
Flunitrazepam	106.24	4.87	4.59	94.13
Flurazepam	87.98	4.98	5.66	113.66
Lorazepam	99.86	5.39	5.40	100.14
Midazolam	94.52	6.79	7.18	105.80
Nitrazepam	104.48	6.63	6.35	95.71
Nordiazepam	107.38	5.32	4.96	93.13
Oxazepam	91.62	9.29	10.14	109.15
Temazepam	93.66	3.12	3.33	106.77
Triazolam	107.80	5.05	4.68	92.76

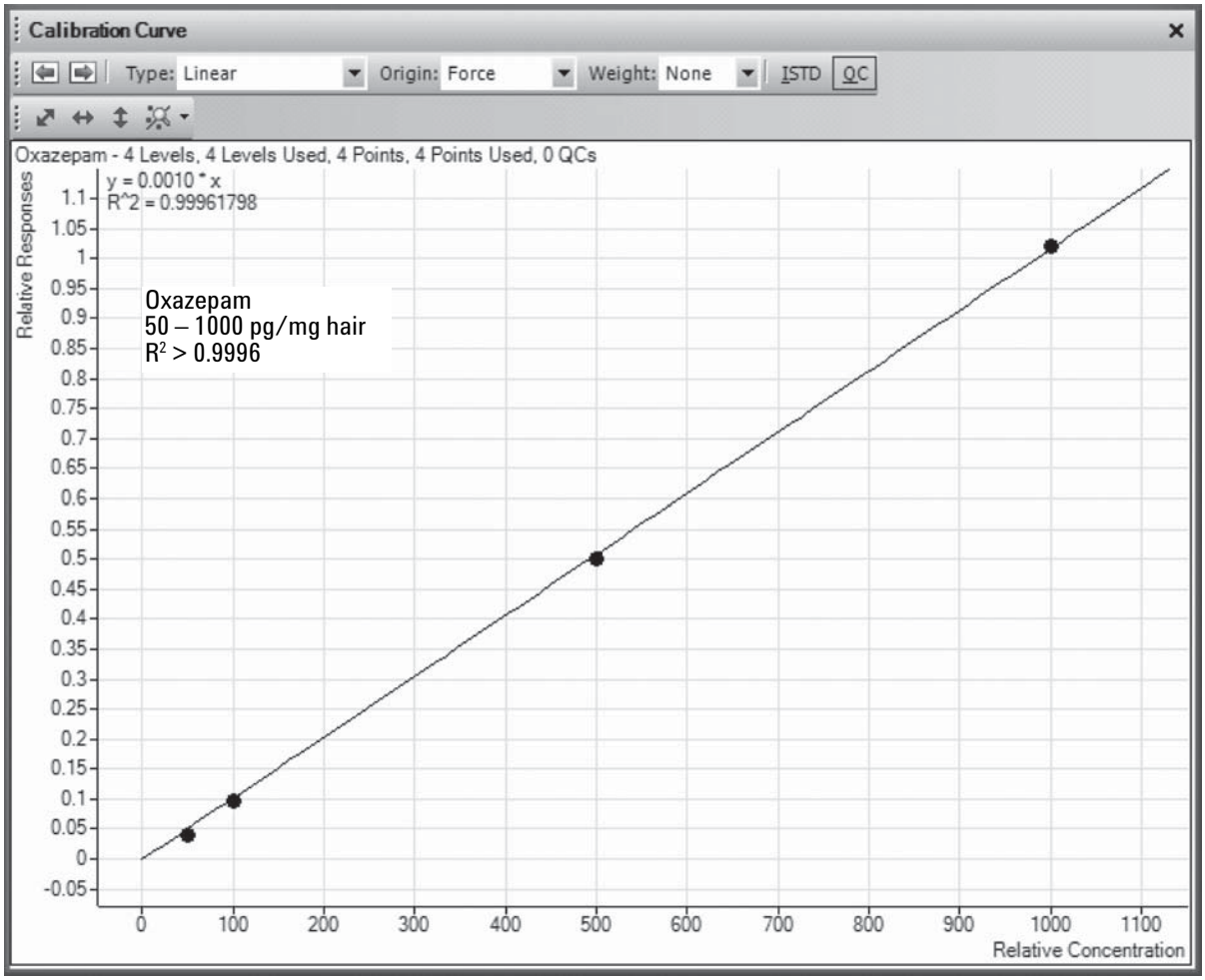

Figure 1. Calibration curve for oxazepam using a linear fit, forced origin, and no weighting.

