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Optimization of triple quadrupole mass spectrometer for quantitation of trace degradants of pharmaceutical compounds

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Abstract

LC/MS–MS is a sensitive analytical technique capable of quantifying impurities and degradation products of pharmaceutical compounds at very low concentrations. Obtaining reproducible and reliable signals near the lower limit of quantitation (LLQ) from an LC/MS–MS system requires careful tuning of the instrument as there are significantly more tuning parameters for optimization of an LC/MS–MS than for an instrument with a single mass analyzer. Using response surface methodology in conjunction with sequential design strategy, a Micromass Quattro LC triple quadrupole mass spectrometer was optimized for LLQ based on the signal-to-noise ratio (*S*/*N*) using the model pharmaceutical compound Azithromycin, (2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*,14*R*)-13-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-(alpha)-L-ribo-hexopyranosyl) oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-hepta-methyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-(beta)-D-xylo-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one. Statistical analyses determined that the tuning parameters impacting the signal-to-noise ratio were cone, capillary, extractor, radio frequency (RF) lens voltages and high mass resolution (HM Res1). A mathematical model was derived that can be used to predict the signal-to-noise ratio of Azithromycin $[M + H]^+$ over a wide tuning range and to identify a setting which maximizes the signal-to-noise ratio. Based on this model, the optimal instrument parameters were found at low extractor voltage (17 V), medium capillary voltage (3 kV), medium cone voltage (48 V), high RF voltage (0.9 V), and medium HM Res1 (2.5). Using the optimized settings determined for Azithromycin, a standard curve for the analysis of a structurally related trace degradant (pseudo-aglycone Azithromycin) was generated from which the LLQ was determined to be 39.1 ng/mL, suggesting that the initial rate method of shelf-life prediction of pharmaceutical compounds may be tenable using LC/MS–MS. (Int J Mass Spectrom 216 (2002) 209–218) © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Triple quadrupole MS; Experimental design; Azithromycin; Quantitative analysis; Optimization

1. Introduction

LC/MS–MS is a sensitive and highly selective analytical technique that has been widely used for quantitative analyses in fields such as environmental science [1], toxicology [2], forensics [3], and pharmacokinetics [4], where determinations of trace amounts of analytes are required. Zhang and Henion [5] recently reported that estrogen sulfates at concentrations as low as 0.2 ng/mL could be detected in human urine by using an LC/MS–MS. Sutherland et al. [6] showed that

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a lower limit of quantitation (LLQ) of 0.7 ng/mL for linsidomine in human plasma was achievable with a tandem LC/MS equipped with an electrospray ionization (ESI) interface. As a powerful technique for the monitoring, characterization and identification of impurities, LC/MS–MS has been used for development and quality control of drug substances and pharmaceutical dosage forms, and applied to support manufacturing and safety evaluation of batches used in clinical trials [7]. In many reported studies, the high resolution and sensitivity of LC/MS–MS aided the structural elucidation, as well as quantitation of degradation products and impurities in pharmaceutical dosage forms [8,9].

Compared to single MS, the MS/MS technique uses sequential mass analyzers to separate and identify ions in a single instrument. In the triple quadrupole mass spectrometer, ionization takes place in the source region. These ions are sampled through a series of orifices into the first quadrupole where they are filtered according to their mass to charge ratio, *m*/*z*. The mass-separated ions then pass into the hexapole collision cell where they either undergo collision-induced decomposition (CID) or pass unhindered to the second quadrupole. The fragment ions are then mass analyzed by the second quadrupole. Finally, transmitted ions are detected by a conversion dynode, phosphor and photomultiplier detection system. With two mass analyzers in one instrument, many data acquisition methods that cannot be performed by a single MS become available: e.g., constant neutral loss (or gain), as well as parent and daughter modes. These acquisition modes can be used to effectively suppress background noise from the sample matrix and greatly enhance sensitivity if the instrument is properly tuned and operated.

Many parameters have to be considered for tuning when a new LC/MS method is developed for unknown structural analysis or quantitative analysis of known compounds [10,11]. There are many more tuning parameters in a triple quadrupole mass spectrometer than a regular mass spectrometer. In addition to capillary, cone, extractor, and radio frequency (RF) lens voltages that are common tuning parameters for an ESI interface, the parameters associated with two mass analyzers (low mass resolution (LM Res), high mass resolution (HM Res)), ionization energy (IEnergy), and collision cell (CID energy, entrance and exit voltages) must also be considered during system optimization. Finally, desolvation temperature, desolvation gas flow rate, nebulizer flow rate, and source block temperature should be taken into account in order to achieve the optimum operative condition.

