



***Ionization Enhancement in APCI and  
Suppression ESI between Target Drugs and  
Stable Isotope-Labeled Internal Standards in  
Quantitative LC/MS and LC/MS/MS***

**Hairui Liang, Rodger L. Foltz, Min Meng, and Patrick K. Bennett**

**Tandem Labs, Salt Lake City, UT**

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**Tandem Labs-Salt Lake City**

1121 East 3900 South  
Salt Lake City, UT 84124  
(801) 293-2400  
(801) 313-6495 Fax

**Tandem Labs-New Jersey**

115 Silvia Street  
West Trenton, NJ 08628  
(609) 434-0044  
(609) 434-0033 Fax

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## INTRODUCTION

Stable isotope-labeled analogs are commonly used as internal standards (IS) in quantitative Liquid Chromatography/Mass Spectrometry (LC/MS) and LC/MS/MS. They normalize the responses of target drugs, thus compensating for variations in injection, sample preparation, instrumental parameters and matrix effects. Previous reports have noticed that the peak areas of the co-eluting labeled IS decreased with increasing drug concentrations in calibration curves. We also noticed this phenomenon when using electrospray ionization (ESI). However, with Atmospheric Pressure Chemical Ionization (APCI) the peak areas of stable labeled IS increased when drug concentrations increased. To further explore these phenomena and their possible influence on assay reproducibility, sensitivity and linearity, we investigated the effects of ionization suppression in ESI and enhancement in APCI between seven drugs and their labeled internal standards.

## METHODS

The target drugs and labeled IS were chromatographed using a Synergi Hydro-RP or Prodigy Phenyl-3 column (30 x 2 mm, 5  $\mu$ m particle size), or a chiral column (50 x 2 mm, 5  $\mu$ m particle size) and were detected by either ESI or APCI using either selected-ion monitoring (SIM) or selected-reaction monitoring (SRM). The extent of ionization

suppression or enhancement was measured by comparing the peak areas of drug or IS from solutions containing only drug or IS or both of them. We also used a post-column infusion system in which a constant flow of a drug (or its IS) was infused post-column and the IS (or the drug) injected on-column.

## RESULTS AND DISCUSSION

The seven investigated drugs included basic, neutral and acidic compounds. The basic compounds contained primary, secondary or tertiary amine groups. The internal standards were deuterium or <sup>13</sup>C labeled analogs of their corresponding target drugs (Table 1.).

**Table 1. Investigated 7 Target Drugs and their Corresponding Isotope-Labeled IS**

<b>Drugs (Molecular Ions)</b>	<b>Labeled IS (Molecular Ions)</b>	<b>Isotopic Contribution*</b>	<b>Structure type</b>
<i>Drug I, 409</i>	<sup>13</sup> C <sub>6</sub> , 415	<0.2%	Primary amine
<i>Drug II, 478</i>	<sup>13</sup> C <sub>2</sub> , D <sub>2</sub> , 482	<0.2%	Secondary amine
<i>Drug III, 506</i>	<sup>13</sup> C <sub>2</sub> , D <sub>2</sub> , 510	<0.2%	Tertiary amine
<i>Drug IV, 310</i> <i>(R, S-Methadone)</i>	D <sub>3</sub> , 313 (R, S-Methadone-D <sub>3</sub> )	0.2%	Tertiary amine
<i>Drug V, 474</i>	D <sub>4</sub> , 478	0.2%	Acidic compound
<i>Drug VI, 249</i>	D <sub>3</sub> , 252	0.5%	Neutral compound
<i>Drug VII, 265</i>	D <sub>3</sub> , 268	0.5%	Neutral compound

\* Isotopic Contribution indicates contribution of natural occurring isotopic abundance of drugs to their corresponding isotope-labeled IS molecular ions

