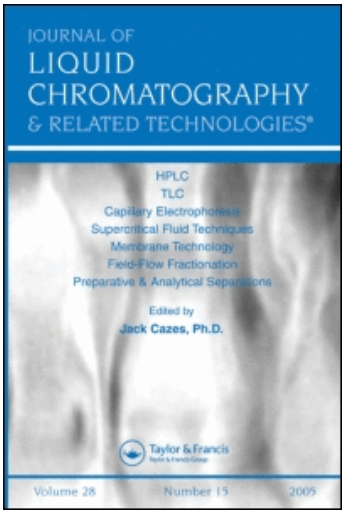

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Comparison of Time-of-Flight Mass Spectrometry to Triple Quadrupole Tandem Mass Spectrometry for Quantitative Bioanalysis: Application to Antipsychotics

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Abstract: Tandem mass spectrometry (MS/MS) is frequently used for the detection of small molecules in biological samples because of the high specificity and sensitivity associated with these instruments. Time-of-flight mass spectrometers (TOF-MS) are typically used qualitatively for accurate mass determination; however, recently they have gained more attention for quantitation. A selective and sensitive TOF-MS method was validated to determine risperidone, its active metabolite paliperidol, haloperidol, clozapine, and olanzapine in rat plasma using midazolam as an internal standard (IS). Comparisons were made between the use of LC-TOF and LC-MS/MS using a triple quadrupole for these compounds for specificity, linearity, precision, accuracy, matrix effects, and recovery. A focus of this study was the evaluation of recent instrumental and software improvements, which have been made to time-of-flight mass analyzers to increase the linearity and quantitative capabilities of these instruments.

Keywords: Antipsychotics, LC-MS, Method validation, Time-of-flight mass spectrometry

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INTRODUCTION

Quantitation of small molecules in biological matrices requires analytical methods with high selectivity and sensitivity. HPLC, used with mass spectrometry (MS) detection, has advantages for this type of analysis including, improved limits of quantitation, high selectivity, small sample volumes, and higher throughput.[1] Even though triple quadrupole mass spectrometers have high specificity, they cannot ordinarily provide accurate mass data. Many groups currently use time-of-flight mass spectrometers (TOF-MS) for accurate mass data due to their high resolving power (up to 10,000 at full width half maxima peak height) and ability to provide better than 5 ppm mass accuracy. Therefore, many groups have both of these instrument types to cover the range of measurements that are needed in early drug discovery.

One of the major advantages of the triple quadrupole in the multiple reaction monitoring mode of operation is its ability to dramatically decrease the background noise during quantitation. Therefore, even though there is a loss in absolute signal going from Q1 to Q3, the even greater decrease in noise yields better overall sensitivity. It is also possible to use the high resolution capabilities of TOF-MS to discriminate against noise and provide enhanced capabilities for the determination of analytes.

This fact has caused an increase over the past several years in TOF-MS applications involving quantitation.[2,3] Another advantage of TOF mass spectrometers is that they are not scanning instruments. Therefore, a wide mass range can be acquired at a rapid pace without losing significant sensitivity. Methods using TOF-MS have been reported to have similar results for accuracy and precision versus MS/MS.[4–6] These methods also reported limits of quantitation (LOQs) 5–10 times higher when using TOF-MS versus MS/MS.

The most significant issue to overcome for TOF-MS has been the narrow linear dynamic range. This is caused because the detector becomes saturated at higher concentrations. Recent software and hardware improvements have targeted increasing the dynamic range of these instruments. This has been accomplished by charging the Z-focus lens to decrease the intensity of the primary ion beam. Alternate scans are taken at the normal and attenuated Z-focus lens settings, and peaks are sketched in using software algorithms when the intensity reaches a threshold. However, it is unclear what affect these improvements have on the ability of LC-TOF-MS to provide quantitative data.