Results and Discussion

The Agilent instrumentation allowed the rapid determination of 14 benzodiazepines and 5 metabolites in hair. The chromatographic separation produced by the small-particle analytical column allowed separation of the peaks in each group segment (Figure 2). 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).

In Figure 3 is shown the confirmation of midazolam in hair at the 50 pg/mg level. The requirement for confirmation used in this work is that the peak area ratio of the quantifier and the qualifier ions must be within a tolerance of ± 20% of the expected ratio. For this calibration level the expected ratio is 31%, which is within the tolerance of the 35% found.

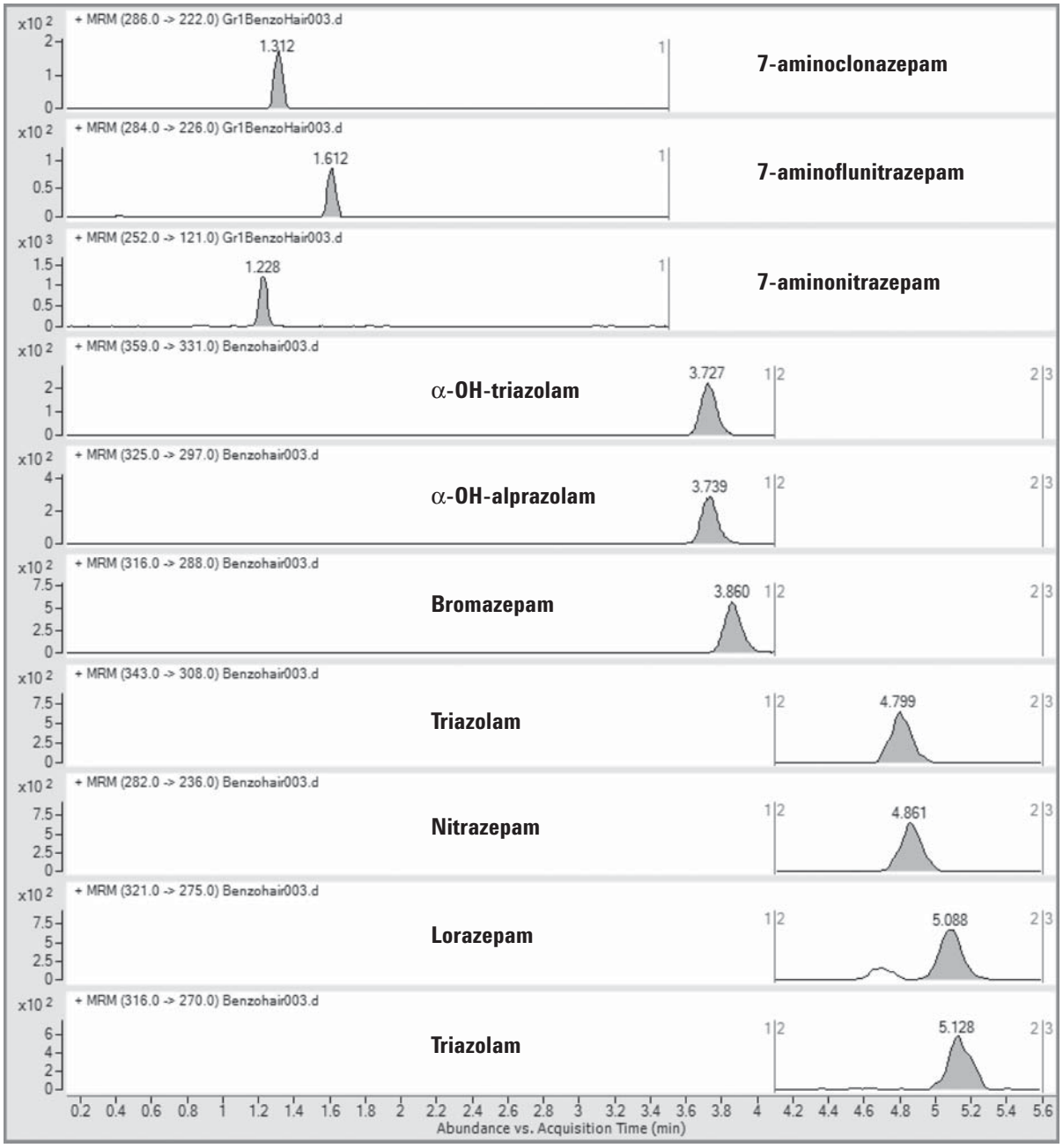

Figure 2. Benzodiazepines extracted from hair (100 pg/mg): primary transitions (quantifiers).

