

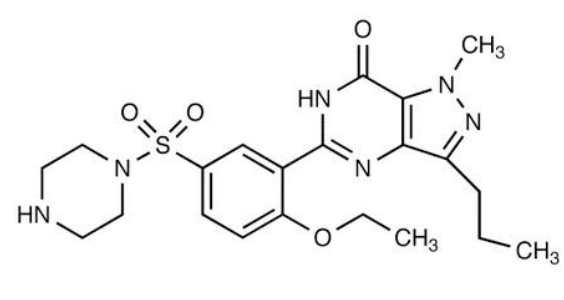
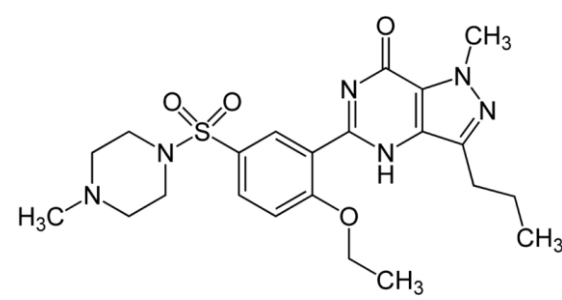


Resolution of Sildenafil-d₈ Ionization Dependence on Sildenafil Concentrations

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Introduction

Sildenafil is a selective phosphodiesterase type 5 (PDE5) inhibitor. PDE5 is responsible for breaking down cyclic guanyl monophosphate (cGMP), a vasodilator produced in response to nitric oxide. Internal standard ionic suppression was observed when Sildenafil and Desmethyl Sildenafil upper limit of quantitation was raised from 250 ng/mL up to 1500 ng/mL and 125 ng/mL up to 750 ng/mL, respectively. This ionic suppression gave a difference of approximately 20% (up to 30%) in internal standard area response between a LLOQ and a ULOQ sample. Because of this important variation, modifications were brought to chromatography and ionization mode was performed to solve this issue.



Method

Original chromatography used a Zorbax SB-C18 50x4.6 analytical column and ionization was performed with a turbo ion spray source operated in positive mode. Ion spray voltage was set at 4500 under a temperature of 400°C. Nebulizer and auxiliary gases as well as mass spectrometer parameters were set to achieve the best analyte and internal standard response. Internal standard for N-desmethyl sildenafil was added for better quantitation of the metabolite. Transitions monitored were 475.3 → 283.1, 461.4 → 283.2, 483.2 → 283.1 and 469.2 → 283.1 for Sildenafil, Desmethyl Sildenafil and internal standards respectively. The new chromatography uses an ACE 3 C18 30x4.6 analytical column. The TurbolonSpray source was changed for APCI. Most of the MS parameters are slightly modified to increase signal response.

Extraction Procedure

	Original Method	Improved Method
Matrix	EDTA K ₃	EDTA K ₃
Analytical Range	1-250 ng/mL for Sildenafil 0.5-125 for N-Desmethyl Sildenafil	1-1500 ng/mL for Sildenafil 0.5-750 for N-Desmethyl Sildenafil
Internal Standard	Sildenafil-d ₈	Sildenafil-d ₈ N-Desmethyl Sildenafil-d ₈
Sample Volume	0.200 mL	0.250 mL
Extraction Type	Automated Solid-phase Extraction	Liquid-Liquid Extraction
Dilution Factor	1.11	1

LC-MS/MS Analysis

	Original Method	Improved Method
Chromatographic Mode	Reverse Phase	Reverse Phase
Analytical Column	Zorbax SB C18	ACE 3 C18
Elution Mode	Isocratic	Isocratic
Mobile Phase A	MeOH/Water/Ammonium formate	Methanol/Water/Acetic Acid
Flow Rate	1.00 mL/min	1.00 mL/min
Injection Volume	25 µL	20 µL
Retention Time	2.22 min for Sildenafil 2.18 min for N-Desmethyl Sildenafil	1.52 min for Sildenafil 1.55 for N-Desmethyl Sildenafil
Acquisition Time	3.00 min	4.20 min
Detector	API 4000	API 4000
Source	TurbolonSpray	APCI
Ion Monitored	475→283 for Sildenafil 461→283 for N-Desmethyl Sildenafil	475→283 for Sildenafil 461→283 for N-Desmethyl Sildenafil

