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Influence of number of calibration standards within a defined range on pharmacokinetic disposition—case studies with omeprazole and clopidogrel carboxylic acid

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ABSTRACT: While the practice of using a smaller number of non-zero standards (typically seven to eight) has not been entertained in routine bioanalytical work, it is important to innovate and be pragmatic about minimizing the number of calibration standards to promote cost-effective and speedy assessment. In this exercise, two important compounds, omeprazole and clopidogrel carboxylic acid, were considered. Additionally, both analytes offered a 1000-fold calibration curve range with eight non-zero standards to permit a systematic evaluation. Accordingly various scenarios of *post-hoc* analysis of the calibration data were formulated which included step-wise reduction of the number of calibration standards from a maximum of $n = 8$ to a minimum of $n = 3$. In all the scenarios evaluated in this exercise, a calibration curve was reconstructed and both quality control samples and *in vivo* pharmacokinetics were calculated in each instance. Based on the data generated in this exercise, a minimum of three non-zero calibration standards were adequate to predict the quality control samples with the predefined accuracy and precision estimates for both omeprazole and clopidogrel carboxylic acid. Additionally, the *in vivo* pharmacokinetic characterization of the chosen compounds was not hampered by the reduction of calibration standards (from $n = 8$ to $n = 3$). Hence, consideration for reducing number of calibration standards in bioanalytical work may provide a viable alternative in several situations such as formulation screening strategies, routine therapeutic drug monitoring and sparse sample analyses. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: calibration curve; regression; omeprazole; clopidogrel carboxylic acid; pharmacokinetics

Introduction

The pharmacokinetic characterization of novel chemical entities (NCEs) in preclinical and clinical development is achieved by employing non-zero standards (typically $n = 8$) to cover a well-defined range of concentrations. As part of the assay validation, it is customary to define the lower bound (denoted as lower limit of quantitation, LLQ) and the upper bound (denoted as upper limit of quantitation, ULQ) of the calibration curves. While the lower bound is greatly influenced by factors such as method of sample extraction (protein precipitation, solid-phase extraction or liquid–liquid extraction), detection platform and inherent attributes of the analyte etc., the upper bound is generally dictated by the saturation of the instrument's dynamic response. It is not uncommon to construct calibration curves that cover a more than 100-fold range in order to characterize the pharmacokinetics from starting low human doses to the anticipated maximum tolerated dose (MTD) in first-in-man clinical protocols and also for application in bioequivalence/pharmacokinetic studies (Vijaya Bharathi *et al.*, 2009; Zhang and Chen, 2009; Tang *et al.*, 2009; Zeng *et al.*, 2009; Jiang *et al.*, 2009; Arnold *et al.*, 2008; Handy *et al.*, 2008; Minkin *et al.*, 2008; Jain *et al.*, 2008; Xue *et al.*, 2007; Zeng *et al.*, 2007; Xu *et al.*, 2007; Shen *et al.*, 2004; Upreti *et al.*, 2003). Additionally, the existence of a 100-fold range may be useful when dealing with NCEs that are substrates for polymorphic cytochrome P450 (CYP) isozymes wherein log-orders of differences in the plasma concentrations of the parent and/or metabolite are expected to occur between the extensive metabolizer and poor metabolizer phenotypes (Preskorn *et al.*, 2009; Shao *et al.*, 2009; Davies *et al.*, 2008; Stamer *et al.*, 2007; Shilbayeh and Tutunji, 2006; Inomata *et al.*, 2005).

Rationale

It is important that common yardsticks, unbiased assessment and rigor need to be strictly adhered during assay validation of any analyte(s) and/or its associated metabolite(s) since it will be used

