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The use of calibration approaches for quantitative GC/MS analysis —secobarbital example

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ABSTRACT

One-point, Linear, Hyperbolic and Polynomial calibration approaches were adopted to elucidate the quantitative effectiveness of standard solutions of 10 to 800 ng/mL secobarbital by comparing theoretical and observed concentrations. Different concentrations of internal standard（IS）, 50 and 200 ng/mL, were added to standard solutions for comparison purpose. Two GC column temperature programs, a high ramp rate of 20 ℃ per minute and a low ramp rate of 2 ℃ per minute, were used to generate different degrees of peak-overlap and ion cross-contributions to evaluate the most appropriate application for each calibration approach.

The ion cross-contribution deriving from IS to analyte leads to the significantly positive observed concentration at low concentration levels while using One-point calibration. This phenomenon is clearly exhibited by ion pairs containing the higher ion cross-contribution, the larger added IS magnitude, and the higher ramp rate of temperature programming. One-point calibration is appropriately used for the quantitation that concentrations of analytes are close to that of the calibrator. In addition to the ion cross contribution, the "non-proportional over-all change in ionization efficiency" phenomenon will also affect the effectiveness under Linear calibration. Thus, it can be adopted by using appropriate derivatization, IS and/or its magnitude, and temperature programming along with the suitable concentration range of standard solutions. The Hyperbolic calibration can be generally used for determining analytes at various concentration levels without interference from ion cross-contribution factor. The Polynomial approach can completely fit in ion-pair intensity ratios of standard solutions for each ion-pair yielding the ideal observed concentrations.

Keywords: Non-linear calibration, GC/MS quantitation, Secobarbital, One-point, Linear, Hyperbolic, Polynomial

Introduction

Isotopic analogs used as internal standards (ISs), and selected ion monitoring (SIM) GC/MS procedures are used in a state-of-the-art method for quantitatively analyzing drugs and their metabolites [1]. A fuller understanding of the performance characteristics of IS and the calibration approaches is important for the accurate quantitation. Ion cross-contribution and the "overall non-proportional change in ionization efficiency" have been regarded as the underlying causes of changes in the theoretical analyte/IS ratios [2]. Although linear calibration has been employed routinely in quantitation by GC/MS analysis [3], interference factors, including the appropriate ion-pair, IS magnitude, reconstituted volume and temperature programming, must also be evaluated before the linear approach can be applied [4,5]. Non-linear approaches to quantitation have been evaluated with reference to the inevitable overlap of the peaks that correspond to isotopic IS and the analyte, and the "over-all non-proportional change in ionization efficiency" [6].

This work is focused on evaluating the features of calibration curves that correspond to the use of the $^{2}H_{5}$-analog as IS for quantitatively determining secobarbital

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in urine. One-point, Linear, Hyperbolic and Polynomial calibration approaches were adopted to elucidate the quantitative effectiveness based on specific parameters, such as (a) ion-pair, （b）IS magnitude, and (c) column temperature programming, which may influence the use of the calibration approach.

Experimental

Reagents

Secobarbital and $^2H_5$-analogs (1 mg/mL methanol solution; 99% purity) were purchased from Radian Corp. (Austin, TX). Tetramethylammonium hydroxide (TMAH) was provided by Acros Organics (USA). Dimethyl sulfoxide was purchased from Hayashi Pure Chemical Industries Ltd. (Japan). Iodomethane and sodium acetate were obtained from Wako Pure Chemical Industries, Ltd. (Japan). Drug-free urine, used in the preparation of standard drug solutions, was provided by a member of the research group.

Solid-Phase Extraction and Derivatization

The solid-phase extraction (SPE) procedures specified by the Varian Bond Elut Certificate II [7], were followed to process standard solutions of 10, 20, 50, 100, 200, 400 and 800 ng/mL, using 2 mL urine specimens. Two amounts of IS, 50 and 200 ng/mL, were respectively added to standard solutions for comparative quantitation. Extracts were derivatized as methyl-derivatives [8]. The final product was reconstituted with 20 µL ethyl acetate prior to GC/MS analysis.

GC/MS Analysis

A Thermo Finnigan Trace GC/MS (Column: 15-M, 0.25-mm ID, 0.25-mm film thickness) was used for collecting full-scan and SIM mass spectrometric data. These data were used preliminarily to select the following analogous ion pairs that are apparently free of that exhibit minimal cross-contribution between the analyte and the isotopic analog for the quantitative analyses. The ion pairs of m/z 196/201, 195/200, and 138/143 all derived as methyl-derivatives were selected to investigate quantitative when using different calibration approaches.