Antipsychotics have been used for treating psychological disorders since the 1950's. Antipsychotics are typically classified into two groups. The first generation antipsychotics, such as haloperidol, are still widely used but have a greater level of extrapirimidal side effects. The second generation antipsychotics (SGAs) such as olanzapine, risperidone, and

clozapine, in addition to having less side effects, have also been shown to be more effective in treating positive and negative symptoms in schizophrenia.[7] It is not unusual for patients to be on multiple antipsychotics simultaneously. The therapeutic ranges of these drugs in plasma are low (ng/mL levels)[8–10] and, therefore, require analytical methods with high sensitivity for therapeutic monitoring. These needs make antipsychotics a good class of compounds to use to compare instrument platforms.

EXPERIMENTAL

Chemicals and Reagents

Risperidone (RISP), clozapine (CLOZ), olanzapine (OLAN), and haloperidol (HAL) were donated by Eli Lilly & Co. (Indianapolis, IN, USA). Paliperidone (PAL) was donated by Janssen Research Foundation (Beers, Belgium). Leucine enkephalin and the internal standard midazolam (IS) were obtained from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Isopropyl ether was purchased from Acros Organics (Morris Plains, NJ, USA). Ammonium formate and ammonium phosphate were obtained from Sigma (St. Louis, MO, USA). Formic acid was purchased from J.T. Baker (Phillipsburg, NJ, USA). Deionized water was generated from a Continental deionized water system (Natick, MA, USA).

Liquid Chromatography

An Agilent 1100 Series HPLC system (Palo Alto, CA, USA) equipped with a degasser, binary pump, autosampler, and thermostated column compartment was used. A separate Agilent 1100 Series binary pump was used to introduce the leucine enkephalin into the reference probe. A Waters Atlantis^{TM} dC-18 (2.1×30 mm, 3 μm) (Milford, MA, USA) with a 4.0×2.0 mm Phenomenex Security Guard C8 column (Torrance, CA, USA) was used for the chromatographic separation. The compounds were separated by gradient elution using mobile phases of 5 mM ammonium formate in water, pH 6.1 (mobile phase A), and acetonitrile (mobile phase B). The flow rate was 0.30 mL/minute using the following gradient (minutes, % B): (0,15) (1,15) (5,50) (10,72) (10.5, 80) (13.5, 80) (14, 15) (20, 15). A 15 μL injection was loaded onto the column. The first 5 minutes of the LC flow was diverted to waste, while data was acquired up until 12 minutes. The last 8 minutes of each run was also diverted to waste.

Mass Spectrometry

A Waters LCT Premier^{TM} time-of-flight mass spectrometer was operated in electrospray ionization (ESI) positive V-optics mode. The capillary voltage was 3500 V and the cone voltage was 35 V. The source temperature and desolvation temperatures were 130°C and 350°C, respectively. Cone gas and desolvation gas flows were 50 and 500 L/hr, respectively. The MCP plate voltage was 2700 V. Mass spectra were acquired from m/z 100–600 at a rate of 1 sec/spectrum. The dynamic range enhancement (DRE) function was used and leucine enkephalin was used as the reference spray. A 0.50 μg/mL solution in 50:50 acetonitrile:water was infused into the reference probe at a rate of 10 μL/min. The protonated C_{13} isotope peak was used for the lock mass (m/z 557.2804) and the protonated C_{12} peak was used for the attenuated lock mass (m/z 556.2117). One scan from the reference probe was taken for every 5 scans from the analyte probe. Ten scans from the reference were averaged for the calculation of the dynamic range enhancement (DRE) corrected peaks. The m/z 557.2804 corrected for the accurate mass, and the m/z 556.2117 was used for the DRE function.

Calibration Standards and Quality Control Standards

Stock solutions were prepared by dissolving the RISP, OLAN, PAL, HAL, and CLOZ in methanol to yield a mixture with a final concentration of 1 mg/mL (see Figure 1 for structures and MW). Standard solutions