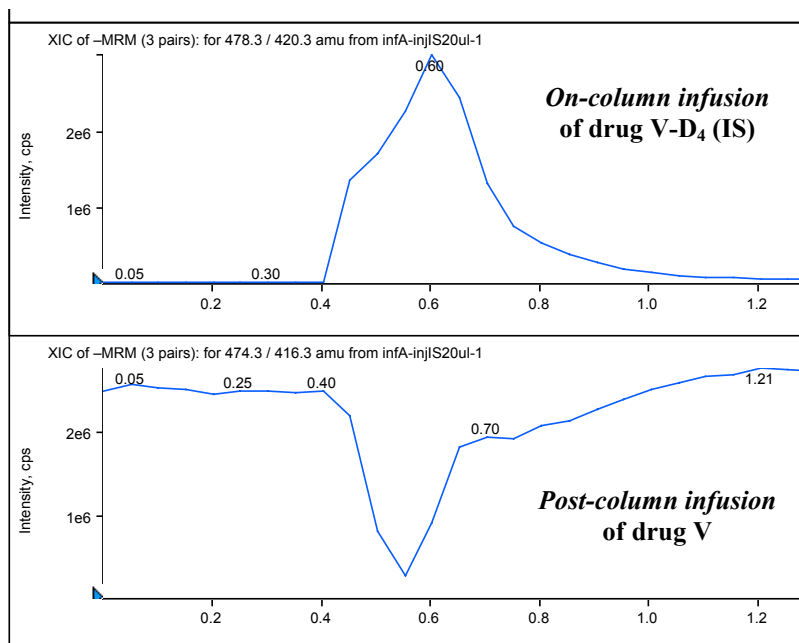
## IONIZATION SUPPRESSION BY ESI

### ***Ionization Suppression between Target Drugs and their Stable Isotope-Labeled IS in SIM and SRM Modes***

The results showed that all target drugs suppressed the ionization responses of their co-eluting labeled IS in both SIM and SRM modes, and likewise the labeled IS suppressed

the ionization responses of the target drugs under ESI. Figure 1 shows a representative of the results. This suppression can be explained by Enke's model of ESI ion generation.

**Figure 1. Ionization response of drug V suppressed ~80% by its D<sub>4</sub>-IS during the Retention Time of D<sub>4</sub>**



The extent of suppression % was calculated as:  $100 * \{(A - B) / A\}$   
 A: intensity of D<sub>0</sub> by post-column infusion; B: intensity of D<sub>0</sub> suppressed due to the on-column injection of D<sub>4</sub>. Concentrations of drug V and its D<sub>4</sub>: 10 µg/mL; injection volume: 20 µL; infusion rate: 20 µL/min. For other experimental conditions see methods.

**Table 2. Ionization Suppression in ESI between Methadone-D<sub>0</sub> and Methadone-D<sub>3</sub>**

<b>Peak Areas of methadone-D<sub>0</sub> or methadone-D<sub>3</sub> from solutions containing only D<sub>0</sub> or D<sub>3</sub></b>				
(n=10)	<b>R-Methadone-D<sub>0</sub></b>	<b>S-Methadone-D<sub>0</sub></b>	<b>R-Methadone-D<sub>3</sub></b>	<b>S-Methadone-D<sub>3</sub></b>
Mean	262000	277000	267000	285000
SD	53300	58300	49300	48000
CV%	20.4	21.0	18.5	16.9
<b>Peak Areas of methadone-D<sub>0</sub> or methadone-D<sub>3</sub> from solutions containing both D<sub>0</sub> and D<sub>3</sub></b>				
(n=10)	<b>R-Methadone-D<sub>0</sub></b>	<b>S-Methadone-D<sub>0</sub></b>	<b>R-Methadone-D<sub>3</sub></b>	<b>S-Methadone-D<sub>3</sub></b>
Mean	222000	234000	207000	218000
SD	28700	32300	27700	29500
CV%	12.9	13.8	13.4	13.5
<b>Suppression %</b>	<b>15.27</b>	<b>15.52</b>	<b>22.47</b>	<b>23.51</b>

