Determination of Benzodiazepines in Oral Fluid Using LC/MS/MS

Application Note

Forensic Toxicology

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Abstract

A rapid, simple, highly sensitive procedure for the simultaneous analysis of 14 benzodiazepines in oral fluid, using the Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ) in electrospray mode, is described. Sample preparation includes 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 for the treatment of anxiety and insomnia, particularly in the elderly [1]. They are also used as muscle relaxants and anticonvulsants. They are commonly detected in incidents of driving under the influence of drugs (DUID), often in combination with other medications [2,3]. Oral fluid is becoming increasingly used as a specimen in many areas of forensic interest, including collection at the roadside during traffic stops. Its ease of collection, difficulty of adulteration, and applicability to routine testing has promoted its use as a valid test specimen. However, the detection of benzodiazepines in particular in oral fluid is not without difficulty since the saliva:plasma ratio for most of the drug class is low.

One of the main issues with the quantitation of drugs in oral fluid is the difficulty of collection in terms of specimen volume. Many of the currently available devices do not give an indication of how much oral fluid is collected, thereby rendering any quantitative results meaningless without further manipulation in the laboratory [4,5]. Further, devices incorporating a pad or material for the saliva collection do not always indicate how much of each drug is recovered from the pad before analysis, again calling into question any quantitative result. The drug concentration reported is dependent on the collection procedure used [6].

This work employs the Quantisal oral fluid collection device, which collects a known amount of neat oral fluid. The efficiency of recovery of the benzodiazepines from the collection pad into the trans-

portation buffer is determined, in order to increase confidence in the quantitative value.

Several publications have addressed the issue of the analysis of benzodiazepines in oral fluid. Quintela et al. [7] determined nine benzodiazepines in neat oral fluid using an LC/MS procedure. They included lormetazepam and tetrazepam, which were not in our profile; however, clonazepam, chlordiazepoxide, nordiazepam, temazepam, oxazepam, flurazepam, and nitrazepam were not included.

A recent publication from Oiestad et al reported the screening of oral fluid using tandem LC mass spectrometry for several drugs, including benzodiazepines [8]. They analyzed fenazepam and some benzodiazepine metabolites, which we did not include (see below); but they did not include the prescribed drugs triazolam, temazepam, midazolam, flurazepam, or chlordiazepoxide. Smink et al. [9] analyzed urine and oral fluid for 33 benzodiazepines using LC/MS/MS. With the exception of diazepam, where a limit of quantitation (LOQ) of 0 ng/mL was reported, the lower limit of quantitation for the other analytes was significantly higher than in our application. In their study, five oral fluid samples were found to be positive; two for oxazepam (concentrations of 18 and 1,659 ng/mL) and three for alprazolam (concentrations of 5, 6, and 9 ng/mL).

In our research, we did not include the metabolites such as 7-aminoflunitrazepam, 7-aminoclonazepam, 7-aminonitrazepam, α-hydroxy alprazolam, α-hydroxytriazolam, or desalkylflurazepam because the parent drug is more often in higher concentration than metabolites in oral fluid. We did, however, include metabolites such as nordiazepam, temazepam, lorazepam, and oxazepam.

Experimental

Materials and Methods

Oral Fluid Collection Devices

Quantisal devices for the collection of oral fluid specimens are obtained from Immunalysis Corporation (Pomona, CA). The devices contain a collection pad with a volume adequacy indicator, which turns blue when one milliliter of oral fluid (± 10%) has been collected. The pad is then placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). This is specifically advantageous in cases where the specimen is positive for more than one drug and the volume of specimen available for analysis may be an issue. The oral fluid concentration is diluted 1:3 when using Quantisal collection devices, and drug concentrations detected were adjusted accordingly.