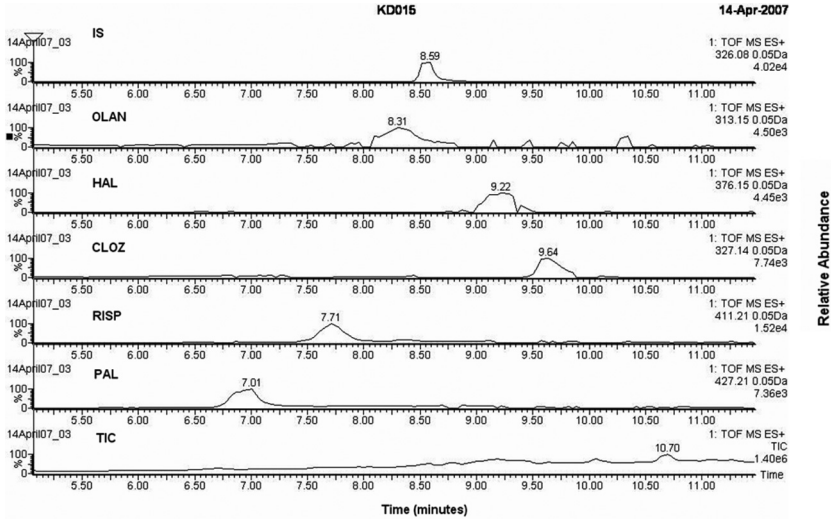

Figure 1. Total ion and reconstructed ion chromatograms for plasma at 2 ng/mL.

were prepared by diluting the stock solutions in 5 mM ammonium fomate:acetonitrile (70:30, v/v). The final concentrations were 20, 100, 250, 500, 1,000, and 2,000 ng/mL. The final concentrations of the quality control (QC) standards were 50, 300, and 1,500 ng/mL. The IS was prepared in the same manor and the final concentration was 100 ng/mL. Blank plasma samples were spiked with the standards to yield final concentrations of 2, 10, 25, 50, 100, and 200 ng/mL. The spiked concentration of the IS was 10 ng/mL. The final concentrations of the QC samples were 5, 30, and 150 ng/mL. Stock solutions were stored at -20°C. Fresh standard solutions were prepared each validation day.

Sample Collection

All procedures were reviewed and approved by the Medical College of Georgia Committee on Animal Use for Research and the Veterans Affairs Medical Center Subcommittee on Animal Use, and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by US National Institute of Health. Male albino Wistar rats (Harlan, Indianapolis, IN, USA) 2–3 months old were housed individually in a temperature controlled room (25°C), maintained on a 12 h light/dark cycle with free access to food. Rats were thus treated with HAL (2.0 mg/kg/day), RISP (2.5 mg/kg/day) and OLZ (10.0 mg/kg/day) orally in drinking water for periods greater than two weeks to reach steady state concentrations. These doses were chosen based on previous studies in which behavioral and time dependent neurochemical effects were detected.[11–13] These doses also resulted in plasma levels that approximated the levels often associated with antipsychotic effects in humans.[14] Plasma samples were collected and kept frozen at -70°C until analysis. The analytes were reported to be stable under these storage conditions.[15,16]

Sample Preparation

The extraction method was adapted from the method published by Zhang, et al.[15–17] To 250 μL of rat plasma, 25 μL of the standard mix, 25 μL of the IS, and 200 μL of 0.5 mM ammonium phosphate were added. After briefly vortexing, 3 mL of isopropyl ether was added for extraction. The samples were vortexed for 5 minutes, followed by centrifugation for 10 minutes at 2,000 × g. The organic layer was evaporated using a vacuum centrifuge. The residue was reconstituted in 100 μL of methanol:20 mM ammonium formate (pH 3.86 with formic acid)

(70:30, v/v). The samples were sonicated for 1 minute, briefly vortexed, and centrifuged for 5 minutes at 16,000 × g before LC injection.

Method Validation

Six-point calibration curves were acquired by plotting peak area ratios of RISP, OLAN, PAL, HAL, and CLOZ to the IS against analyte concentration. The curves were acquired daily for 3 days (n = 5 for intra-day and n = 15 for inter-day). Precision and accuracy were determined for the 3 QC points (5, 30, and 200 ng/mL) and the LOQ (2 ng/mL). The LOQ values were determined by observing where precision and accuracy values did not exceed 20%. Precision was reported as percent relative standard deviation (% RSD) and accuracy was reported as percent error (% Error).