Typically, instrument optimization is achieved by adjustment of each factor independently and repeatedly until a perceived optimum is reached. However, it is well documented that the standard one-variable-at-a-time approach may only lead to a local optimum, especially when explanatory variables interact statistically. Conversely, response surface methodology and sequential experimental design strategy can be very successful in obtaining the desired information by using the least resources, screening the important variables from many possible variables, identifying potential interactions between variables, and optimizing the experimental system.

In this study, a conditional two-stage experimental design approach was employed in order to efficiently optimize a triple quadrupole mass spectrometer for the purpose of trace degradant quantitation. Accurate quantitation of trace degradants should allow for the utilization of initial rate method of shelf-life estimation and is currently under investigation in our laboratories.

2. Experimental

A Micromass Quattro LC triple quadrupole mass spectrometer (Micromass, Beverly, MA) equipped with an electrospray probe and a Z-spray interface was used for this study. The ESI technique allows rapid, accurate and sensitive analysis of a wide range of analytes from low molecular weight polar compounds (<200 Da) to high molecular weight biopolymers (>100 kDa).

Azithromycin produced by Pfizer Inc. (Groton, CT) was chosen as the model compound for LC/MS–MS optimization. Azithromycin has a molecular weight of 748.99 Da and its chemical structure is shown in

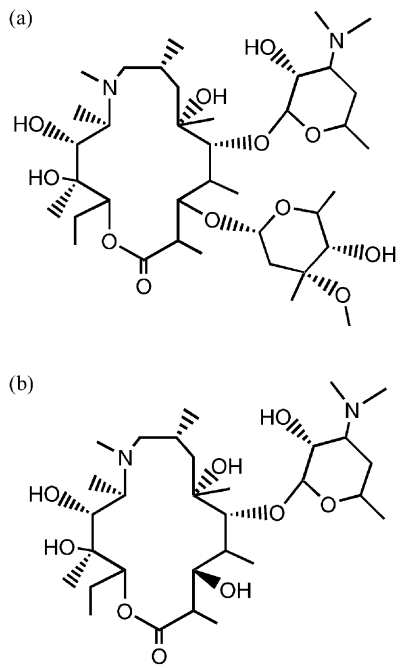

Fig. 1. Chemical structure of (a) Azithromycin, (2*R*,3*S*,4*R*,5*R*,8*R*, 10*R*,11*R*,12*S*,13*S*,14*R*)-13-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-(alpha)-L-ribo-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6, 8,10,12,14-hepta-methyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-(beta)-D-xylo-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one, and (b) the low pH degradation product of Azithromycin, pseudo-aglycone Azithromycin.

Fig. 1a. Generation of the standard curve was achieved using the low pH trace degradant of Azithromycin, pseudo-aglycone Azithromycin (Fig. 1b). The compound was dissolved in a 10 mM ammonium acetate solution (pH 4.5) at concentrations of 0.1–2.5 μg/mL. Infusion into the mass spectrometer was via a syringe infusion pump (Harvard Apparatus, Model 11, Holliston, MA) at a constant rate of 20 μL/min and ionized with positive ion mode in the source region. For each tuning condition, $[M + H]^+$ were acquired at the following fixed settings: scan function → mass scan (MS); data acquisition format → continuum; inter scan time → 0.10 s; run duration → 2 min. Total ion current was recorded and the signal-to-noise ratio (*S*/*N*) of $[M + H]^+$ was used as the measure of sensitivity.

Prior to the statistical design phase, all previously discussed parameters were screened for inclusion in the study. Results from these experiments showed a wide range of potential optimal settings for some of the variables, as displayed for capillary, cone and extractor voltages in Fig. 2. In order to simplify the statistical design and minimize the number of experiments, variables that were deemed to have a negligible impact on tuning optimization for Azithromycin were held constant. These included the ionization energy of both mass analyzers, source block temperature, infusion rate and concentration of the model compound, CID gas energy, collision cell entrance and exit voltages. As a result, 9 of the original 25 tuning parameters were initially evaluated for their effects on the sensitivity of the instrument in the stage-1 experiment. The variables and associated ranges were capillary voltage (2.3–3.7 kV), cone voltage (55–105 V), extractor voltage (23–37 V), RF lens voltage (0.35–0.65 V), desolvation gas flow rate (360–640 mL/min), HM Res of mass analyzer 1 and 2 (1.6–3.4 Da), LM Res of mass analyzer 1 and 2 (8–17 Da).