Standards and Reagents

Deuterated internal standards: D5-diazepam; D5-temazepam; D5-alprazolam and D4-clonazepam, as well as unlabeled drug standards: 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 (CSDAU020) were purchased from United Chemical Technologies (Bristol, PA)

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

Calibrators and Controls

Calibration standards and controls were prepared from synthetic oral fluid and diluted with Quantisal transportation buffer. Throughout the development of the assay, multiple Quantisal collection devices were selected from different lots. In this experiment, the drug concentration used to fortify the synthetic oral fluid was adjusted according to the dilution factor for all calibration standards and controls. In this way, the final result obtained from the instrument did not need to be recalculated for dilution factors. For each analysis, a four-point calibration curve (1, 10, 20, and 40 ng/mL) was run with each batch; the internal standard concentration was 100 ng/mL.

Extraction Procedure

Quantisal buffer (1 mL) was measured and the calibration curve was prepared at the following concentrations:

Negative:	100 µL of deuterated stock solution (100 ng/mL)
0.5 ng/mL:	100 µL of deuterated stock solution (100 ng/mL) 12.5 µL of 10 ng/mL stock solution
1 ng/mL:	100 µL of deuterated stock solution (100 ng/mL) 5 µL of 10 ng/mL stock solution
10 ng/mL:	100 µL of deuterated stock solution (100 ng/mL) 25 µL of 100 ng/mL stock solution
20 ng/mL:	100 µL of deuterated stock solution (100 ng/mL) 50 µL of 100 ng/mL stock solution
40 ng/mL:	100 µL of deuterated stock solution (100 ng/mL) 100 µL of 100 ng/mL stock solution

Sodium phosphate buffer (0.1 M, pH 6.0, 1 mL) was added to the buffer and the samples were mixed. 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) and 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 water (50 µL) for analysis.

Drug Recovery from the Collection Pad

Extraction efficiency of the collection system for benzodiazepines was determined. Oral fluid was fortified with all the drugs at the concentration of 10 ng/mL (n = 6). A collection pad was placed into the fluid until the volume adequacy indicator turned blue, showing that 1 mL (±10%) of oral fluid had been absorbed. The pads were placed into the Quantisal buffer (3 mL), capped, and allowed to remain at room temperature overnight to simulate transportation to the laboratory. The following day, the pads were removed and an aliquot (1 mL) of the specimens was analyzed according to the described procedures.

Analytical Procedure

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

LC Conditions

Column:	ZORBAX Eclipse XDB C18 4.6 x 50 mm x 1.8 µm (PN: 922795-902) A 2.1-mm id column is optimal for a 0.2 mL/min flow rate, but a 1 mL/min column flush is used at the end of the run.
Column temperature:	35°C
Injection volume:	5 µL
Solvent flow rate:	0.2 mL/min
Isocratic pump program:	A = 20 mM ammonium formate (pH = 8.6) B = Acetonitrile 50:50 v,v
Time (minutes)	Flow rate (mL/min)
0	0.2
6.5	0.2
8	1
10	0.2
Post time:	4.5 min

Mass Spectrometer Conditions

Operation:	Electrospray ESI positive mode using Agilent G1948B ESI source
Gas temperature:	300 °C
Gas flow (N_2):	6 L/min
Nebulizer pressure:	15 psi (pressure of 30 to 40 psi recommended)
Capillary voltage:	4,500 V

The precursor and product ions, along with optimized fragmentor and collision energy (CE) voltages, are shown in Table 1. Values pertaining to qualifier ions are in parentheses.