Results

Internal standard ionic suppression was not observed when the assay used a range of 1-250 ng/mL and 0.5-125 ng/mL for Sildenafil and Desmethyl Sildenafil, respectively. This problem became apparent when ULOQ was increased for both analytes. Internal standard area response varied based on the concentration of Sildenafil in the sample. A difference of approximately 25% could be observed between a LLOQ and a ULOQ sample, ranging from 6800000 to 8500000 (Tables 1 and 2, Figures 1 and 2). Although the accuracy for sildenafil was not affected since the IS is Sildenafil-d₈, the high end of the curve for Desmethyl Sildenafil was positively biased up to 20%. An improvement in chromatography was made in order to solve the problem and the analytical column modification gave a shorter retention time, narrower peaks and an increase in peak height, sharpness and signal to noise ratio. Lower injection volume was also tried. These improvements did not solve the ionic suppression problems, but the increase in effectiveness made the use of APCI ionization source possible. However, although this was promising, it didn't solve entirely the issue for N-Desmethyl Sildenafil. The addition of the deuterated IS for the metabolite improved significantly the quantitation (Tables 3 and 4).

Table 1. Internal Standard Variation and Impact on Accuracy of Sildenafil

Standard	Conc. (ng/mL)	Calc. Conc. (ng/mL)	% Nom.	Area	IS Area
CS1 1 1	1.00	0.98	98.00	20037	8559962
CS1 2 1	1.00	1.02	102.00	20493	8504384
CS2 1 1	2.00	2.00	100.00	38114	8358032
CS2 2 1	2.00	1.97	98.50	35715	7959606
CS3 1 1	30.00	32.60	108.67	600185	8419275
CS3 2 1	30.00	32.83	109.43	547317	7622094
CS4 1 1	150.00	157.37	104.91	2884893	8400014
CS4 2 1	150.00	156.74	104.49	2810901	8217452
CS5 1 1	300.00	301.39	100.46	5321461	8092966
CS5 2 1	300.00	295.04	98.35	5218153	8106552
CS6 1 1	600.00	548.31	91.39	8645887	7228334
CS6 2 1	600.00	532.50	88.75	8991637	7740636
CS7 1 1	1200.00	1236.46	103.04	18618194	6903261
CS7 2 1	1200.00	1220.88	101.74	19102690	7173365
CS8 1 1	1500.00	1421.11	94.74	21273326	6862925
CS8 2 1	1500.00	1430.65	95.38	21304885	6827270

Table 2. Internal Standard Variation and Impact on Accuracy of N-Desmethyl Sildenafil

Standard	Conc. (ng/mL)	Calc. Conc. (ng/mL)	% Nom.	Area	IS Area
CS1 1 1	0.50	0.49	98.00	6942	8559962
CS1 2 1	0.50	0.57	114.00	8229	8504384
CS2 1 1	1.00	0.84	84.00	12556	8358032
CS2 2 1	1.00	0.93	93.00	13400	7959606
CS3 1 1	15.00	13.77	91.80	229492	8419275
CS3 2 1	15.00	13.80	92.00	208279	7622094
CS4 1 1	75.00	70.78	94.37	1183065	8400014
CS4 2 1	75.00	73.16	97.55	1196334	8217452
CS5 1 1	150.00	141.78	94.52	2284559	8092966
CS5 2 1	150.00	143.85	95.90	2321722	8106552
CS6 1 1	300.00	266.95	88.98	3842918	7228334
CS6 2 1	300.00	274.76	91.59	4235759	7740636
CS7 1 1	600.00	699.54	116.59	9619318	6903261
CS7 2 1	600.00	715.71	119.29	10226738	7173365
CS8 1 1	750.00	833.86	111.18	11399512	6862925
CS8 2 1	750.00	879.46	117.26	11960517	6827270