A conditional two-stage experimental design approach was utilized. Using information gained from the first set of screening experiments, an experimental region of interest was defined. A central composite design (CCD) was used both to investigate the factors that significantly impacted *S*/*N*, and to determine whether a global maximum for *S*/*N* was within the experimental design region. If optimal parameter settings were identified within the region, a verification study would be completed; otherwise, a new region would be defined and a second set of experiments would be designed that incorporated only the significant parameters from the first experiment. Optimal settings would be derived, given that they were contained within the second region, then verified. For a practical guide to response surface methodology and process optimization, see [12]. All statistical designs and analyses were completed using Design Expert (version 5.0, Stat-Ease, Minneapolis, MN) and SAS (version 6.12, SAS Institute, Cary, NC) software packages.

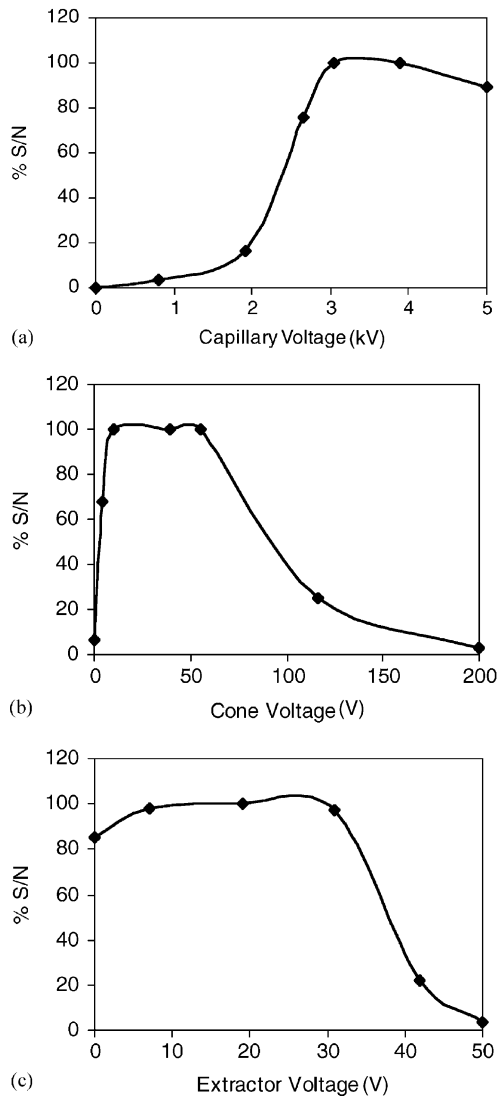

Fig. 2. Screening experiment results for capillary, cone, and extractor parameters. Curves represent interpolative fits of the data. (a) Signal-to-noise ratio of Azithromycin $[M + H]^+$ as function of capillary voltage. Cone and extractor voltages were fixed at 24 and 19 V, respectively. (b) Signal-to-noise ratio of Azithromycin $[M + H]^+$ as function of cone voltage. Capillary and extractor voltages were fixed at 4.23 kV and 19 V, respectively. (c) *S/N* of Azithromycin $[M + H]^+$ as function of extractor voltage. Capillary and cone voltages were fixed at 4.23 kV and 24 V, respectively. Other variables were held constant in plots (a), (b), and (c) at 0.36 V RF lens, 558 L/h desolvation gas flow, 80 °C source block temperature, 100 °C desolvation temperature, 18.0 LM Res1, 6.8 HM Res1, 4.3 V IEnergy1, 20 mV entrance, 0 collision, 20 mV exit, 18.0 LM Res1, 6.6 HM Res2, and 4.3 V IEnergy2.

3. Results and discussion

3.1. Stage-1 experimentation (nine parameters)

A 113-run 2_{IV}^{9-3} CCD was used for the initial experiment and was conducted over three days with 37–39 runs per day. The first and third days were designated to run 1/2 of the 64 factorial points, plus 5 center runs, for a total of 37 experiments. The second day was used to run and replicate the 18 axial points of the CCD, including 3 center runs, for a total of 39 experiments. This design was capable of estimating almost a full second order model for the nine variables; only 6 of the 36 two-way interactions were aliased (in three groups of two interactions); hence, the selection of a resolution IV fraction. A random block effect was included in the model as an additional factor to assess day-to-day variability of the instrument. All analyses, model selection, and diagnostics were done using centered and scaled parameter settings. A summary of results is given below.