Table 1. Benzodiazepine Acquisition Parameters

Compound	Precursor ion	Product ion	Fragmentor (V)	CE (V)
Segment 1 (time = 0 min)				
Bromazepam	316	288 (209)	160	20 (30)
Segment 2 (time = 4.1 min)				
D4-Clonazepam	320	274	120	25
Clonazepam	316	270 (214)	120	25 (35)
Lorazepam	321	275 (229)	140	25 (35)
Nitrazepam	282	236 (180)	160	25 (35)
D5-Alprazolam	314	286	160	25
Alprazolam	309	281 (274)	160	25 (30)
Chlordiazepoxide	300	283 (227)	120	15 (30)
D5-Oxazepam	292	246	120	20
Oxazepam	287	241 (269)	120	20 (20)
Triazolam	343	308 (239)	120	35 (35)

Table 1. Benzodiazepine Acquisition Parameters (Collision energy abbreviated as CE) (continued)

Compound	Precursor ion	Product ion	Fragmentor (V)	CE
Segment 3 (time = 5.4 min)				
Flunitrazepam	314	268 (239)	160	30 (35)
Midazolam	326	291 (249)	200	30 (40)
D5-Temazepam	306	260	120	25
Temazepam	301	255 (177)	120	35 (40)
D5-Nordiazepam	276	140	120	30
Nordiazepam	271	140 (165)	160	30 (30)
Segment 4 (time = 7.2 min)				
D5-Diazepam	290	262	160	25
Diazepam	285	257 (222)	160	25 (25)
Flurazepam	388	315 (288)	160	25 (25)

The analytical method was confirmed according to standard protocols, whereby the limit of quantitation, linearity range, correlation, and intra- and inter-day precision were determined via multiple replicates over a period of 5 days. The study results are presented in Table 2. The slope of the calibration curve was not forced through the origin. The precision of the assays was excellent, with both within-day and between-day variations (CV) being below 7% for all drugs. The limit of quantitation for all drugs was 0.5 ng/mL of neat oral fluid, equivalent to 0.125 ng per mL of buffer solution.

Table 2A. Slope of Calibration Curve and Correlation Coefficient

Analyte	Equation	Correlation (R^2)
Alprazolam	Y = 0.0298x + 0.0114	0.9995
Bromazepam	Y = 0.0096x – 0.0129	0.9909
Chlordiazepoxide	Y = 0.0146x – 0.0032	0.9998
Clonazepam	Y = 0.0278x – 0.0108	0.9991
Diazepam	Y = 0.0305x – 0.0004	0.9996
Flunitrazepam	Y = 0.007x – 0.0002	0.9999
Flurazepam	Y = 0.2984x – 0.0024	0.9993
Lorazepam	Y = 0.0189x – 0.008	0.9986
Midazolam	Y = 0.0156x – 0.0143	0.9960
Nitrazepam	Y = 0.0551x + 0.018	0.9987
Nordiazepam	Y = 0.011x – 0.0013	0.9999
Oxazepam	Y = 0.0228x – 0.0065	0.9996
Temazepam	Y = 0.0149x – 0.0034	0.9998
Triazolam	Y = 0.0225x + 0.0073	0.9995

Table 2B. Inter-Day Precision (10 ng/mL control specimens; n = 5)

Drug	Mean recovery (ng/mL)	SD	Precision (%)	Acuracy (%)
Alprazolam	9.48	0.19	2.03	105.49
Bromazepam	9.72	0.66	6.8	102.88
Chlordiazepoxide	10.08	0.23	2.26	99.21
Clonazepam	9.44	0.3	3.14	105.93
Diazepam	9.84	0.59	6.04	101.63
Flunitrazepam	9.84	0.5	5.11	101.63
Flurazepam	9.84	0.49	5.01	101.63
Lorazepam	8.88	0.33	3.68	112.61
Midazolam	9.18	0.54	5.94	108.93
Nitrazepam	10.48	0.115	1.42	95.42
Nordiazepam	9.9	0.32	3.27	101.01
Oxazepam	9.94	0.3	3.07	100.6
Temazepam	10	0.3	3	100
Triazolam	9.86	0.25	2.55	101.42

Table 2C. Intra-Day Precision (n = 5)