Changes in ion-pair intensity ratios and the observed concentrations in standard solutions, caused by the variation in peak overlap, were examined under the following two temperature programming conditions in GC to study the ion cross-contribution and the "non-proportional over-all change in ionization efficiency" associated with the difference between the retention times difference of the analyte and its IS.

2℃ ramp rate: 100 ℃ initial temperature, 2℃ /min ramp to 128 ℃ for 2 min, 40℃/min ramp to 280 ℃, end temperature for 3 min.

20℃ ramp rate: 100 ℃ initial temperature, 20℃ /min ramp to 160 ℃ for 2 min, 40℃/min ramp to 280 ℃, end temperature for 3 min.

The following regression equations associated with the calibration approaches were applied to the quantitations where Y is the observed ion-pair intensity ratio and X is the concentration of the analyte or that of the calibrator.

A. One-point Calibration: $(Y_{analyte}/Y_{calibrator})\,X_{calibrator} = X_{analyte}$

B. Linear Calibration: $Y = b_0 + b_1 X$

C. Hyperbolic Calibration: $Y = (b_0 + X)/(b_1X + b_2)$

D. Polynomial Calibration: $y = b_0 + b_1X + b_2X^2 + b_3X^3$

Results and Discussions

Cross-contribution data on the characteristics of the calibration curves

The ion-pairs designated for the analyte and the IS must be evaluated based on their quantitative effectiveness, to select an appropriate IS for the quantitation. The ion cross-contribution is the underlying cause of interference with the quantitative results associated with the selected ion-pair. The SIM ion intensity and cross-contribution data (in parentheses in %) in Table 1 for ions designed for secobarbital and its labeled analogs, were measured by "direct measurement" and "normalized direct measurement" methods. The authors' earlier work revealed that ion cross-contribution could be most accurately evaluated by multiple methods for verification. These methods include direct measurement or/and normalized direct measurement, internal standard or/and standard addition methods [9].

Table 1. SIM ion intensity and cross-contribution data (in parentheses in %) for ions designed for secobarbital and its labeled analogs — "direct measurement" and "normalized direct measurement" methods.

Ion (m/z)	SIM ion intensity derived from secobarbital and % contribution (in parentheses) by analog	SIM ion intensity derived from $^2H_5$-secobarbital and % contribution (in parentheses) by analog	Normalized SIM ion intensity derived from $^2H_5$-secobarbital and % contribution (in parentheses) by analog
201	153216	58867712 (0.26%)	46399488 (0.33%)
200	136576	29380608 (0.46%)	23457774 (0.59%)
143	450496	12128256 (3.7%)	9559786 (4.7%)
196	46399488 (2.4%; 1.9%)	1107200	872694
195	30932992 (17%; 13%)	5215232	4110642
138	9494528 (8.8%; 7.0%)	839168	661432

Experimental results demonstrate that ^2H-analogs contribute more to the intensity of ions designated for secobarbital than secobarbital contributes to the intensity of ions designated for ^2H-analogs. A low concentration of added IS, therefore, significantly increases the ion intensity of the analyte. The low mass ions were practically excluded as candidates because they contributed more to the corresponding ions than did the high mass ions. Higher mass ion-pairs have been typically selected for quantitation [10].

Effect of IS magnitude to the quantitative effectiveness

The results in Table 2 show that ion-pair m/z 196/201 was used as an example of quantitative effectiveness, using various added IS magnitudes. Based on the more ion cross-contribution from IS, positive deviation of observed concentration at lower concentration levels using 200 ng/mL are higher than those using 50 ng/mL under One-point and Hyperbolic calibrations. Negative deviations of the observed concentrations reveal that analytes could be easily affected by Linear calibration at low concentrations. This trend might be caused by the "over-all non-proportional change in ionization efficiency", which is responsible for the lower ion intensity ratios at higher concentration levels. The amount of IS added seems not to affect the quantitative effectiveness given Polynomial calibration.

Table 2. Comparison of quantitation results at different amounts of IS — ion-pair m/z 196/201.