The low *p*-value for the model (<0.0001) and the high R^2_{adj} (>93%) imply that the model fits the data very well. The high R^2_{pred} value (>91%) indicates that this model would be expected to predict the data values in future experiments conducted over a similar design region reasonably well. Lack-of-fit was statistically significant ($p \approx 0.0015$), but not important in practice, since the observed *F*-statistic was approximately 2.8 with a very large number of degrees of freedom.

Table 1 provides the terms in the regression model that best fit the data in the first stage of experimentation. Standard backward variable selection was used to delete statistically insignificant terms. Based on the model, capillary, cone, extractor, RF voltages and HM Res1 were strong factors that impacted the sensitivity. The contour plot (Fig. 3) suggests that the optimum settings were not present in the initial experimental region, but rather lower values for cone (<55 V) and extractor (<23 V) were needed. Non-constant variance and non-normality of residuals were also detected, primarily due to the nature of the ratio response. The square root transformation was used to smooth this non-homogeneity in variance. Residuals from the

Table 1
Stage-1 regression statistics and measures of model adequacy for response surface modeling of $\sqrt{S/N}$

Parameter	Centered and scaled values				Actual values coefficient
	Coefficient	S.E.	*t*-statistic	*p*-value	
Intercept	3.24	0.062			15.09
Capillary	0.42	0.040	10.50	<0.0001	4.60
Cone	−0.82	0.040	−20.25	<0.0001	−0.21
Extractor	−0.85	0.040	−21.19	<0.0001	−0.62
RF lens	0.23	0.041	5.60	<0.0001	4.35
HM Res1	−0.10	0.040	−2.44	0.0167	−0.11
Capillary^2	−0.20	0.025	−7.84	<0.0001	−0.40
Cone^2	0.23	0.026	8.70	<0.0001	0.000365
Extractor^2	0.18	0.025	7.20	<0.0001	0.00371
RF lens^2	−0.06	0.029	−2.20	0.0299	−2.82
Capillary × cone	−0.13	0.049	−2.58	0.0114	−0.00725
Capillary × extractor	−0.16	0.049	−3.32	0.0013	−0.033
Cone × extractor	0.82	0.049	16.75	<0.0001	0.00471

$R^2 = 0.9392$, $R^2_{adj} = 0.9318$, $R^2_{pred} = 0.9146$.

models that used the square root transformation were much closer to being normally distributed than were residuals from the untransformed models. The logarithmic transformation was also considered, which is a typical statistical approach when the response is a ratio of two independent variables. However, the square root transformation was ultimately chosen simply because the Shapiro–Wilk test for normality [13] of the residuals was slightly stronger than for the logarithmic transformation.

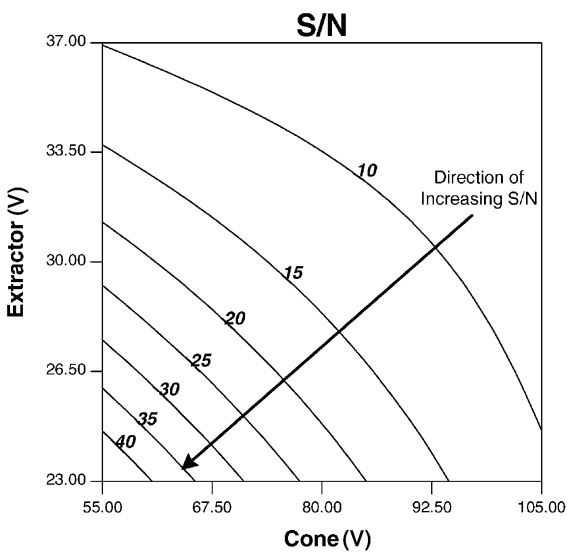

Fig. 3. Contour plot for Azithromycin *S/N* ratio from the stage-1 experiment, where *S/N* is expressed as a function of cone (abscissa) and extractor (ordinate) voltages. Other variables were held constant at the following levels: capillary = 3.70 kV, RF lens = 0.65 V, desolvation gas flow = 500 L/h, LM Res1 = 12.50, LM Res2 = 12.50, HM Res1 = 2.50, HM Res2 = 2.50.

Fig. 4 displays the within-day and between-day variability in *S/N* at the center point of the CCD from this experiment. These data show that the within-day and between-day variability were negligible, implying that the experiment was both repeatable and rugged.