Drug	Mean recovery (ng/mL)	SD	Precision (%)
Alprazolam	9.64	0.27	2.80
Bromazepam	10.08	0.62	6.13
Chlordiazepoxide	10.14	0.68	6.71
Clonazepam	9.18	0.39	4.25
Diazepam	9.48	0.69	7.29
Flunitrazepam	9.94	0.46	4.64
Flurazepam	9.74	0.68	6.95
Lorazepam	9.24	0.34	3.64
Midazolam	9.26	0.30	3.29
Nitrazepam	10.40	0.46	4.41
Nordiazepam	9.84	0.36	3.71
Oxazepam	9.58	0.40	4.20
Temazepam	10.12	0.39	3.85

Commonly encountered drugs were extracted and analyzed at high concentrations and found not to interfere with the assays. Figure 1 shows a typical calibration curve for alprazolam, with a correlation coefficient of 0.9995. The recovery of the various benzodiazepines from the collection system is shown in Table 3.

Table 3. Percentage Recovery of Benzodiazepines from Oral Fluid Collection System Following Overnight Incubation at Room Temperature (fortified at 10 ng/mL; n = 6)

Drug	Mean recovery (%)	CV (%)
Alprazolam	86.76	8.85
Bromazepam	88.42	14.01
Chlordiazepoxide	89.41	6.33
Clonazepam	88.10	2.97
Diazepam	82.82	4.42
Flunitrazepam	85.10	4.46
Flurazepam	81.57	2.85
Lorazepam	83.44	2.52
Midazolam	81.48	5.32
Nitrazepam	90.17	3.64
Nordiazepam	83.28	3.80
Oxazepam	84.65	2.82
Temazepam	84.19	2.96
Triazolam	85.45	8.71

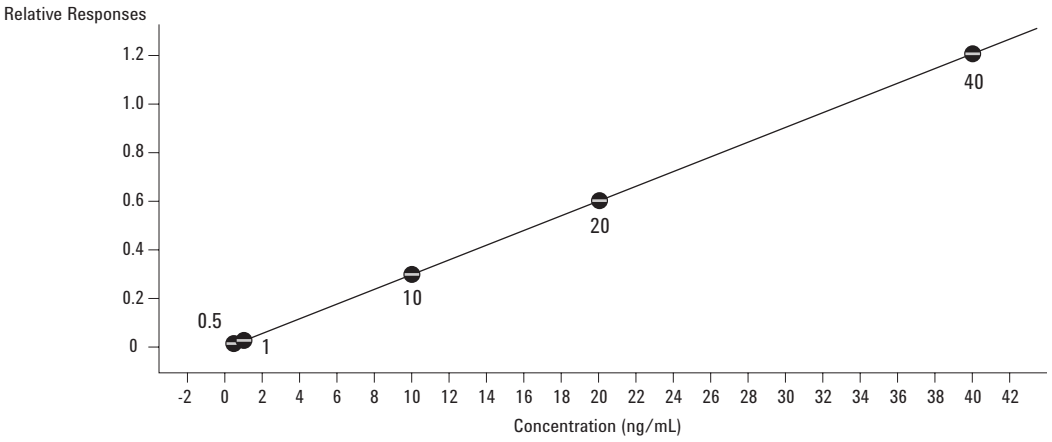

Figure 1. Calibration curve for alprazolam in oral fluid (0.5, 1, 10, 20, and 40 ng/mL).

Results and Discussion

The Agilent instrumentation allowed the rapid determination of 14 benzodiazepines in oral fluid at an extremely low concentration. The chromatography afforded by the small-particle analytical column allowed sepa-ration of the peaks in each of the four group seg-ments (Figure 2).