Recoveries for each analyte were also determined. Absolute recovery was determined by dividing the analyte/IS ratio in the plasma by the response of the standard solution in methanol:20 mM ammonium formate (pH 3.86 with formic acid) (70:30, v/v). Relative recovery was determined by dividing the response of the analyte/IS ratio in the plasma by the response of the analyte/IS ratio in plasma that was spiked after the extraction process. The matrix effects were determined using the method reported by Matuszewski, et al.[18] and were calculated to be the response of the plasma spiked after the extraction divided by the response of the standard solution in methanol:20 mM ammonium formate (pH 3.86 with formic acid) (70:30, v/v).

RESULTS AND DISCUSSION

Dynamic Range Enhancement

The normal Z-focus lens was optimized to where the intensity of the HAL ion was the greatest. HAL was chosen because it had the lowest response. The Z-focus lens was set at the highest setting for attenuated mode. A 1 μg/mL mixture of the antipsychotics (in 50:50 acetonitrile:water) was infused into the analyte probe. A PEEK tee was used to introduce the mixture with 85:15 5 mM ammonium formate:acetonitrile.

When calibration data was acquired in normal mode, the response was only linear from 2 to 25 ng/mL. At concentrations above 25 ng/mL, the chromatographic peaks flattened out indicating detector saturation. When the data was acquired in DRE mode, the response was linear from 2 to 200 ng/mL and no saturation was observed. It was also found that increasing the normal Z-focus lens setting resulted in further increases in the dynamic range. When the normal Z-focus lens was increased to

a higher setting, the response became linear up to 1,000 ng/mL. However, the LOQ was increased to 5 ng/mL. Therefore, the Z-focus lens is a parameter that can be optimized to produce the linear range that is desired according to the samples that are going to be analyzed. We chose to set the normal Z-focus lens to obtain the lowest LOQ value possible since this was needed to cover the therapeutic range of all of the analytes.

Method Validation

Specificity

Standard mixtures were injected to determine the retention times of the analytes. The retention times were 7.0, 7.7, 8.3, 8.6, 9.2, and 9.6 for PAL, RISP, OLAN, IS, HAL, and CLOZ, respectively. Reconstructed ion chromatograms were extracted from the total ion chromatograms (TIC) for each analyte. The TIC and reconstructed ion chromatograms for a spiked plasma sample at the LOQ (2 ng/mL) can be found in Figure 1. The specificity for each analyte was improved when a smaller mass window was used. When a mass window of 100 mDa was used, we observed cross talk between CLOZ (m/z 327.1376) and the IS (m/z 326.08). When a mass window of 50 mDa was chosen there was no cross talk observed and, therefore, the method specificity was improved. Figure 2 shows a blank plasma chromatogram and the respective blank

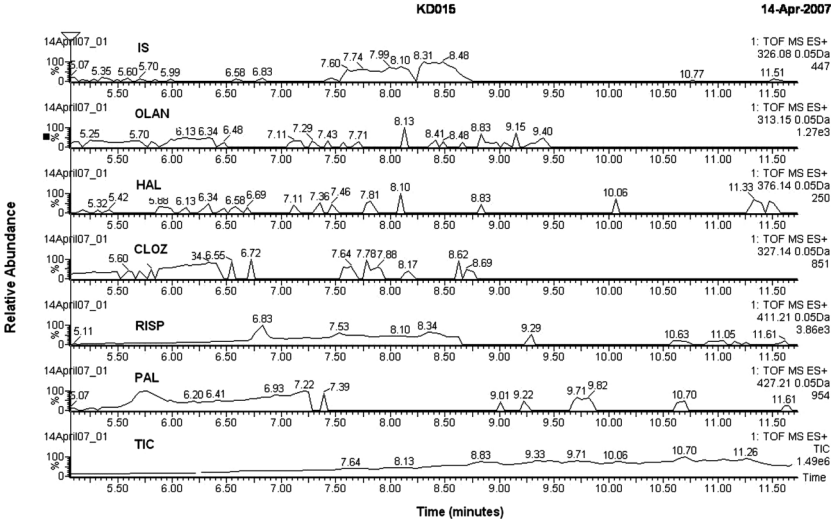

Figure 2. Total ion and reconstructed ion chromatograms from blank plasma.

extracted ion chromatogram for each analyte. It can be seen that there are no responses arising from endogenous compounds in the plasma.