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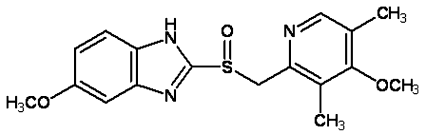
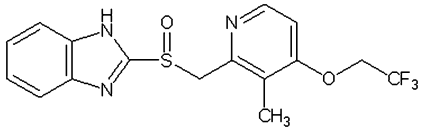
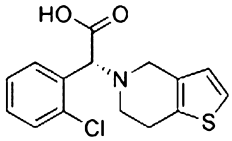
Abbreviations used: ANDA, abbreviated new drug application; AUC_{0-t}, area under the plasma concentration vs time curve up to time = t; $AUC_{0-\infty}$, area under the plasma concentration vs time curve up to time = infinity; C_{max}, peak plasma concentration; CYP, cytochrome P450 isozyme; EM, extensive metabolizer; IS, internal standard; HQC, high quality control; LLQ, lower limit of quantitation; LQC, low quality control; MTD, maximum tolerated dose; MQC, medium quality control; NCE, new chemical entity; NDA, new drug application; PM, poor metabolizer; QC, quality control sample; TDM, therapeutic drug monitoring; $t_{1/2}$, elimination half-life; ULQ, upper limit of quantitation.



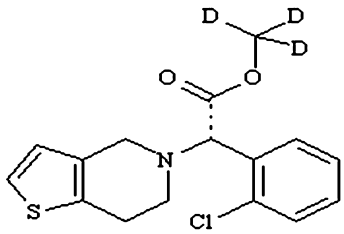
Scheme 1. Omeprazole.



Scheme 2. Lansoprazole.



Scheme 3. Clopidogrel carboxylic acid.



Scheme 4. D_3 clopidogrel.

to generate the pharmacokinetic data for regulatory submissions such as new drug application (NDA) or abbreviated NDA (ANDA) filings, etc. However, in other situations where scientific question(s) needs to be addressed, such as the feasibility of a newer formulation, alternative route of administration, formulation screening strategy, therapeutic drug monitoring (TDM), etc., it may be important to minimize the bioanalytical work such as reducing the number of calibration standards to promote cost-effective and speedy assessment. It is needless to mention that it is important that, if any departure is attempted from the regular practice of generating a standard calibration curve data, it needs to be carefully examined so that it does not compromise data quality and/or meaningful data interpretation for the stated objective(s). We were interested in minimizing the number of calibration standards to a point where it would still give a meaningful data quality and also would not provide challenges in data interpretation. Using two case studies of two important analytes, namely omeprazole and clopidogrel carboxylic acid, we performed *post-hoc* analysis of the analytical data and report various scenarios of reduction of calibration standards (from a maximum of $n = 8$ to a minimum of $n = 3$). In each of the scenarios, we also computed quality control (QC) prediction and characterization of pharmacokinetics in a chosen subject to ensure objectivity in our assessment protocol.

Methods

Assay Methodology

The details of the liquid chromatographic–mass spectrometric assays including internal standard selection with chromatographic conditions and monitoring of transition pairs for both omeprazole and clopidogrel carboxylic acid are provided in Table 1.

Creation of Discrete Sets of Calibration Standards

Both the assays of omeprazole and clopidogrel carboxylic acid employed $n = 8$ non-zero calibration standards covering a range of approximately 1000-fold (omeprazole, 5.081–5002.500 ng/mL; clopidogrel carboxylic acid, 4.071–4309.742 ng/mL). For each analyte discrete sets, ranging from $n = 7$ to $n = 3$, of calibration standard sets were created as shown in Table 2. After fixing the high and low ends of calibration curve, sequential and systematic steps were employed that ensured removal of one calibration standard, alternating from the bottom and upper portions of the calibration curve. Hence, using this approach there were 12 discrete sets of calibration curves for evaluation for both omeprazole and clopidogrel carboxylic acid (one set of $n = 8$; two sets of $n = 7$; one set apiece of $n = 6$, 5 and 4; six sets of $n = 3$). Each discrete set of calibration standards were subjected to linear regression fit with $1/x^2$ as weighting factor.

Prediction of Quality Control Samples

Multiple QC samples available at low, medium and high quartiles for both omeprazole (14.656, 1628.424 and 3618.720 ng/mL) and clopidogrel carboxylic acid (11.275, 1342.239 and 3355.596 ng/mL) were predicted using the regression parameters from the different but discrete calibration sets of the two analytes and the values were compared with that previously obtained from a full set of calibration standards (i.e. $n = 8$).