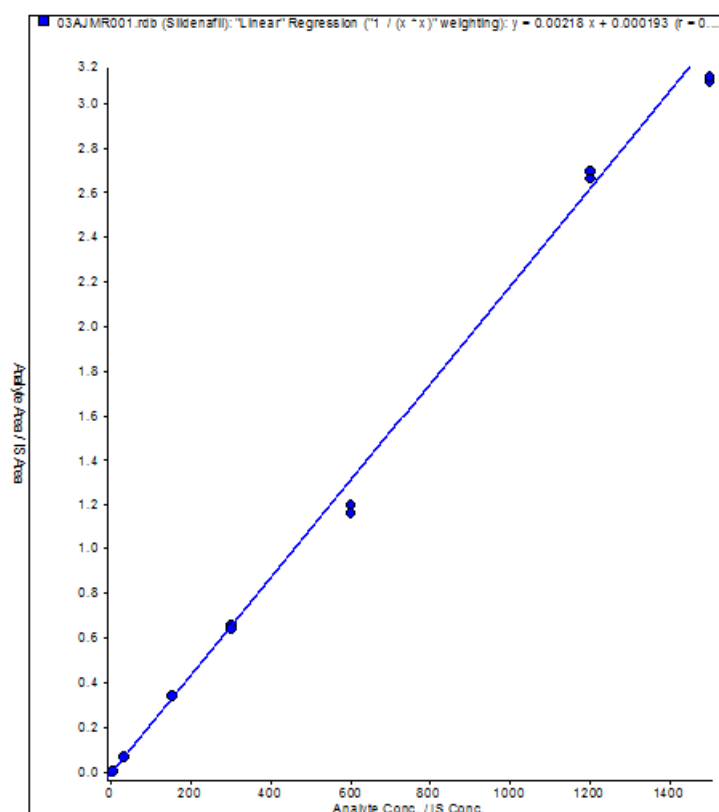


Figure 1. Impact of IS Suppression at High Sildenafil Concentration on Calibration Curve of Sildenafil

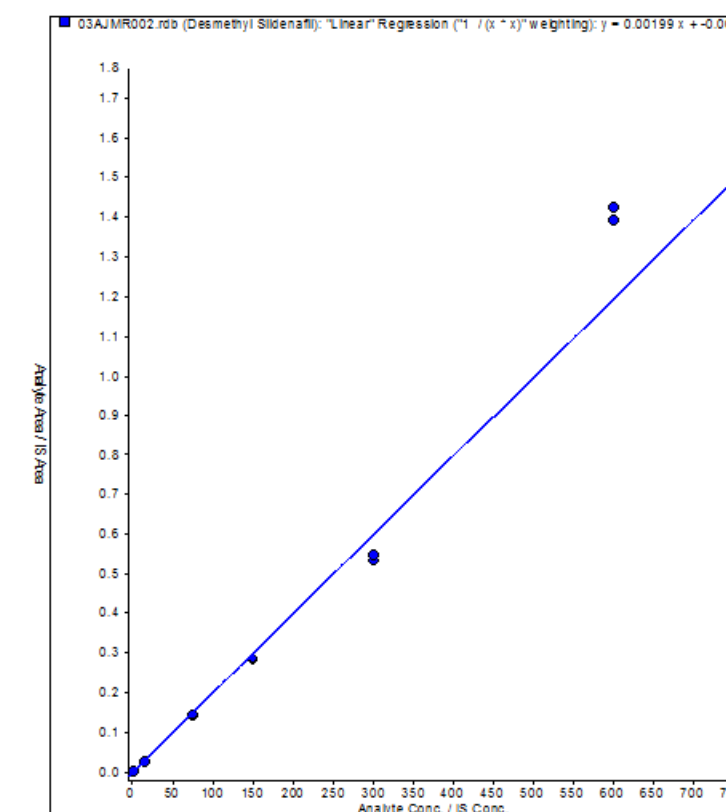


Figure 2. Impact of IS Suppression at High Sildenafil Concentration on Calibration Curve of N-Desmethyl Sildenafil