IS magnitude	Theor. Conc. (ng/mL)	Ion Ratio	Obs'ed Conc. (Dev. %) by One -point	Obs'ed Conc. (Dev. %) by Linear	Obs'ed Conc. (Dev. %) by Hyperbolic	Obs'ed Conc. (Dev. %) by Polynomial
50 ng/mL	10	0.2528	11.31(13)	7.969(-20)	10.94(9.4)	9.509(-4.9)
	20	0.4256	19.03(-4.8)	16.74(-16)	19.33(-3.8)	18.33(-8.3)
	50	1.118	Calibrator	51.89(2.8)	53.05(6.1)	53.48(7.0)
	100	2.019	90.29(-9.7)	97.62(-2.4)	97.13(2.9)	98.79(-1.2)
	200	4.051	181.2(-9.4)	200.8(0.38)	197.4(-1.3)	199.8(-0.10)
	400	8.13	363.6(-9.1)	407.8(1.9)	402.6(0.65)	401.9(0.49)
	800	15.74	703.9(-12)	794.1(-0.73)	799.5(-0.061)	812.1(1.5)
200 ng/mL	10	0.0799	14.17(41)	8.000(-20)	13.60(36)	10.59(5.9)
	20	0.1266	22.44(12)	16.65(-16)	21.55(7.8)	19.47(-2.6)
	50	0.2881	51.08(2.2)	46.56(-6.9)	49.15(-1.7)	49.77(-0.46)
	100	0.5573	98.81(-1.2)	96.41(-3.6)	95.58(-4.4)	99.04(-0.96)
	200	1.128	Calibrator	202.1(1.0)	195.8(-2.1)	199.9(-0.052)
	400	2.279	404.1(1.0)	415.2(3.8)	405.6(1.4)	396.9(-0.75)
	800	4.290	760.6(-4.9)	787.6(-1.5)	798.8(-0.15)	767.6(-4.0)

Effect of ion-pair to the quantitative effectiveness

Table 3. Comparison of quantitation results obtained using ion-pairs m/z 196/201, 195/200 and 138/143.（IS: 50 ng/mL）

Ion pair（m/z）	Theor. Conc. (ng/mL)	Ion Ratio	Obs'ed Conc. (Dev. %) by One-point	Obs'ed Conc. (Dev. %) by Linear	Obs'ed Conc. (Dev. %) by Hyperbolic	Obs'ed Conc. (Dev. %) by Polynomial
196/201	10	0.2528	11.31(13)	7.969(-20)	10.94(9.4)	9.509(-4.9)
	20	0.4256	19.03(-4.8)	16.74(-16)	19.33(-3.8)	18.33(-8.3)
	50	1.118	Calibrator	51.89(2.8)	53.05(6.1)	53.48(7.0)
	100	2.019	90.29(-9.7)	97.62(-2.4)	97.13(2.9)	98.79(-1.2)
	200	4.051	181.2(-9.4)	200.8(0.38)	197.4(-1.3)	199.8(-0.10)
	400	8.13	363.6(-9.1)	407.8(1.9)	402.6(0.65)	401.9(0.49)
	800	15.74	703.9(-12)	794.1(-0.73)	799.5(-0.061)	812.1(1.5)
195/200	10	0.8005	21.62(116)	10.58(5.8)	14.36(44)	11.73(17)
	20	0.9984	26.97(35)	17.45(-13)	20.82(4.1)	18.90(-5.5)
	50	1.851	Calibrator	47.06(-5.9)	48.71(-2.6)	49.41(-1.2)
	100	3.276	88.49(-12)	96.54(-3.5)	95.62(-4.4)	99.21(-0.79)
	200	6.312	170.5(-15)	201.9(0.97)	196.8(-1.6)	201.8(0.88)
	400	12.39	334.7(-16)	413.0(3.2)	404.7(1.2)	401.0(0.26)
	800	23.37	631.3(-21)	764.2(-0.72)	799.0(-0.13)	790.6(-1.2)
138/143	10	0.5711	18.72(87)	11.83(18)	13.27(33)	8.290(-17)
	20	0.8959	29.37(47)	23.82(19)	24.98(25)	22.35(12)
	50	1.525	Calibrator	47.03(-5.9)	47.68(-4.6)	48.73(-2.5)
	100	2.842	93.18(-6.8)	95.63(-4.3)	95.32(-4.6)	101.1(1.1)
	200	5.511	180.7(-10)	194.1(-2.9)	192.3(-3.9)	199.6(-0.22)
	400	11.39	373.4(-6.6)	411.1(2.8)	408.0(2.0)	403.0(0.75)
	800	21.84	716.1(-10)	796.7(-0.42)	798.5(-0.19)	862.9(7.9)