3.2. Stage-2 experimentation

Five tuning parameters were identified in the first stage of experimentation as significant effects on *S/N*: capillary, cone, extractor, RF, and HM Res1. Since HM Res1 had only a negative linear effect on *S/N*: this variable was held constant at its lowest desirable level (2.5) during the second set of experiments. The remaining four variables were studied at levels suggested by the results of the first experiment. A 37-run 2^4 CCD was selected for the second stage, which consisted of the 16 factorial points, eight replicated axial points, and five center runs. Because the optimum levels for two of the variables could have been near

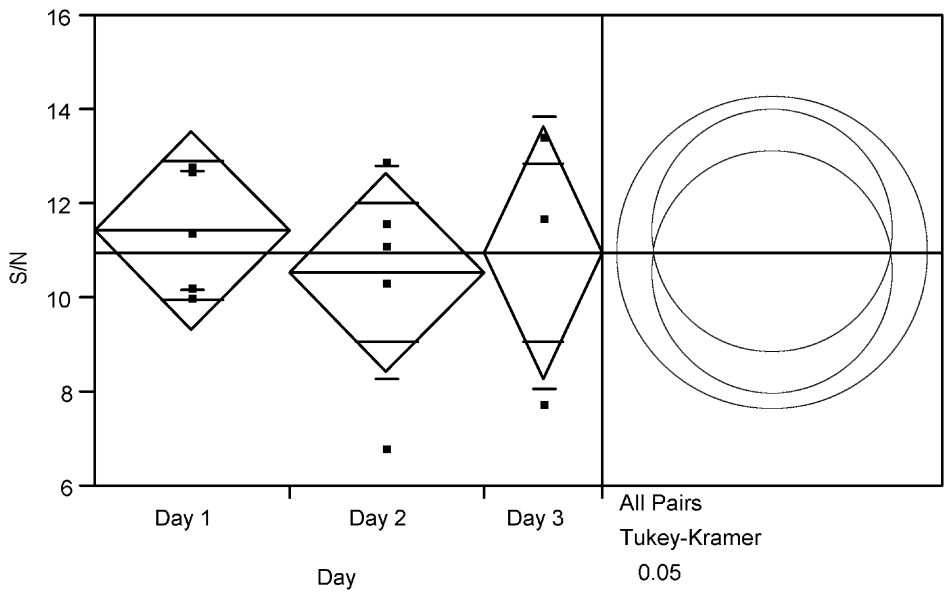

Fig. 4. Within-day and across-day variability in *S*/*N* at the center point from the stage-1 experiment. (plot generated by JMP version 3.2.6, Cary, NC).

zero, a cuboidal design region was selected to thoroughly cover that area. This design allowed fitting a full second-order model for *S*/*N*, which could be used to predict the combination of tuning parameters that would yield the maximum *S*/*N*. As with the first experiment, the square root transformation was used for these data and normality of the residuals verified. The regression statistics from the second experiment are given in Table 2.

The experimental regions for stage-1 and stage-2 did not overlap. Predictive models describing response surfaces over two non-overlapping regions may be quite different in form and are not expected to be identical. Nevertheless, the stage-1 and stage-2

Table 2
Stage-2 regression statistics and measures of model adequacy for response surface modeling of $\sqrt{S/N}$

Parameter	Centered and scaled values				Actual values coefficient
	Coefficient	S.E.	*t*-statistic	*p*-value	
Intercept	8.09	0.16			15.28
Capillary	−1.28	0.13	−9.87	<0.0001	−6.39
Cone	2.95	0.13	22.73	<0.0001	0.49
Extractor	0.66	0.13	5.10	<0.0001	0.20
RF lens	0.31	0.13	2.37	0.0250	1.54
Capillary^2	0.72	0.25	2.89	0.0075	0.72
Cone^2	−0.88	0.25	−3.55	0.0015	−0.00450
Extractor^2	−0.66	0.25	−2.67	0.0128	−0.010
Capillary × cone	−0.53	0.15	−3.66	0.0011	−0.038
Cone × extractor	0.35	0.15	2.39	0.0240	0.00310

$R^2 = 0.9627$, $R^2_{adj} = 0.9502$, $R^2_{pred} = 0.9255$.