Table 3. Summary Table of Calibration Curve of Sildenafil with Improved Assay

Standard	Conc. (ng/mL)	Calc. Conc. (ng/mL)	% Nom.	Area	IS Area
CS1 1 1	1.00	0.96	96.00	3286	71980
CS1 2 1	1.00	1.09	109.00	2775	53262
CS2 1 1	2.00	1.99	99.50	6832	71357
CS2 2 1	2.00	1.84	92.00	5251	59303
CS3 1 1	30.00	28.57	95.23	94441	68308
CS3 2 1	30.00	28.93	96.43	79946	57095
CS4 1 1	150.00	141.76	94.51	475922	69346
CS4 2 1	150.00	141.57	94.38	398048	58076
CS5 1 1	300.00	278.31	92.77	933877	69309
CS5 2 1	300.00	296.12	98.71	841165	58672
CS6 1 1	600.00	605.92	100.99	2106470	71905
CS6 2 1	600.00	617.98	103.00	1873311	62610
CS7 1 1	1200.00	1229.27	102.44	4669882	78465
CS7 2 1	1200.00	1270.27	105.86	4061100	66032
CS8 1 1	1500.00	1637.21	109.15	5193803	65522
CS8 2 1	1500.00	1655.29	110.35	5134488	64066

Table 4. Summary Table of Calibration Curve of N-Desmethyl Sildenafil with Improved Assay

Standard	Conc. (ng/mL)	Calc. Conc. (ng/mL)	% Nom.	Area	IS Area
CS1 1 1	0.50	0.50	100.00	3760	103901
CS1 2 1	0.50	0.53	106.00	2932	76190
CS2 1 1	1.00	0.96	96.00	7488	103306
CS2 2 1	1.00	0.94	94.00	6106	85985
CS3 1 1	15.00	14.19	94.60	108830	97491
CS3 2 1	15.00	14.57	97.13	95955	83753
CS4 1 1	75.00	73.83	98.44	602001	103437
CS4 2 1	75.00	72.72	96.96	528690	92239
CS5 1 1	150.00	145.82	97.21	1212431	105454
CS5 2 1	150.00	148.20	98.80	1118938	95761
CS6 1 1	300.00	302.34	100.78	2823134	118411
CS6 2 1	300.00	305.18	101.73	2581762	107281
CS7 1 1	600.00	607.36	101.23	6390081	133409
CS7 2 1	600.00	614.88	102.48	5768704	118964
CS8 1 1	750.00	808.89	107.85	6398530	100302
CS8 2 1	750.00	806.37	107.52	7099703	111642

Results

Table 5. Summary of Inter-Run Accuracy and Precision

Analyte		LLQC 1.0 ng/mL		QC1 3.0 ng/mL		QC2 750.0 ng/mL		QC3 1125.0 ng/mL	
		Conc. Found (ng/mL)	% Bias	Conc. Found (ng/mL)	% Bias	Conc. Found (ng/mL)	% Bias	Conc. Found (ng/mL)	% Bias
Sildenafil	N	24	24	24	24	24	24	24	24
	Mean	1.01	0.63	2.94	-1.90	802.91	7.05	1131.05	0.54
	SD(±)	0.074		0.130		28.628		30.594	
	CV(%)	7.35		4.42		3.57		2.70	
N-Desmethyl Sildenafil	N	24	24	24	24	24	24	24	24
	Mean	0.50	0.75	1.46	-2.81	394.15	5.11	551.28	-1.99
	SD(±)	0.036		0.056		9.499		16.776	
	CV(%)	7.09		3.86		2.41		3.04	

Conclusion

The new method was validated as per the most recent regulatory guidelines. The use of the APCI source improved the quantitation of Sildenafil, specifically suppression of the internal standard response was inhibited. Moreover, the addition of a deuterated internal standard for N-Desmethyl Sildenafil solved the accuracy issue. The assay was used for study sample analysis and was shown to be reproducible with reanalysis confirmation rate near 100%.