Comparison of quantitation results in Table 3 using different ion-pairs of m/z 196/201, 195/200, 138/143 and calibration approaches. Standard solutions below the concentration level of calibrator were significantly affected when m/z 195/200 and 138/143 ion-pairs, but not the m/z 196/201 ion-pair, were used under the One-point approach. This finding reveals that the higher ion cross-contribution is responsible for the greater intensity of the ion-pair ratio. Hence, m/z 195/200 and 138/143 ion-pairs were inappropriate to use for quantitation, based on the One-point calibration. This result is consistent with the determination of ion cross-contribution in Table 1 for three counterpart ions. However, quantitation results obtained using m/z 196/201, 195/200 and 138/143 ion-pairs under Linear, Hyperbolic and Polynomial approaches exhibit similar deviations with most standard solutions, except 10 ng/mL for m/z 195/200 and 10 and 20 ng/mL for m/z 138/143. Although m/z 195/200 and 138/143 ion-pairs are responsible for a greater ion cross-contribution to each other, both can be used in quantitation under appropriate calibrations. Comparisons of the quantitation results under four calibrations for the m/z 196/201 ion-pair, obtained using the $^{2}H_{5}$-analog as IS, are preferred for the quantitative GC/MS analysis of secobarbital.

Effects of temperature programming conditions to the quantitative effectiveness

Table 4. Comparison of quantitation results obtained under different temperature programming conditions — m/z 195/200 ion-pair. （IS: 50 ng/mL）

Temp. Ramp	Theor. Conc. (ng/mL)	Ion Ratio	Obs'ed Conc. (Dev. %) by One-point	Obs'ed Conc. (Dev. %) by Linear	Obs'ed Conc. (Dev. %) by Hyperbolic	Obs'ed Conc. (Dev. %) by Polynomial
2 ℃	10	0.4298	13.18(31)	8.544(-14)	7.998(-20)	9.981(-0.19)
	20	0.7075	21.69(8.4)	17.99(-10)	17.52(-12)	18.90(-5.5)
	50	1.631	Calibrator	49.40(-1.2)	49.19(-1.6)	48.86(-2.3)
	100	3.347	102.6(2.6)	107.8(7.8)	107.9(7.9)	105.8(5.8)
	200	6.01	184.2(-7.9)	198.3(-0.82)	199.0(-0.48)	196.8(-1.6)
	400	11.84	362.9(-9.3)	396.6(-0.84)	397.8(-0.55)	403.2(0.81)
	800	23.73	727.5(-9.1)	801.0(0.13)	800.5(0.066)	804.8(0.60)
20 ℃	10	0.7008	19.68(96)	11.52(15)	9.558(-4.4)	11.41(14)
	20	0.9275	26.05(30)	19.45(-2.7)	17.73(-11)	19.05(-4.8)
	50	1.78	Calibrator	49.25(-1.5)	48.40(-3.2)	47.99(-4.0)
	100	3.346	93.99(-6.0)	104.0(4.0)	104.6(4.6)	102.0(2.0)
	200	6.084	170.9(-15)	199.7(-0.13)	202.1(1.1)	198.8(-0.58)
	400	11.62	326.4(-18)	393.3(-1.7)	397.1(-0.71)	400.0(-0.26)
	800	23.35	655.9(-18)	803.5(0.43)	800.5(0.07)	798.9(-0.13)

Table 4 depicts the observed concentrations and percentage deviations under different temperature programming conditions. Ion intensity ratios and percentage deviations at low concentrations increase with the rate of the temperature ramp under One-point calibration, suggesting that the increase of ion intensity ratios especially at lower concentration levels results from the increases in the ion cross-contribution with the peak-overlap. The ion intensity ratios at concentrations 10, 20 and 50 ng/mL are 0.4298, 0.7075 and 1.631 for a 2℃ ramp rate, and 0.7008, 0.9275 and 1.980 for a 20℃ ramp rate. The ion intensity ratios at both ramp rates above a concentration of 50 ng/mL are similar. The temperature program substantially affects the quantitation results under the One-point approach. The lower ramp rate is preferred for determining the lower concentrations of unknown analytes.

Effect of calibration approach to the quantitative effectiveness

The m/z 195/200 ion-pair was adopted as an example to elucidate the quantitative effectiveness under various calibrations. Compared to the theoretical values, quantitation using One-point calibration yields lower observed concentrations at high concentration levels and higher observed concentrations at low concentrations levels. The "over-all non-proportional change in ionization efficiency" and ion cross-contribution may be two underlying causes of this trend. The analyte with high molecular population at the ion source has lower ionization efficiency than the IS for the standard solution at high concentration levels. Thus, ion intensity ratios at high concentrations are lower than the theoretical values. Molecular abundance strongly affects these ratios when ^{2}H-analogs are used as ISs [4]. This trend in Fig. 1 reveals that the concentration ranges of unknown analytes should be close to concentration of the calibrator when One-point calibration is used. Linear, Hyperbolic and Polynomial calibration curves completely fit the determined ion intensity ratios.