Further, the Agilent software 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 QQQ mass spectrometer, which is extremely important in forensic analysis, where court challenges to labo-

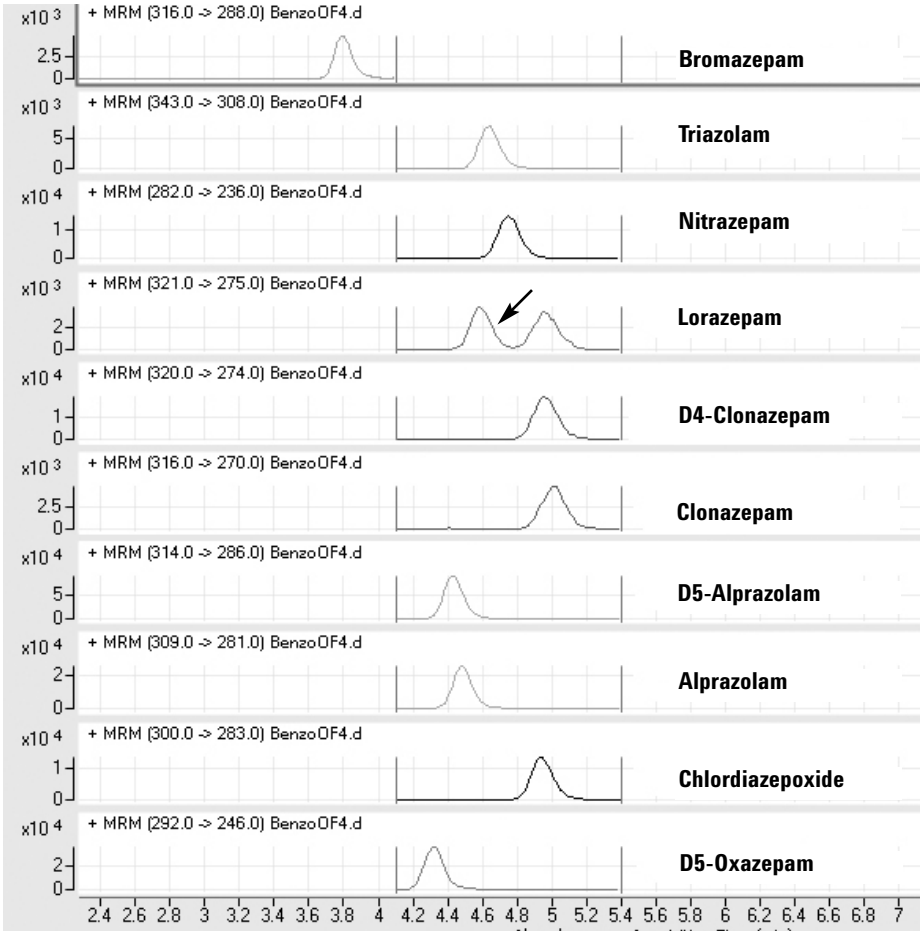

Figure 2. Primary transitions for benzodiazepines in oral fluid.