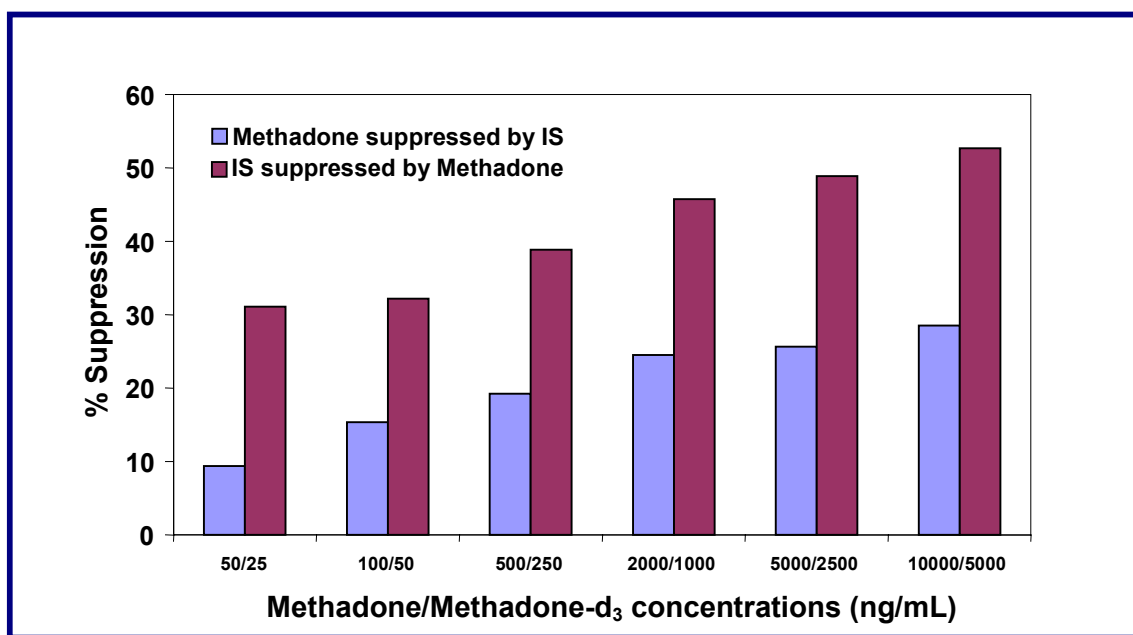
The suppression (%) of methadone D<sub>0</sub> (or D<sub>3</sub>) was measured by comparing the peak areas of D<sub>0</sub> (or D<sub>3</sub>) from solutions containing only D<sub>0</sub> (or D<sub>3</sub>), or both D<sub>0</sub> and D<sub>3</sub>. Concentrations of R-Methadone and S and their D<sub>3</sub>-IS: 1000 ng/mL; injection volume: 10 µL.

The enantiomers of methadone and its D<sub>3</sub> were chromatographed using a Chiral AGP column (50 x 2 mm, 5 µm particle size). For other experimental conditions see methods.

## **Extent of Suppression and Concentrations of Investigated Drugs**

The extent of suppression in each drug-IS pair was concentration-dependent in a nonlinear fashion. Figure 2 shows that the ionization suppression between methadone and its D<sub>3</sub>-IS. The results demonstrated that the higher the concentrations of its D<sub>3</sub>-IS, the higher the suppression of methadone D<sub>0</sub> by its D<sub>3</sub>-IS, and vice versa.