Linearity

Standard solutions were made fresh each validation day and a linear calibration curve was generated for each analyte. The linear range was 2 to 200 ng/mL for each analyte. The ratio of the analyte peak area to the internal standard peak area was plotted against concentration. A weighting factor of $1/x^2$ was used to fit each curve. The slopes, intercepts, and correlation coefficients are reported in Table 1. The correlation coefficients were lower than those observed in our studies using the triple quadrupole (0.94–0.98 versus >0.99).[15] However, even with these lower correlation coefficients the method was still easily validated.

Accuracy and Precision

Precision and accuracy measurements were acquired for the 3 QC points and the LOQ for each compound. The accuracy and precision data is summarized in Table 2. Microsoft Excel was used to calculate the % RSDs and % Errors. Values for the intra-day precision and accuracy ranged from 3.32–9.62% and 4.00–12.61%, respectively. Inter-day precision and accuracy ranged from 4.12–13.55% and 5.53–12.97%, respectively. These results were acceptable according to the current FDA bioanalytical validation guidelines.

Recovery and Matrix Effects

Absolute recovery, relative recovery, and matrix effects data are summarized in Table 3. Absolute recoveries ranged from 58.1 to 91.1% while the relative recoveries ranged from 50.7–99.0%. The peak areas of each analyte and the IS were used to calculate the matrix effects. Matrix effects were somewhat higher for CLOZ, HAL, and OLAN, however they did

Table 1. Slopes, intercepts, and correlation coefficient (mean ± SD) of antipsychotics in rat plasma

Compound	Slope	Intercept	R^2
PAL	0.13 ± 0.02	0.05 ± 0.03	0.9716 ± 0.0032
RISP	0.21 ± 0.04	0.09 ± 0.02	0.9573 ± 0.0023
CLOZ	0.09 ± 0.01	0.10 ± 0.02	0.9422 ± 0.0277
HAL	0.08 ± 0.01	0.08 ± 0.02	0.9676 ± 0.0183
OLAN	0.09 ± 0.02	0.04 ± 0.04	0.9837 ± 0.0077

Table 2. Intra-day (n = 5) and their-day (n = 15) precision (% RSD) and accuracy (% Error) of drugs in rat plasma

		Intra-day			Inter-day		
Drug	Theoritical concentration (ng/mL)	Measured concentration (ng/mL)	RSD (%)	Error (%)	Measured concentration (ng/mL)	RSD (%)	Error (%)
PAL	2	1.9 ± 0.1	4.3	6.5	1.9 ± 0.1	7.0	7.6
	5	5.0 ± 0.3	5.8	4.0	5.3 ± 0.3	6.5	7.3
	30	31.5 ± 1.4	4.3	5.6	32.8 ± 1.7	5.2	9.6
	150	145.8 ± 8.0	5.5	4.6	134.9 ± 9.7	7.2	10.7
RISP	2	1.9 ± 0.1	3.3	7.6	2.0 ± 0.2	10.0	7.9
	5	5.1 ± 0.3	6.2	5.5	5.5 ± 0.4	7.4	10.9
	30	32.3 ± 1.4	4.3	7.7	33.2 ± 1.4	4.1	10.5
	150	135.8 ± 9.8	7.2	9.6	130.8 ± 8.5	6.5	12.8
CLOZ	2	2.0 ± 0.2	7.5	6.6	2.0 ± 0.3	13.6	11.1
	5	5.5 ± 0.3	5.1	10.5	5.7 ± 0.4	6.5	13.0
	30	32.3 ± 1.4	4.2	7.5	32.9 ± 1.6	4.9	9.6
	150	131.1 ± 7.7	5.9	12.6	130.9 ± 6.3	4.8	12.7
HAL	2	1.8 ± 0.1	7.7	10.8	1.8 ± 0.2	10.2	10.2
	5	5.4 ± 0.5	8.6	10.5	5.6 ± 0.3	5.5	11.9
	30	33.0 ± 1.4	4.2	10.0	33.1 ± 1.5	4.4	10.3
	150	134.3 ± 5.4	4.0	10.5	135.6 ± 10.3	7.6	10.8
OLAN	2	2.0 ± 0.2	9.6	7.1	2.0 ± 0.2	9.9	8.2
	5	4.7 ± 0.3	5.3	5.9	5.3 ± 0.6	11.6	10.7
	30	28.5 ± 1.2	4.1	5.1	30.3 ± 0.6	6.9	5.5
	150	156.3 ± 8.0	5.1	5.1	145.8 ± 147.7	10.1	8.4

not prevent the method from passing validation or from covering the therapeutic range of the analytes.