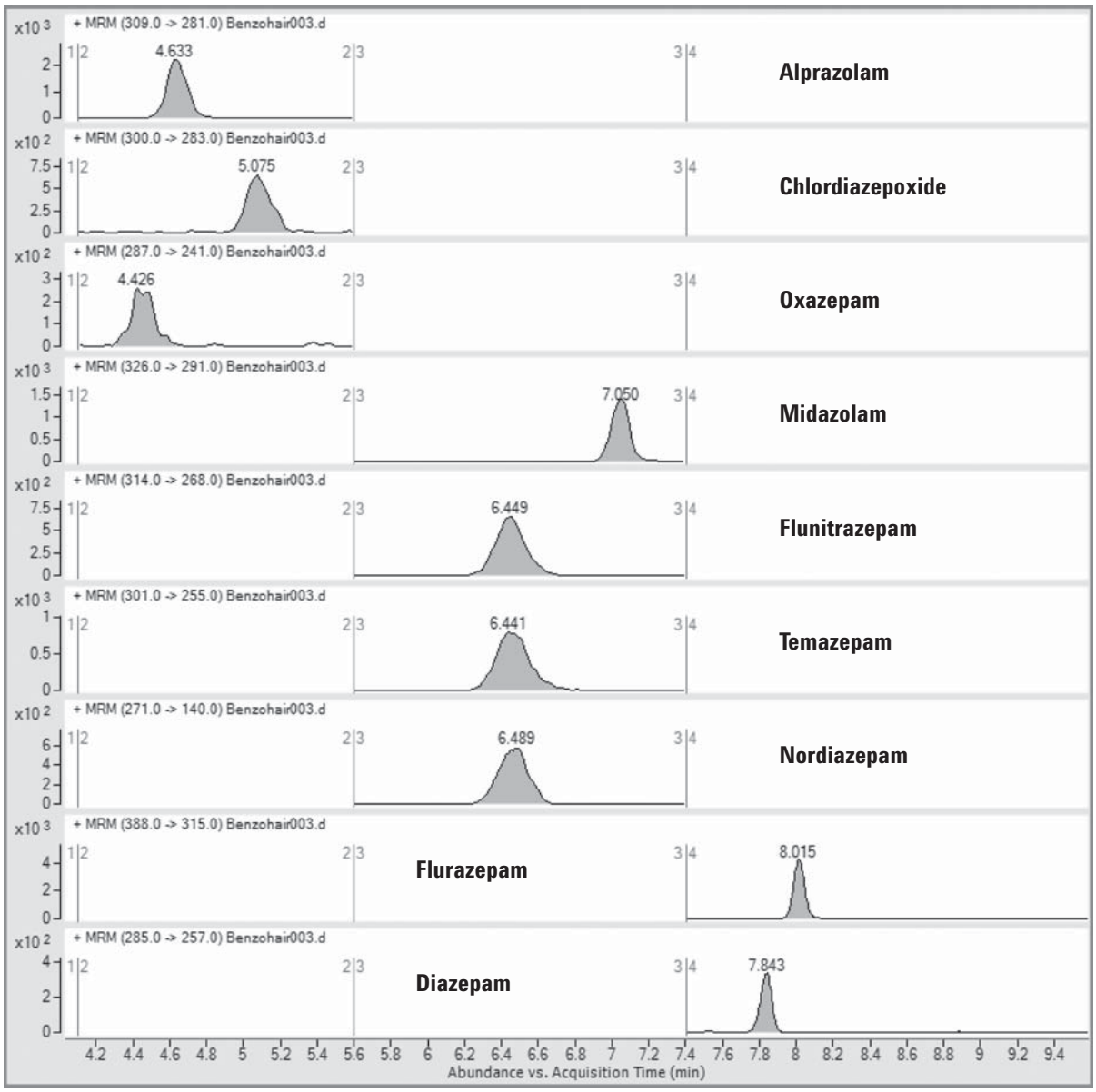

Figure 2. Benzodiazepines extracted from hair (100 pg/mg): primary transitions (quantifiers). (continued)