**Figure 2. Ionization suppression between methadone and its D<sub>3</sub>-IS**



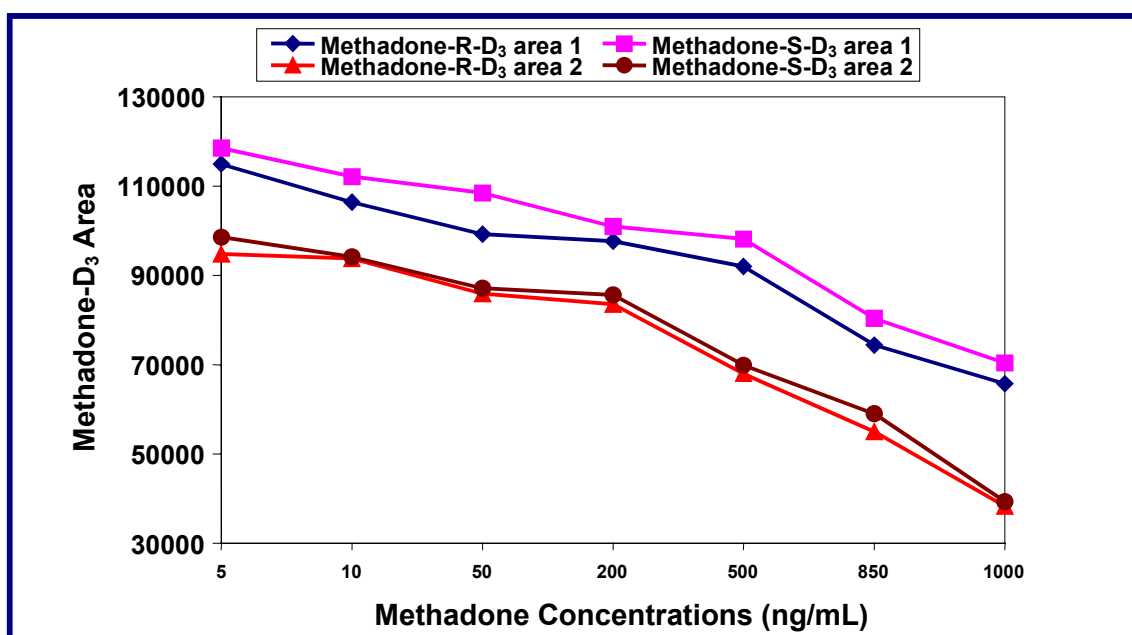
Concentrations of D<sub>0</sub>: 50-10000 ng/mL; Concentrations of D<sub>3</sub>: 25-5000 ng/mL.

The blue bars indicate the extent of suppression of D<sub>0</sub> ionization by D<sub>3</sub> by comparing the peak areas of D<sub>0</sub> from the solutions containing only D<sub>0</sub>, or both D<sub>0</sub> and D<sub>3</sub>. The red bars indicate the extent of suppression of D<sub>3</sub> ionization by D<sub>0</sub> by comparing the peak areas of D<sub>3</sub> from the solutions containing only D<sub>3</sub>, or both of them. For other experimental conditions see methods.

## ***Effect of Ionization Suppression on Assay Sensitivity, Reproducibility and Linearity***

In calibration curves, the ionization suppression of drugs by the IS may influence assay sensitivity because the signals of drugs at low concentrations were suppressed more than that at high concentrations. The suppression of IS signals depended upon the drug concentrations. Figure 3 shows the peak areas of co-eluting labeled methadone-D<sub>3</sub> decreased with increasing methadone concentrations in calibration series.

**Figure 3. Peak areas of co-eluting labeled methadone-D<sub>3</sub> IS decreasing with increasing methadone concentrations in calibration series**



Areas 1 and 2 were from different day's results.

The suppression of IS signals by its target drug caused the poor reproducibility of IS. However, calibration curves were still linear if the response factor is kept constant by selecting an appropriate internal standard concentration for the desired calibration range. Table 3 shows that the overall CV% of methadone-D<sub>3</sub>-IS was 28-34%. However, the overall CV% of response factor was <6% and the calibration curves of R-Methadone and S were still linear from 5 to 1000 ng/mL (Figure 4).

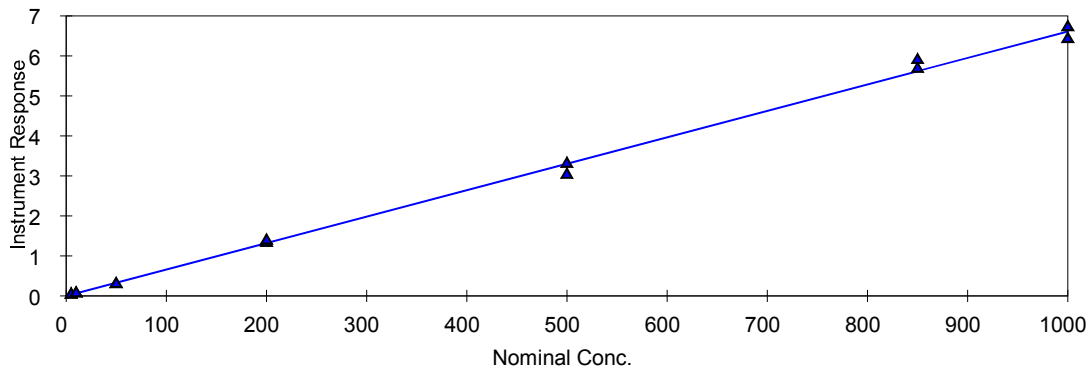
**Table 3. Precision of IS Area, Response Factor and Accuracy of R-Methadone and S in Calibration Curves (n=2)**