Accurate Mass Measurement

Accurate mass measurements were acquired by averaging ten scans across each chromatographic peak in a 25 ng/mL spiked plasma sample. The measured masses were within 1.2 mDa of the theoretical masses and within 3.2 ppm mass accuracy. The protonated ion mass spectra are found in Figure 3 and the accurate mass data is summarized in Table 4.

Cross Validation

Finally, we conducted a direct comparison of samples that had been previously analyzed using LC-MS/MS on a triple quadrupole instrument.[15]

Table 3. Absolute recovery, relative recovery, and matrix effects (mean ± SD) of drugs in rat plasma (n = 5)

Drug	Concentration (mg/mL)	Abs. recovery (%)	Relative recovery (%)	Matrix effect (%)	Type of effect
PAL	5	68.1 ± 2.7	77.6 ± 3.7	87.9	12.1 % suppression
	30	84.6 ± 5.5	87.4 ± 5.9	96.8	3.2 % suppression
	150	79.4 ± 1.4	88.1 ± 2.8	90.2	9.8 % suppression
RISP	5	79.6 ± 3.9	82.0 ± 5.1	97.4	2.6 % suppression
	30	88.7 ± 6.5	85.8 ± 4.4	103.3	3.3 % enhancement
	150	90.5 ± 1.4	91.9 ± 1.6	98.5	1.5 % suppression
CLOZ	5	64.5 ± 5.2	83.4 ± 7.1	77.7	22.3 % suppression
	30	78.6 ± 3.6	91.0 ± 3.0	86.4	13.6 % suppression
	150	77.2 ± 1.7	95.1 ± 2.6	81.2	18.8 % suppression
HAL	5	61.3 ± 2.9	86.2 ± 6.6	71.5	28.5 % suppression
	30	69.4 ± 3.6	87.1 ± 2.3	79.6	20.4 % suppression
	150	67.4 ± 3.9	99.0 ± 4.4	75.9	27.1 % suppression
OLAN	5	58.1 ± 2.7	50.7 ± 3.3	114.9	14.9 % enhancement
	30	75.0 ± 4.5	59.8 ± 2.3	125.4	25.4 % enhancement
	150	67.4 ± 3.9	62.9 ± 2.6	107.3	7.3 % enhancement
IS	10	91.1 ± 6.1	93.9 ± 0.5	97.0	3.0 % suppression

This experiment allowed us to determine if the quantitative data acquired using LC-TOF would be equivalent to data acquired from a triple quadrupole. Three sets of five samples were analyzed. The first set of samples contained the compound RISP along with its active metabolite PAL. The second set of samples contained HAL and the third set of samples contained OLAN. The data from the cross validation is shown in Table 5 and representative mass chromatograms are shown in Figure 4. A two sample t-test ($\alpha = 0.05$) for each analyte resulted in calculated T values less than the $T_{critical}$ of 2.3. Therefore, there was no significant difference between the results obtained from the LC-TOF and the LC-MS/MS methods, demonstrating that at the 95% confidence level the methods provided equivalent results.

Comparison of Time-of-Flight to Triple Quadrupole Mass Spectrometry

A direct comparison between the Waters Quattro Micro and the Waters LCT Premiere has several significant advantages. The ion sources and electrospray probes for these instruments are interchangeable meaning that differences noted between the instruments are directly attributable to the mass analyzers. In each case the instruments also had identical HPLC systems.