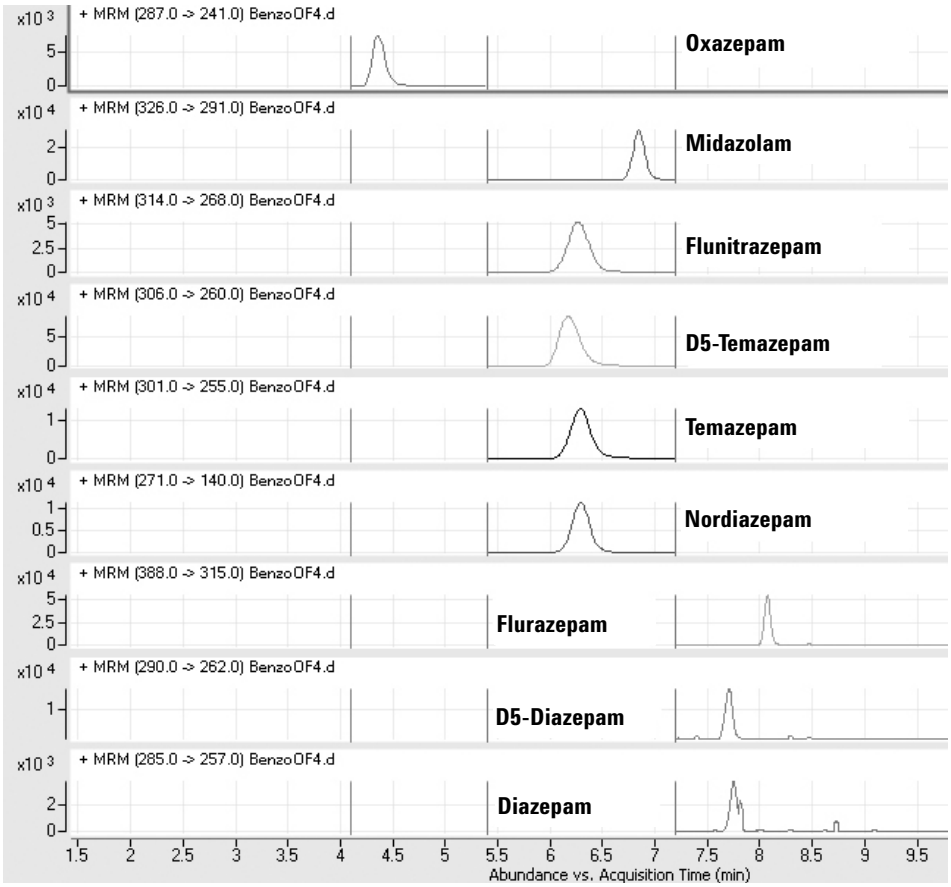

Figure 2. Primary transitions for benzodiazepines in oral fluid. (continued)

ratory 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 oral fluid. The software plots the ratio in the chromatographic window, so the operator is able to assess positivity visually (Figure 3).

Conclusions

The procedure described is suitable for the detection of benzodiazepines in oral fluid using an Agilent Technologies QQQ LC/MS/MS system. The sensitivity of the assay is a significant improvement over other methods. This is the first method that includes qualifying ions for the identification

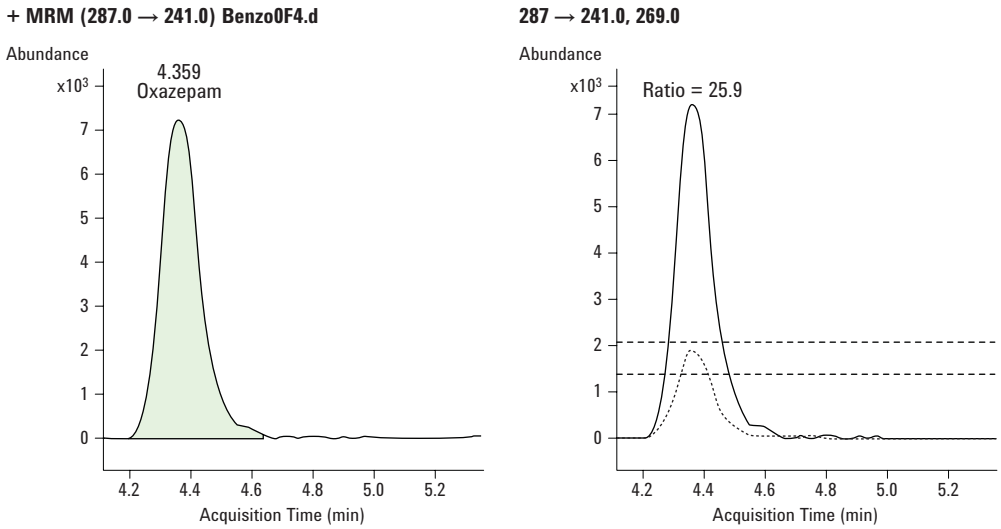

Figure 3. Oxazepam extracted from oral fluid (10 ng/mL).

of benzodiazepines at low concentration in oral fluid, and is in routine use in our laboratory.

Author's note: This work has been accepted for publication in the *Journal of Analytical Toxicology*.

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Printed in the USA
August 15, 2007
5989-7201EN

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