Prediction of Concentrations from *in Vivo* Pharmacokinetic Samples

The regression parameters from the individual calibration sets were used to predict the value of the unknown *in vivo* samples gathered from one or two human subjects. The computed pharmacokinetic parameters (peak plasma concentration, C_{max}; area under the plasma concentration versus time curve up to time = t, AUC_{0-t}, or up to time = infinity, $AUC_{0-\infty}$; and elimination half-life, $t_{1/2}$) for the 11 discrete calibration sets were compared with the value obtained from the full set of calibration standards ($n = 8$).

Results

As observed in Table 3, the slope and intercept values for omeprazole were found to be extremely close across the 12 calibration sets evaluated. Although numerically not as tight as omeprazole, the regression parameter values for clopidogrel carboxylic acid were found to be consistent in the 12 calibration sets (Table 3). As would be expected from the regression parameters, the QC predictions (LQC, MQC or HQC samples) across the range of the calibration curve were found to be similar for the various calibration sets for either omeprazole or clopidogrel carboxylic acid (Table 4). The prediction of consistent and overlapping pharmacokinetic parameters for omeprazole carried out from the various calibration sets suggested the utility of lesser number of calibration standards (Table 5). Similarly, the prediction of pharmacokinetic parameters for clopidogrel carboxylic acid was found to be similar from the various calibration sets, reconfirming the applicability of a fewer calibration standards (Table 6).

Table 1. Assay particulars for the analysis of omeprazole and clopidogrel carboxylic acid in human plasma samples

Particulars	Omeprazole	Clopidogrel carboxylic acid
Instrument	API 2000 LC/MS/MS	API 4000 LC/MS/MS
Column	X-Terra RP_{18}, 4.6 × 50 mm (5 µm particle size)	Zorbax SB-C_8, 4.6 × 100 mm (3.5 µm particle size)
Mobile phase	Binary mixture comprising 5 mM ammonium acetate solution adjusted to pH 10–acetonitrile, 20 : 80,v/v	Binary mixture comprising 2 mM ammonium acetate solution adjusted to pH 3.5–acetonitrile, 20 : 80, v/v
Extraction Process	To 500 µL sample, add 50 µL IS; extraction involves liquid–liquid extraction with MTBE and basic buffer	To 500 µL sample, add 50 µL IS; extraction involves liquid–liquid extraction with MTBE and acidic buffer
Calibration curve range	5.081–5002.500 ng/mL	4.071–4309.742 ng/mL
IS (concentration)	Lansoprazole (30 µg/mL)	d_3 Clopidogrel (1 µg/mL)
Transition pair monitoring	Omeprazole: *m/z* 346.2 to *m/z* 198.20 Lansoprazole (IS): *m/z* 370.40 to *m/z* 252.00	Clopidogrel carboxylic acid: *m/z* 308.1 to *m/z* 198.0 d_3 Clopidogrel: *m/z* 326.1 to *m/z* 215.0
Accuracy/precision	Accuracy 92.36–107.41% Precision 1.00–18.10%	Accuracy 92.55–105.59% Precision 0.97–4.30%
Matrix effect	Negligible matrix effect	Negligible matrix effect
Stability	Stable under various conditions: 154 days long-term storage 3 freeze–thaw cycles 5.00 h RT stability 16.00 h injector stability	Stable under various conditions: 116 days long-term storage 3 freeze–thaw cycles 6.00 h RT stability 25.00 h injector stability
Recovery	89.03–95.27% for omeprazole 94.81% for Lansoprazole	63.98–70.98% for clopidogrel carboxylic acid 66.31% for d_3 clopidogrel

Table 2. Various sets of calibration curve sets generated using omeprazole and clopidogrel carboxylic acid standards