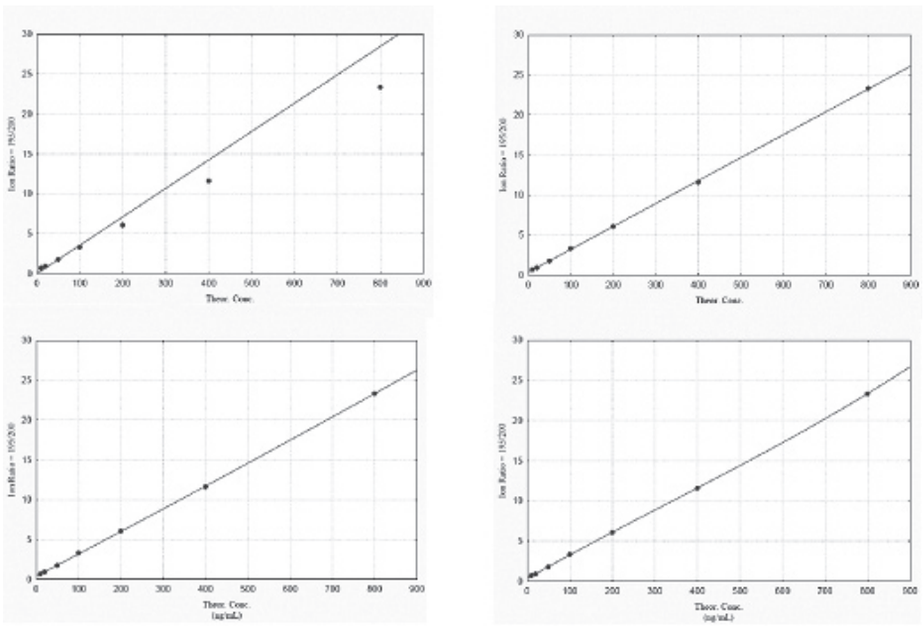

Fig 1. Calibration curves obtained using the m/z 195/200 ion-pair, obtained by different calibration approaches.（A） One-point — 50 ng/mL calibrator; （B）Linear; （C） Hyperbolic;（D） Polynomial .

The observed low concentrations in the standard solutions obtained by Linear calibration were obviously lower than those obtained by the One-point approach, revealing that the ion cross-contribution is responsible for that higher ion-pair ratios at low concentrations can be adjusted according to the lower ion-pair ratios derived from the "non-proportional overall change in ionization efficiency" at high concentrations. Thus, the linearity of the calibration curve increases, especially at low concentrations. On the other hand, the slight increase of the ion cross-contribution with the peak-overlap makes the temperature program with the high ramp rate responsible for the observation of a higher concentration by Linear calibration at low concentrations.

Quantitation results obtained using Hyperbolic approach demonstrate different phenomena. The standard solutions with the higher ion cross-contribution at low concentrations exhibit lower observed concentrations and deviations, suggesting that the characteristic of Hyperbolic curve is associated with standard solutions with a high ion cross-contribution. Therefore, the quantitative effectiveness obtained by the Hyperbolic approach at low concentrations with a high ramp rate exceeds that obtained at a low ramp rate in the GC temperature program. The resulting data obtained using Polynomial calibration reveal that all of the ion-pairs offer ideal quantitation without interference by ion cross-contribution and GC temperature programming. Polynomial curves could appropriately fit the ion-pair ratio of each standard solution .

Conclusions

Ion cross-contribution is the underlying cause of interference in the quantitative determination of secobarbital by the One-point approach at low concentrations. This interference can be improved by adjusting derivativzation, IS and/or its magnitudes and GC temperature programming conditions. A high mass ion-pair ratio of m/z 196/201 is preferred for quantitation of secobarbital using One-point calibration. It can be appropriately used when the concentration of the analyte is close to that of the calibrator. Quantitations that using Linear calibration were simultaneously affected by the ion cross-contribution and the "over-all non-proportional change in ionization efficiency" at high concentrations, associated with the analyte and IS. Negative deviations of observed concentrations were thus observed at low concentration levels. It might be effectively improved when adopting not only the ion-pair of analyte/^{2}H-analog with low ion cross-contribution but also the short concentration range of standard solutions [2]. In contrast, ion cross-contribution and the "over-all non-proportional change in ionization efficiency"

factors do not considerably affect quantitation under Hyperbolic calibration. However, some ion-pairs generate larger deviations at the low concentration levels. Polynomial calibration has been clearly shown as an ideal approach by giving quantitative results deriving from all ion-pairs under different parameters.

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