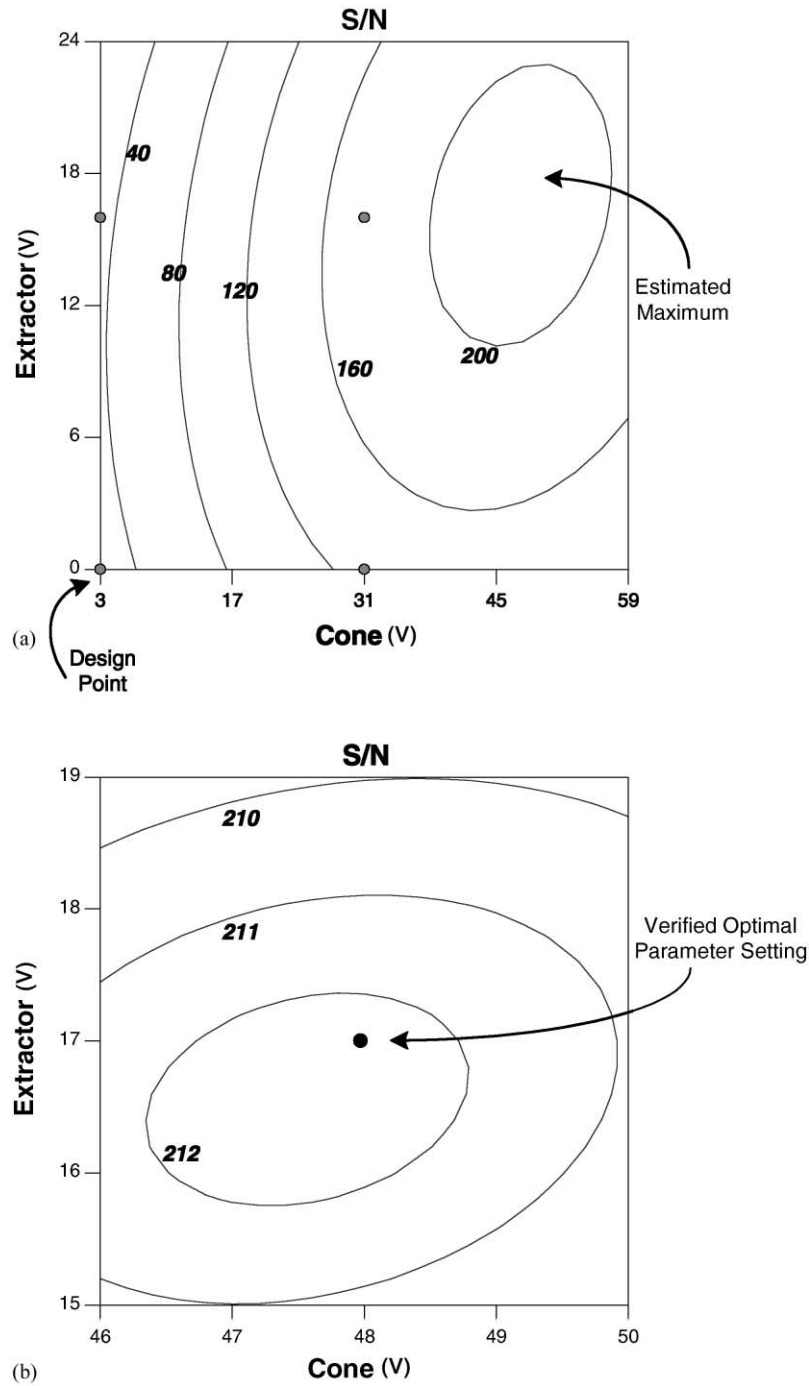

Fig. 5. Two contour plots and one three-dimensional response surface plot from stage-2 experiment. (a) Contour plot for Azithromycin *S*/*N* ratio expressed as function of cone and extractor voltages in area of optimal parameter settings. Capillary and RF lens voltages were held constant at 3.00 kV and 0.90 V, respectively. (b) Enlarged optimal region of the contour plot (a). (c) Three-dimensional response surface plot of the enlarged optimal region shown in (b).