Standard curve	Number of standards	Nominal concentrations of calibration curve (ng/mL) for omeprazole and clopidogrel carboxylic acid (in parentheses)							
		A 5.081 (4.071)	B 10.162 (8.142)	C 282.291 (99.296)	D 564.582 (413.725)	E 1254.627 (1034.338)	F 2201.100 (2068.676)	G 4002.000 (3447.794)	H 5.002.500 (4309.742)
1	8	✓	✓	✓	✓	✓	✓	✓	✓
2	7	✓		✓	✓	✓	✓	✓	✓
3	7	✓	✓	✓	✓	✓	✓		✓
4	6	✓		✓	✓	✓	✓		✓
5	5	✓			✓	✓	✓		✓
6	4	✓			✓	✓			✓
7	3	✓			✓				✓
8	3	✓				✓			✓
9	3	✓	✓						✓
10	3	✓		✓					✓
11	3	✓					✓		✓
12	3	✓						✓	✓

Table 3. Regression curve parameters for various calibration sets for omeprazole and clopidogrel carboxylic acid

Curve	Number of standards	Omeprazole			Clopidogrel carboxylic acid		
		Slope	Intercept	*r*	Slope	Intercept	*r*
1	8	0.000642	0.000483	0.9998	0.000449	–0.0000104	0.9984
2	7	0.000644	0.000511	0.9998	0.000448	–0.0000127	0.9983
3	7	0.000640	0.000500	0.9998	0.000446	0.00000104	0.9981
4	6	0.000641	0.000524	0.9998	0.000446	–0.00000253	0.9980
5	5	0.000646	0.000500	0.9999	0.000442	0.0000119	0.9978
6	4	0.000643	0.000516	0.9999	0.000436	0.0000363	0.9973
7	3	0.000643	0.000516	0.9999	0.000426	0.0000767	0.9966
8	3	0.000648	0.000492	1.0000	0.000423	0.0000868	0.9971
9	3	0.000645	0.000468	0.9997	0.000401	0.000217	0.9984
10	3	0.000637	0.000546	0.9997	0.000426	0.0000809	0.9964
11	3	0.000654	0.000461	1.0000	0.000425	0.0000782	0.9968
12	3	0.000654	0.000458	1.0000	0.000426	0.0000771	0.9967

Discussion

The importance of calibration curves, which is a primary driver for the validation parameters such as accuracy, precision and lower limit of quantitation, has been covered by previously published review articles (Srinivas, 2006; Srinivas, 2008). We were interested in exploring scenarios where one could reduce the number of non-zero calibration standards within the defined upper and lower limits of the calibration curve without compromising the end result of the quantitation (i.e. parameter values of the curve, QC predictions and *in vivo* pharmacokinetic characterization of the selected compounds).

In order to establish various scenarios for a systematic analysis there was the need to apply a 3–4 log order calibration curve ranges. Therefore, after inspecting our archives, we selected omeprazole and clopidogrel carboxylic acid since it provided a 1000-fold range in the calibration curve. Additionally, selection of omeprazole had a specific connotation from genetic polymorphism since omeprazole, a CYP2C19 substrate, is shown to exhibit differences in the pharmacokinetic disposition between extensive metabolizers and poor metabolizers (Desta *et al.*, 2002; Chang *et al.*, 1995).

In spite of a 1000-fold range, the data unequivocally confirmed the applicability of reduced number of non-zero calibration standards (i.e. $n = 3$) to predict the *in vivo* pharmacokinetics of both omeprazole and clopidogrel carboxylic acid. Although conservatively we have considered a wide calibration curve range in the present exercise, such minimization strategies of calibration curve standards should practically be feasible for a narrower calibration curve ranges (i.e. 50- to 100-fold).

The proposed strategy of minimizing number of calibration standards may be useful in situations where data evaluation may be required to answer some scientific questions such as: (1) TDM—in this situation, one has to ascertain if the *in vivo* plasma/serum level of the compound has met the required threshold concentration; (2) sparse sampling protocols—in this situation one has to determine the plasma/serum concentrations at the specified time points across a population pool; (3) formulation screening strategies—in this situation, one has to rank order the formulations using exposure data as the benchmark;

Table 4. Predictions of quality control samples for various calibration sets for omeprazole and clopidogrel carboxylic acid (average values)