<b>R-Methadone</b>			
<b>Concentration</b>	<b>IS Area (R-Methadone D<sub>3</sub>)</b>	<b>Response Factor</b>	<b>Accuracy</b>
5 (ng/mL)	94769.75	0.0061	105.14
10 (ng/mL)	93774.36	0.0067	108.30
50 (ng/mL)	85851.90	0.0060	91.54
200 (ng/mL)	83530.86	0.0069	104.82
500 (ng/mL)	66706.51	0.0066	100.08
850 (ng/mL)	38320.14	0.0069	104.98
1000 (ng/mL)	37896.89	0.0067	101.64
MEAN	77158.92	0.0066	102.36
SD	21541.07	0.0004	5.46
CV%	<b>27.92</b>	<b>5.80</b>	<b>5.33</b>
<b>S-Methadone</b>			
<b>Concentration</b>	<b>IS Area (S-Methadone D<sub>3</sub>)</b>	<b>Response Factor</b>	<b>Accuracy</b>
5 (ng/mL)	98533.69	0.0063	106.71
10 (ng/mL)	94170.30	0.0063	103.06
50 (ng/mL)	87089.01	0.0057	91.02
200 (ng/mL)	85634.00	0.0066	103.89
500 (ng/mL)	67967.35	0.0065	102.23
850 (ng/mL)	39525.31	0.0067	105.77
1000 (ng/mL)	39329.72	0.0068	107.86
MEAN	73178.48	0.0064	102.93
SD	24958.69	0.0004	5.63
CV%	<b>34.11</b>	<b>5.57</b>	<b>5.47</b>

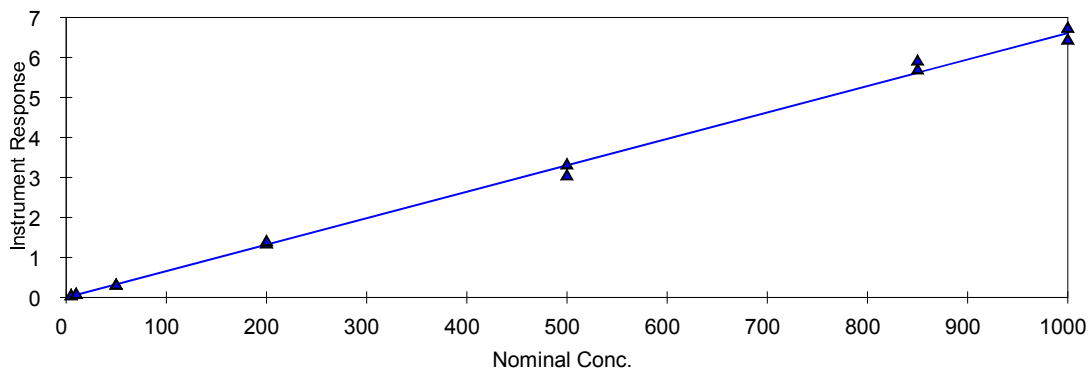
The enantiomers of methadone and its D<sub>3</sub>-IS were chromatographed using a chiral column (50 x 2 mm, 5 μm particle size). For other experimental conditions see methods.

**Figure 4. Calibration curves of R- and S-Methadone from 5 to 1000 ng/mL**

Analytical Run 4 analyzed on 08-Jul-2002 Calibration Standards for R-Methadone (ng/ml)  
 Regression Method = LINEAR - Weighting Factor = 1/X  
 Response = Slope \* Conc + Intercept  
 Slope = 0.006612 Intercept = -0.004187 R-Squared = 0.9983  
 (Protocol NWBS02-079, Assay Validation of (S) and (R)-Methadone in Human Plasma (K3 EDTA))