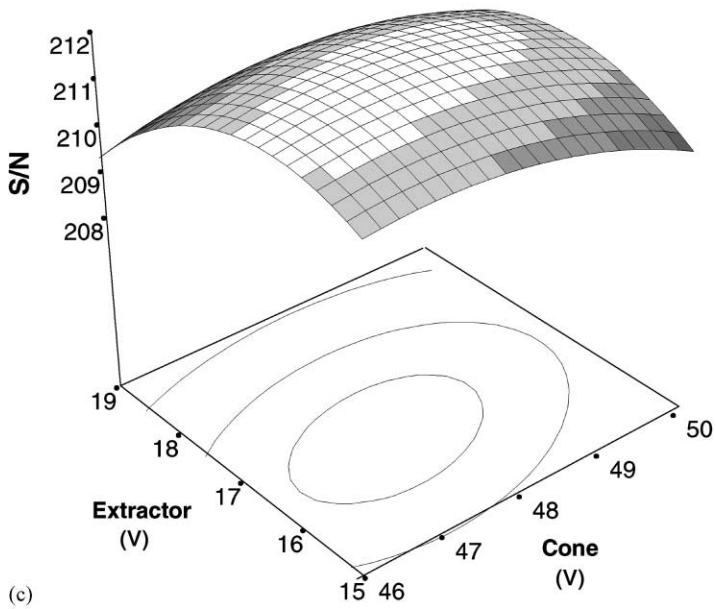

Fig. 5. (*Continued*).

models do share several common traits. RF lens voltage does not interact with any other parameter in either equation, implying that RF lens can be tuned independently of the settings of other variables. Additionally, every statistically significant term in the stage-2 model was also significant in the stage-1 model, providing a large degree of confidence that both design regions were successfully mapped. If the two models had been drastically different from each other, then one might question whether deleting insignificant terms from the stage-1 model was appropriate.

The estimated response surface from the stage-2 experiment predicted that the optimum settings would be slightly outside the design region, as depicted in the two contour plots and one three-dimensional response surface plot in Fig. 5. The first plot shows over the cone-extractor plane the predicted optimum settings, which occur in the middle of the predicted 200-*S*/*N* contour. The second plot is a closer view and suggests that *S*/*N* is very stable when small changes are made to the settings and that the maximum *S*/*N* exceeds 212. The third plot is the 3-D analogue of the second plot. A small verification study was performed at the predicted optimum of capillary = 3.00 kV, cone = 48 V, extractor = 17 V, RF lens = 0.9 V, and HM Res = 2.5 (Fig. 6). Results confirmed the optimal settings with an average *S*/*N* over 270.

The estimated lower limit of detection (LLD) and LLQ for the low pH degradation product of Azithromycin were determined to be 11.7 and 39.1 ng/mL, respectively. LLD and LLQ are defined in Eqs. (1) and (2).

$$\mathrm{LLD} = \frac{3.0\sigma}{S} \tag{1}$$

$$\mathrm{LLQ} = \frac{10\sigma}{S} \tag{2}$$

where σ is the standard deviation of the response at the *Y*-intercept and *S* is the slope of the calibration curve. As it is often the case, minimal quantities of a degradant are available early in a pharmaceutical development program. Thus, the LLD and LLQ were generated for a degradation product based on optimization using the parent molecule in order to demonstrate that, for some cases, LC/MS–MS optimization of the parent may provide suitable sensitivity for the