Curve	Number of standards	Omeprazole			Clopidogrel carboxylic acid		
		Low QC (14.656 ng/mL)	Mid QC (1628.428 ng/mL)	High QC (3618.720 ng/mL)	Low QC (11.275 ng/mL)	Mid QC (1342.239 ng/mL)	High QC (3355.596 ng/mL)
1	8	13.686	1674.225	3617.073	11.928	1416.414	3413.194
2	7	13.621	1671.435	3611.094	11.936	1416.759	3414.020
3	7	13.717	1681.212	3632.197	11.963	1423.594	3430.531
4	6	13.654	1678.108	3625.533	11.977	1424.283	3432.181
5	5	13.586	1665.269	3597.753	12.058	1437.712	3464.586
6	4	13.626	1673.138	3614.781	12.166	1457.293	3511.853
7	3	13.627	1673.261	3615.048	12.354	1490.987	3593.185
8	3	13.562	1660.699	3587.863	12.409	1500.536	3616.380
9	3	13.650	1666.994	3601.422	12.760	1581.902	3812.776
10	3	13.710	1689.147	3664.369	12.341	1490.614	3592.300
11	3	13.484	1645.481	3554.932	12.369	1493.264	3598.676
12	3	13.476	1643.972	3551.667	12.365	1492.425	3596.652

Table 5. Pharmacokinetic parameters computed using various calibration curve sets in two subjects, identified as EM and PM phenotypes of omeprazole

Curve	C_{max} (ng/mL)	AUC_{0-t} (ng h/mL)	$AUC_{0-\infty}$ (ng h/mL)	$t_{1/2}$ (h)
Subject identified as EM phenotype				
1	1007.655	1587.503	1607.018	1.199
2	1008.578	1588.928	1608.450	1.199
3	1017.887	1603.314	1622.921	1.198
4	1025.572	1615.195	1634.871	1.197
5	1035.291	1630.224	1649.991	1.196
6	1021.760	1609.635	1629.392	1.199
7	1025.497	1615.080	1634.757	1.197
8	1020.683	1607.640	1627.273	1.198
9	1028.538	1619.793	1639.502	1.197
10	1024.457	1613.490	1633.162	1.197
11	1030.456	1623.037	1642.860	1.198
12	1026.183	1616.460	1636.255	1.198
Subject identified as PM phenotype				
1	2918.362	14700.553	14964.309	3.525
2	2921.044	14713.966	14977.935	3.525
3	2948.095	14849.352	15115.472	3.525
4	2970.425	14961.122	15229.023	3.524
5	2998.664	15102.483	15372.642	3.523
6	2959.243	14906.192	15173.546	3.525
7	2970.206	14960.029	15227.915	3.524
8	2956.219	14890.027	15156.797	3.524
9	2979.039	15004.281	15272.884	3.524
10	2967.178	14944.928	15212.59	3.524
11	2984.521	15032.592	15301.921	3.524
12	2972.098	14970.500	15238.867	3.525

Table 6. Pharmacokinetic parameters computed using various calibration curve sets in a single subject for clopidogrel carboxylic acid

Curve	C_{max} (ng/mL)	AUC_{0-t} (ng h/mL)	$AUC_{0-\infty}$ (ng h/mL)	$t_{1/2}$ (h)
1	2750.115	9255.260	13569.245	29.393
2	2750.781	9257.603	13572.924	29.394
3	2764.079	9301.722	13635.390	29.386
4	2765.410	9306.367	13642.885	29.389
5	2791.513	9393.542	13768.356	29.380
6	2829.586	9520.506	13950.606	29.366
7	2895.099	9738.992	14264.041	29.342
8	2913.662	9800.941	14352.926	29.335
9	3071.960	10326.753	15100.018	29.256
10	2894.384	9736.379	14259.520	29.339
11	2899.523	9753.797	14285.454	29.340
12	2897.892	9748.367	14277.612	29.341

(4) alternative routes of administration—similar to formulation screening strategies, in this situation one would rank order the dosing routes using guidance from the exposure data.

In conclusion, case studies of both omeprazole and clopidogrel carboxylic acid convincingly demonstrated the applicability of a minimal number of calibration standards ($n = 3$) for a 1000-fold curve range. Therefore, the use of reduced number of calibration standards may find utility in several scientific

applications and would aid in speedy assessment of experimental protocols.

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