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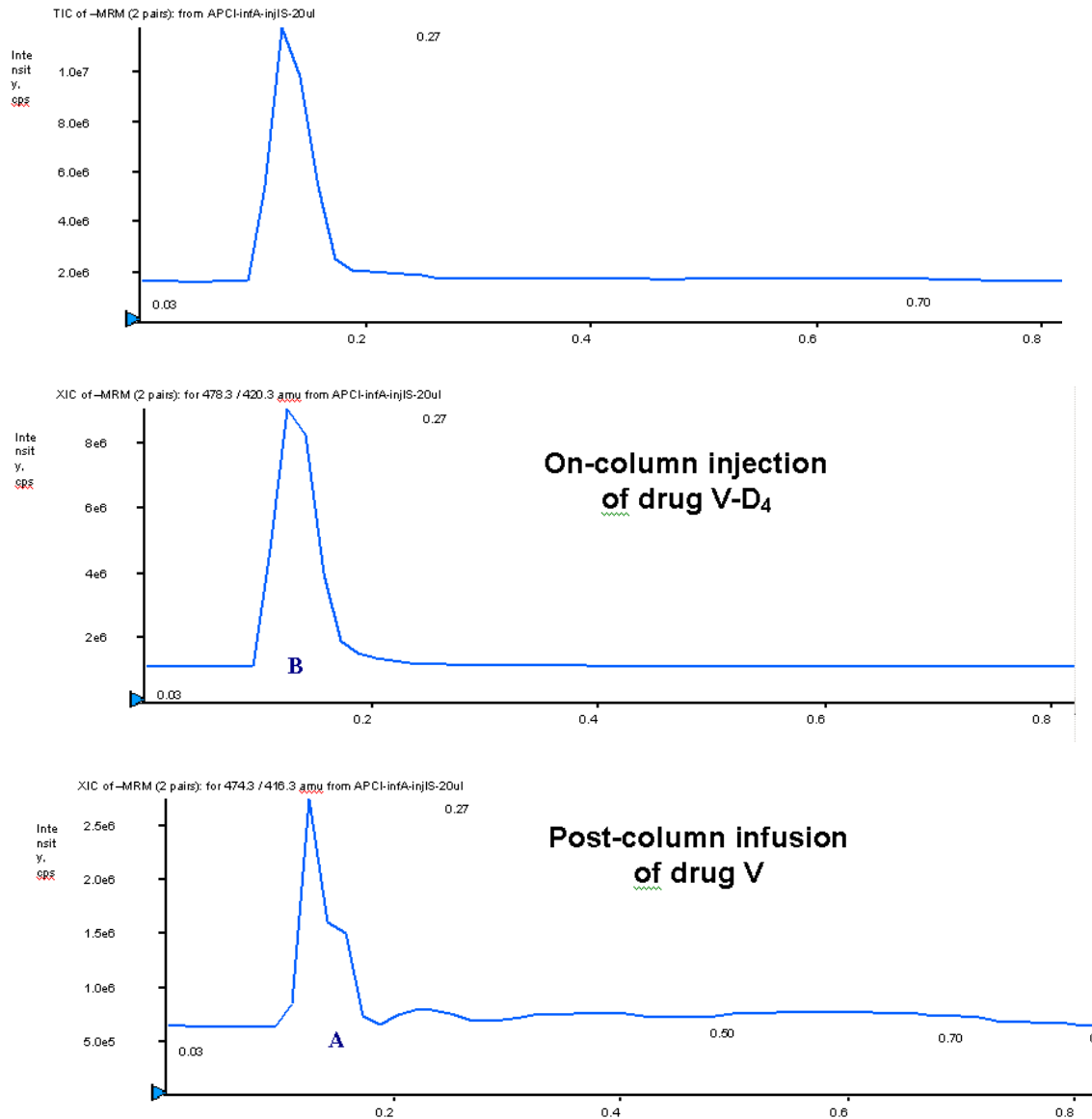
## IONIZATION ENHANCEMENT BY APCI

### ***Ionization Enhancement between Target Drugs and their Stable Isotope-Labeled IS in SIM and SRM Modes***

In contrast to the ESI results, all investigated target drugs enhanced the ionization responses of the corresponding labeled IS in both SIM and SRM modes, and likewise the

labeled IS enhanced the responses of the target drugs with APCI. Figure 5 shows that the response of drug V was enhanced ~ 7 times by its D<sub>4</sub>-IS.