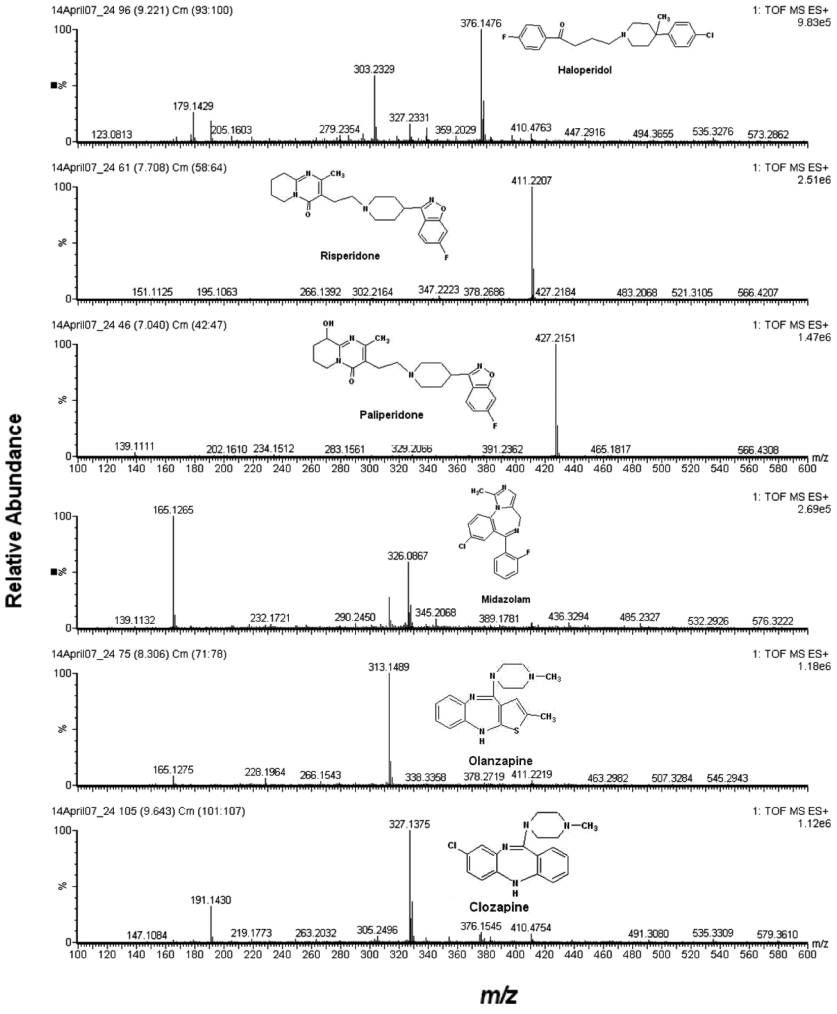

Figure 3. Electrospray positive ion mass spectra of antipsychotics with chemical structures.

One advantage of using the TOF-MS system was the high resolution and accurate mass capabilities. Typically, triple quadrupole instruments have unit mass resolution, whereas many TOF instruments have resolutions of at least 10,000 (FWHM). This high resolving power potentially allows for separation of chromatographic peaks from background interferences, or from other compounds with similar nominal masses. In addition, the accurate mass capability allows for high quality exact mass data to be simultaneously acquired at low concentrations

Table 4. Accurate mass data for antipsychotics in rat plasma (average of 10 scans for 25 ng/mL spiked plasma sample)

Drug	Theoretical Mass	Measured Mass	Mass Difference (mDa)	Mass Difference (ppm)
Olanzapine	313.1487	313.1497	1.00	3.19
Clozapine	327.1376	327.1372	−0.40	−1.22
Haloperidol	376.1479	376.1483	0.40	1.06
Risperidone	411.2196	411.2184	−1.20	−2.92
Paliperidone	427.2145	427.2137	−0.80	−1.87
Midazolam (IS)	326.0860	326.0860	0.00	0.00

(25 ng/mL) demonstrating that the DRE function does not adversely affect this capability. Therefore, TOF-MS is also an excellent tool for screening to verify the presence of compounds of interest in complex matrices, such as plasma.

Another advantage of TOF-MS systems is that they have very fast data acquisition rates allowing them to obtain several full scan mass spectra every second. Therefore, if a question arises later about the presence of other compounds in a sample the data can be reprocessed and searched for additional metabolites or the presence of other compounds. This is in stark contrast to the triple quadrupoles where their high selectivity does not allow reprocessing of data to search for additional compounds unless they serendipitously have the same mass transitions.