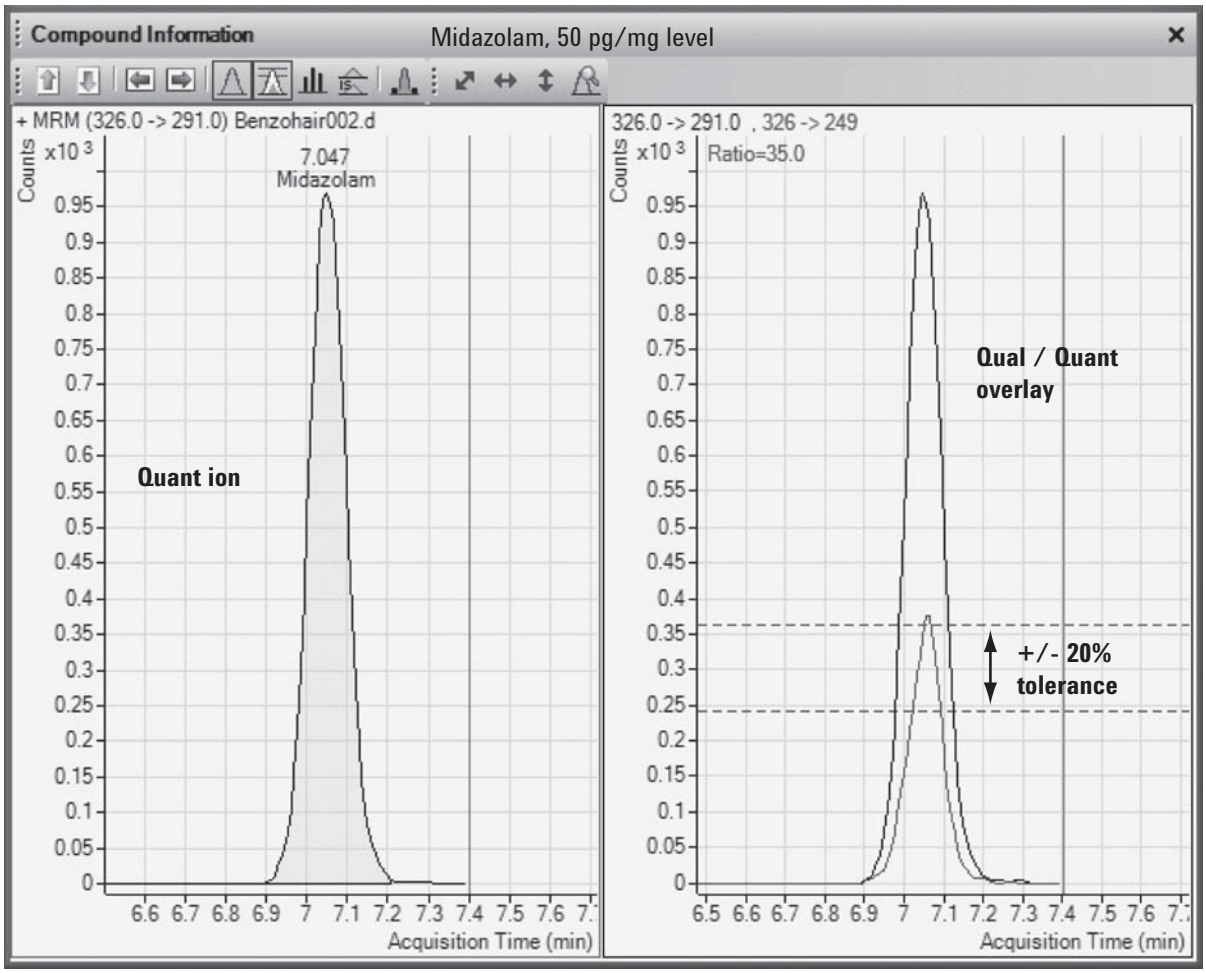

Figure 3. Confirming presence of midazolam using qualifier to quantifier ion peak area ratio.

Conclusions

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

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