**Figure 5. Response of drug V enhanced ~ 7 times by its D<sub>4</sub>-IS during the Retention Time of D<sub>4</sub>**



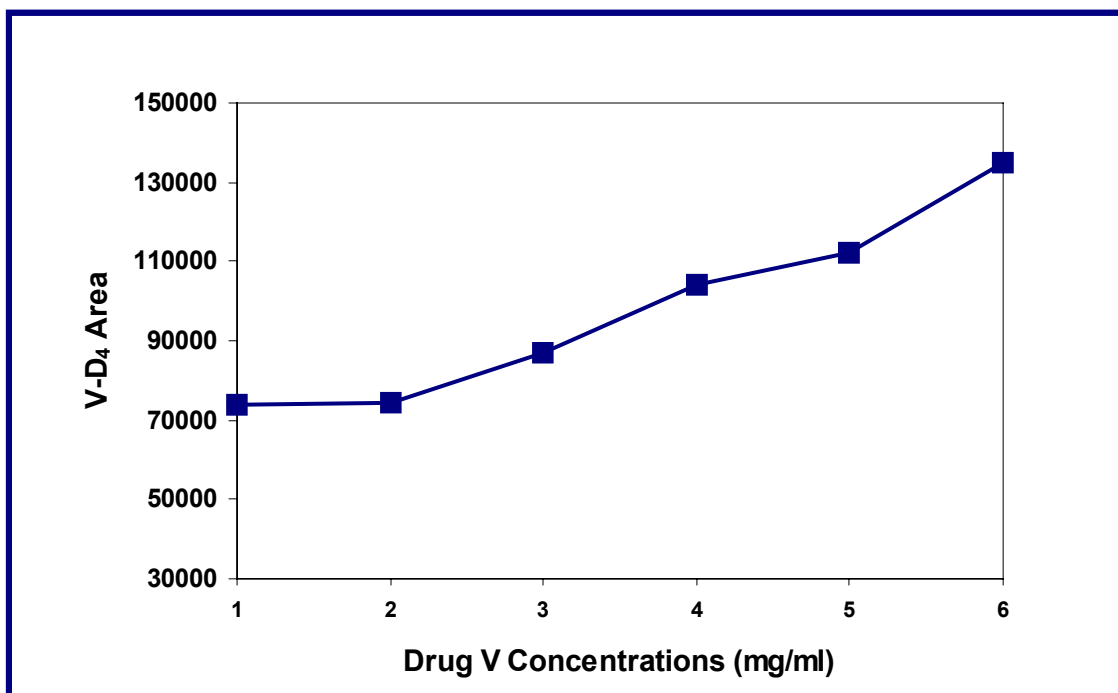
The extent of enhancement was calculated as:  $\{(B - A)/A\}$

A: intensity of D<sub>0</sub> by post-column infusion; B: intensity of D<sub>0</sub> enhanced due to the on-column injection of D<sub>4</sub>. Concentrations of drug V and its D<sub>4</sub>: 10 µg/mL; injection volume: 20 µL; infusion rate: 20 µL/min. For other experimental conditions see methods.

## ***Extent of Enhancement and Concentrations of Investigated Drugs***

The enhancement of IS signals by drugs was also concentration-dependent. **Fig. 6** shows that the peak areas of its co-eluting D<sub>4</sub> IS increased with increasing drug V concentrations.