Both the TOF-MS and triple quadrupole systems were able to generate accuracy and precision that met current US Food and Drug Administration (FDA) guidance for bioanalytical method validation. However, the precision and accuracy numbers from the TOF-MS system ranged from 4–13% and were 50–60% higher than the precision and accuracy number generated from the triple quadrupole system (ranging from 2-8%). Again it does not appear that the DRE function has prevented

Table 5. Rat plasma concentration (mean ± S.D) of the drugs after chronic treatment in drinking water (n = 5)

Analyte	LC-TOF concentration (ng/mL)	LC-MS/MS concentration (ng/mL)
RISP	5.0 ± 4.1	5.9 ± 3.6
PAL	17.9 ± 15.6	27.8 ± 15.5
HAL	10.9 ± 7.0	13.3 ± 7.1
OLAN	81.5 ± 68.0	56.8 ± 63.7

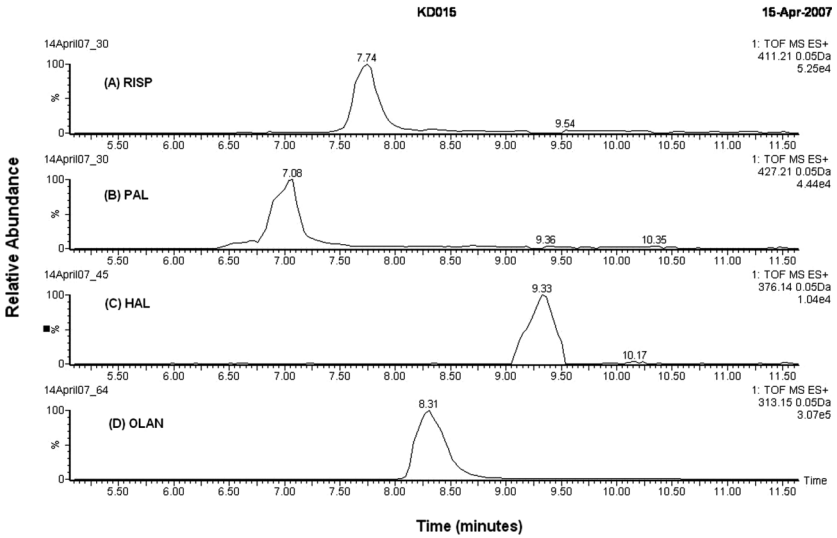

Figure 4. Reconstructed ion chromatograms from rats chronically treated with (A) RISP with the active metabolite (B) PAL, (C) HAL, and (D) OLAN which correspond to concentrations of 7.83, 8.98, 10.80, and 34.02 ng/mL, respectively.

the TOF-MS system from acquiring quantitative data that exceeds current US FDA guidance. When comparing the lower limit of quantitation between the two instruments the TOF-MS system was 2.0 ng/mL and the triple quadrupole system was 0.1 ng/mL. Therefore, it does appear that the triple quadrupole instrument remains about 20 times more sensitive even with the addition of the DRE function to the TOF-MS.

One final advantage of TOF-MS is that precursor to product ion transitions do not have to be determined or optimized as they are when using a triple quadrupole. This may result in somewhat shorter method development times. Overall, the data supports that TOF-MS systems using the DRE function can be used successfully for multianalyte bioanalysis from a complex biological matrix such as plasma.

CONCLUSION

We report a validated method to determine 5 antipsychotic drugs in rat plasma. The linear dynamic range was improved by using the DRE function on the instrument. In addition, the DRE function did not adversely impact the ability of the method to be validated. The linear range was from 2 to 200 ng/mL. The method is specific for RISP, PAL, HAL, CLOZ, and OLAN. The percent errors and RSDs for accuracy and

precision were acceptable and were all less than 13%. This method was successfully applied to plasma samples from rats that were chronically treated with RISP, HAL, and OLAN. These results were compared to those obtained using LC-MS/MS on a triple quadrupole and found not to be statistically different.

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