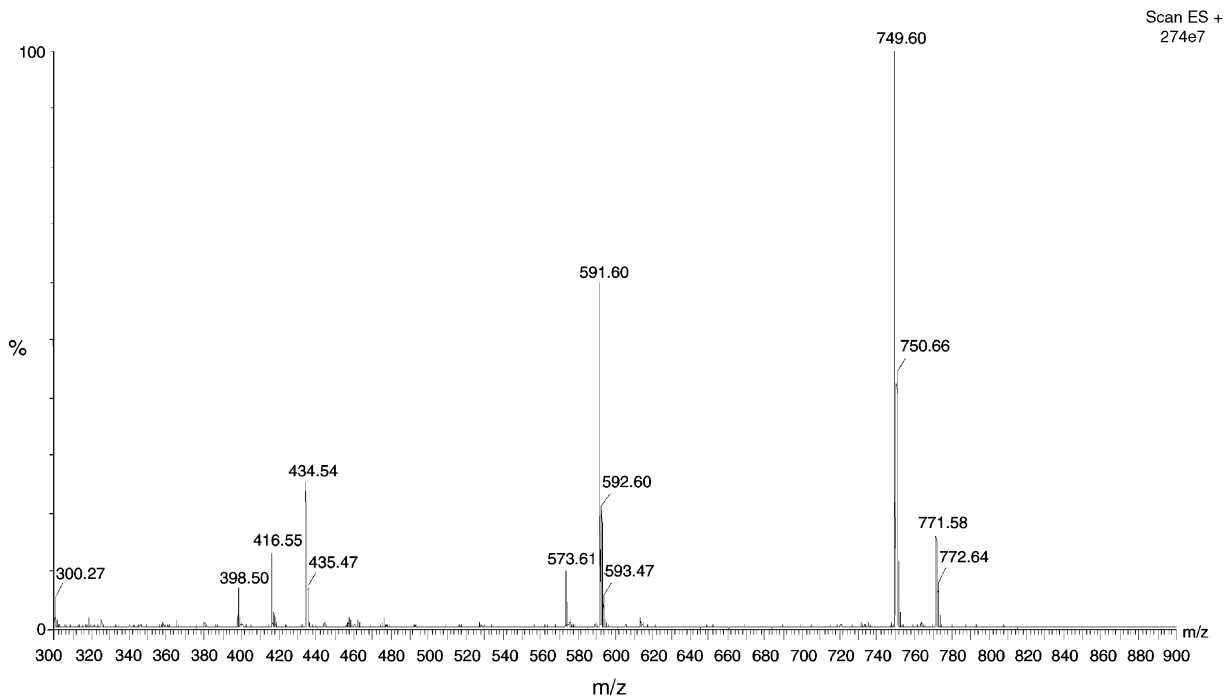

Fig. 6. Mass spectrum of Azithromycin acquired using the optimized tuning parameters (cone = 48 V, capillary = 3 kV, extractor = 17 V, RF lens = 0.9 V, HM Res1 = 2.5).

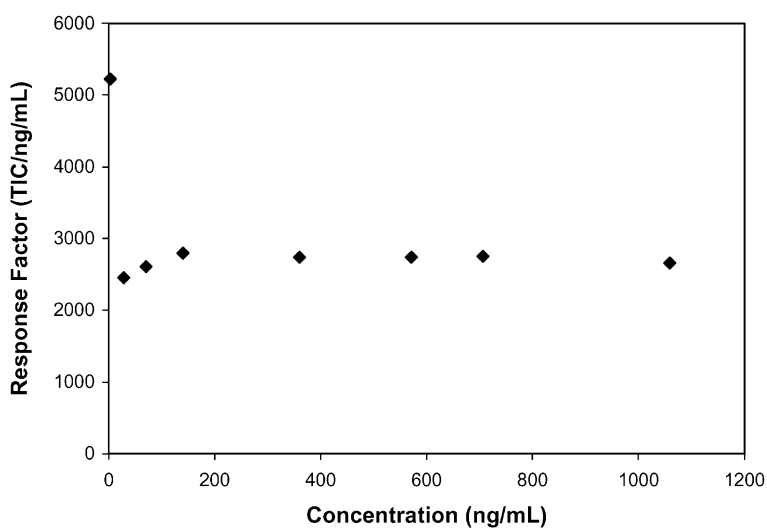

Fig. 7. Standard curve of the low pH degradation product of Azithromycin, pseudo-aglycone Azithromycin, generated with LC/MS–MS settings optimized with the parent molecule, Azithromycin. The response factor is calculated as: (peak area)/(concentration).

degradant. Fig. 7 shows a standard curve for this degradation product (pseudo-aglycone Azithromycin), plotted as response factor (peak area divided by concentration) vs. concentration, and demonstrates excellent linearity from 30–1000 ng/mL, which represents approximately a 40-fold concentration range. This observed wide range of linearity at very low concentrations should allow for the kinetic evaluation of trace levels of degradants over a period of time (initial rates), facilitating the rapid and accurate determination of product shelf-life. Ongoing efforts will report on the utility of LC/MS–MS in determination of pharmaceutical formulation shelf-life using initial rate methods coupled with optimized LC/MS–MS detection [14].

4. Conclusions

A triple quadrupole mass spectrometer equipped with an ESI interface was optimized through statistical experimentation for azithromycin, a model pharmaceutical compound. Five important tuning parameters were identified: capillary, cone, extractor, RF, and HM Res1. The highest sensitivity of the instrument for Azithromycin was found when capillary voltage was set at 3, cone voltage at 48, extractor voltage at 17, RF voltage at 0.9, and HM Res1 at 2.5, respectively. The derived models can be used to predict the *S*/*N* over a relatively wide tuning range. Excellent linearity was achieved for pseudo-aglycone Azithromycin, a low pH degradation product of Azithromycin, using optimization parameters generated with the parent, with over a 40-fold linearity range at levels relevant for degradants that form in pharmaceutical formulations over time (<0.1%). This suggests that with proper instrument optimization, rates of degradant formation (and, hence, product shelf life and/or toxicity qualification strategies) can quickly and accurately be determined.

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