**Figure 6. Peak areas of D<sub>4</sub> IS increasing with increasing drug V concentrations**



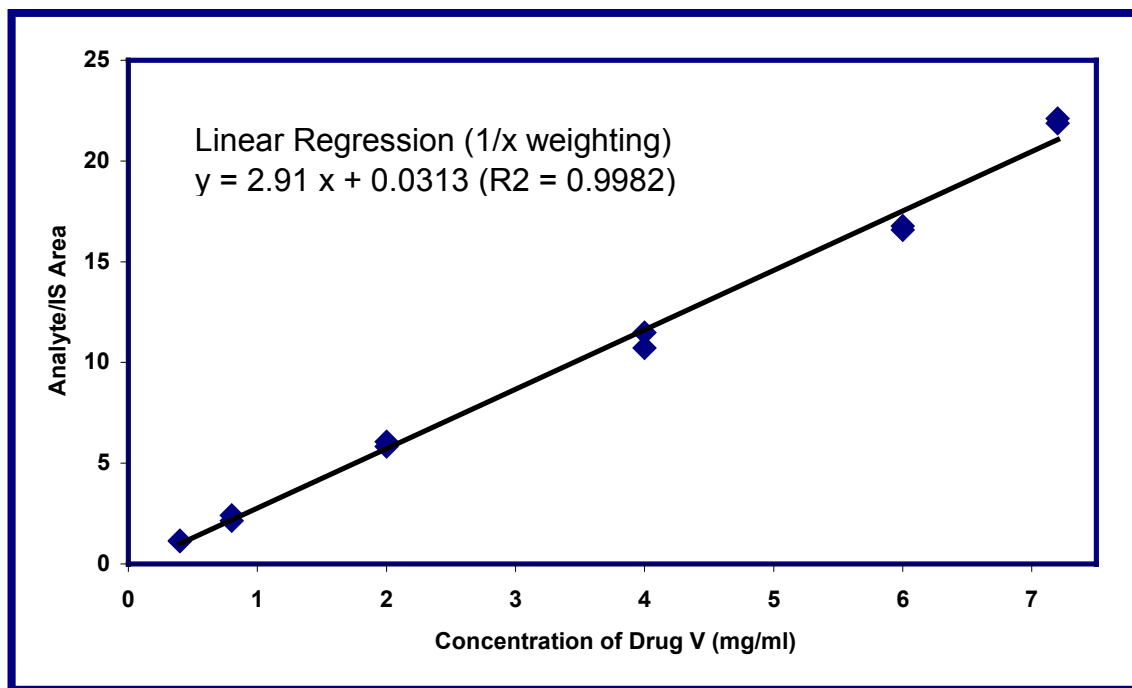
## ***Extent of Ionization Enhancement on Assay Reproducibility and Linearity***

The increase of IS responses with increasing drug concentrations in the calibration series resulted in the poor reproducibility of IS (Table 4). However, calibration curves were still linear (Figure 7) if an appropriate internal standard concentration was selected for the desired calibration range to keep the response factor constant (Table 4).

**Table 4. Precision of IS Area (V-D<sub>4</sub>), Response Factor and Accuracy of Drug V in Calibration Curves (n=2)**

Concentration of Drug V	IS Area	Response Factor	Accuracy
0.400 (mg/mL)	74056	2.81	99
0.800 (mg/mL)	74431	3.03	105
2.00 (mg/mL)	86968	3.03	104
4.00 (mg/mL)	103970	2.87	98
6.00 (mg/mL)	112240	2.76	95
7.20 (mg/mL)	134690	3.07	105
MEAN	97725.83	2.93	101.58
SD	23810.26	0.13	4.38
CV%	24.36	4.48	4.31

**Figure 7. Calibration curves of drug V with a linear range from 0.4 to 7.2 mg/mL**



Experimental conditions see methods.

## ***Effect of Isotopic Contribution from Drugs on Enhancement of IS***

The contributions of natural occurring isotopic abundance of drugs to their corresponding isotope-labeled IS molecular ions are showed in Table 1. The degree of enhancement of isotope-labeled IS was substantially greater than could be due to the natural occurring isotopic contribution from their target drugs.

## ***Effect of Purity of IS on Enhancement of Drugs***

In the present study, all used isotope-labeled IS were over 99% pure. Therefore the presence of any significant D<sub>0</sub>-IS impurities was ruled out.

## **CONCLUSIONS**

- Ionization enhancement under APCI between target drugs and co-eluting isotope-labeled IS was investigated in quantitative LC/MS and LC/MS/MS for the first time.
- All investigated target drugs and their co-eluting isotope-labeled IS were found to suppress each other's ionization responses under ESI, and to enhance each other's ionization responses under APCI.
- Mutual ionization suppression and enhancement between drugs and their isotope-labeled IS can influence assay sensitivity, reproducibility of IS and linearity.
- Calibration curves were still linear if an appropriate IS concentration was selected for the desired calibration range to keep the response factor constant.
- The significance of the findings becomes apparent for situations where metabolites, xenobiotics, or endogenous compounds co-elute with a drug or its IS in quantitative LC/MS and LC/MS/MS.