

# A Fully Automated Symbiosis Online Extraction Method for Determination of Raltegravir in Plasma by LC/MS/MS

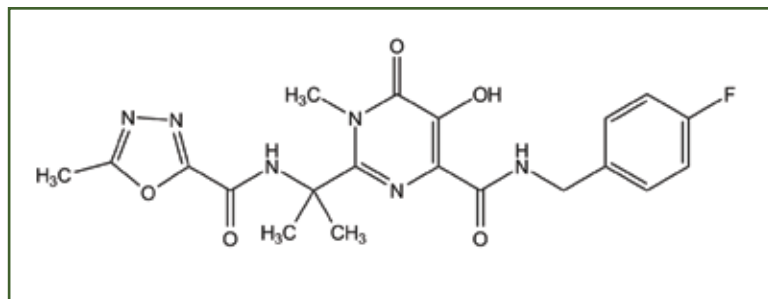
## Authors

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## Introduction

Raltegravir (Figure 1) is a new class of drug used to treat HIV infection. The quantification of raltegravir in human plasma is important to support clinical studies and determine pharmacokinetic parameters. Two methods, solid phase extraction (SPE) and online SPE using Symbiosis Pharma, were developed with LC/MS/MS over a range of 1.00 ng/mL to 1000 ng/mL. The built-in advantage of online extraction is precise liquid handling and fast sample processing, but it also faces the challenge of high carryover from the relatively complicated system design. Our goal is to develop a fully automated SPE-LC/MS/MS method with high throughput capability. The method was GLP validated and proved to be accurate, precise and robust.

FIGURE 1: Chemical Structure of Raltegravir (MRM Transitions: Raltegravir: 443 → 316; Raltegravir-d6: 451 → 324)



## Method – Offline SPE

### 1. LC-MS

Mass Spec:	ABI Sciex API 5000 ESI
Source Temperature:	500 °C
Column:	Waters XBridge Phenyl
Flow Rate:	0.600 mL/min
Mobile Phase:	A: 0.1% formic acid in water B: Acetonitrile
Needle Wash:	1: 0.1% formic acid in water/acetonitrile 2: 0.1% formic acid in water
LC Program:	isocratic
Cycle Time:	3 minutes

### 2. SOLID PHASE EXTRACTION

Cartridge:	Phenomenex StrataX 33µm
Conditioning:	Acetonitrile
Equilibration:	5% formic acid in water
Cartridge Wash:	5% formic acid in water
Elution:	5% formic acid in water/acetonitrile

## Method – Symbiosis Online SPE

### 1. LC-MS

Mass Spec:	ABI Sciex API 4000 ESI
Source Temperature:	500 °C
Column:	None
Flow Rate:	0.600 mL/min
Mobile Phase:	A: 0.1% formic acid in water B: Acetonitrile
Needle Wash:	1: Triethylamine and EDTA in water/DMF 2: 0.1% formic acid in water
LC Program:	Gradient
Cycle Time:	100 seconds

## Method – Symbiosis Online SPE continued

### 2. ONLINE SPE

Cartridge:	Spark Holland HySphere C18 HD 7 $\mu$ m
Conditioning:	Methanol
Equilibration:	5% formic acid in water
Cartridge Wash:	5% formic acid in water/acetonitrile
Elution:	Mobile phase from gradient pumps
Cartridge Flush:	Acetonitrile/methanol

## Results and Discussion

- Representative calibration standard (Table 1 and Figure 2) were linear ( $R^2 > 0.992$  for A;  $R^2 > 0.997$  for B) using weighted  $1/x^2$
- Liquid chromatograms are shown in Figure 3 for the LLOQ set to 1.00 ng/mL and ULOQ set to 1000 ng/mL. Their sensitivity are similar, but the offline method ran on API5000 (vs. API4000 for online method).
- Quality control sample accuracy (Figure 4) and precision (Table 2) from offline and online SPE method are demonstrated at  $n=6$  at LLOQ, Low, Medium, High concentrations over three validation runs.
- Raltegravir-d6 IS response (Figure 5) from both methods is consistent.
- Interference was tested in Low QC at 100  $\mu$ g/mL level for acetaminophen, ibuprofen, caffeine, chlorpheniramine maleate, naproxen, R,R(-)-pseudoephedrine. No ion suppression or enhancement was observed in either extraction method.
- Method selectivity was demonstrated with blank and LLOQ QC concentration in six sources of human plasma.
- The offline method has a better peak shape because the utilization of a HPLC column, while the online method has a shorter cycle time with SPE cartridge as the only chromatographic separation.
- All reagents used in extraction and chromatography are virtually the same except Needle Wash 1. A mixture of triethylamine (TEA) and ethylenediaminetetraacetic acid (EDTA) is the key to eliminate system and SPE cartridge carryover for online method, especially in such a short cycle (wash) time.



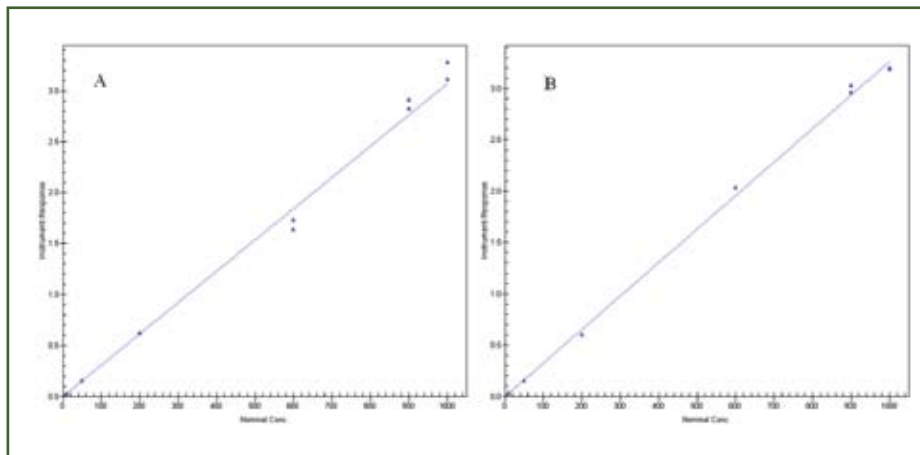
## Advantages of the Symbiosis Online SPE Method

- Online SPE is conducted under high pressure with two syringe pumps, where the C18HD 7 $\mu$ m cartridge provides adequate sample clean-up and chromatographic separation. Thus, the analytical column was omitted to improve the method cycle time and reduce cost.
- The analytical platform was changed from a Sciex API 5000 for the SPE method to an API 4000 for the online SPE method because of the consistently high recovery (Figure 6) of online extraction.
- Sample preparation is as easy as diluting plasma sample with internal standard and buffer solution, which integrate extraction with chromatography and reduce the chances of human error during extraction.
- The diluted sample is extracted and analyzed simultaneously by Symbiosis and mass spectrometer with a cycle time of 100 seconds. The precision of the automation platform is slightly better than that of offline SPE (3% vs 6%).
- Concurrent with samples being processed on the Symbiosis and analyzed in the mass spec, the online SPE cartridge is washed for reuse. Therefore the effective sample preparation time is zero. Reuse of the cartridges for up to five times was validated.

## Limitation of the Symbiosis Online SPE Method

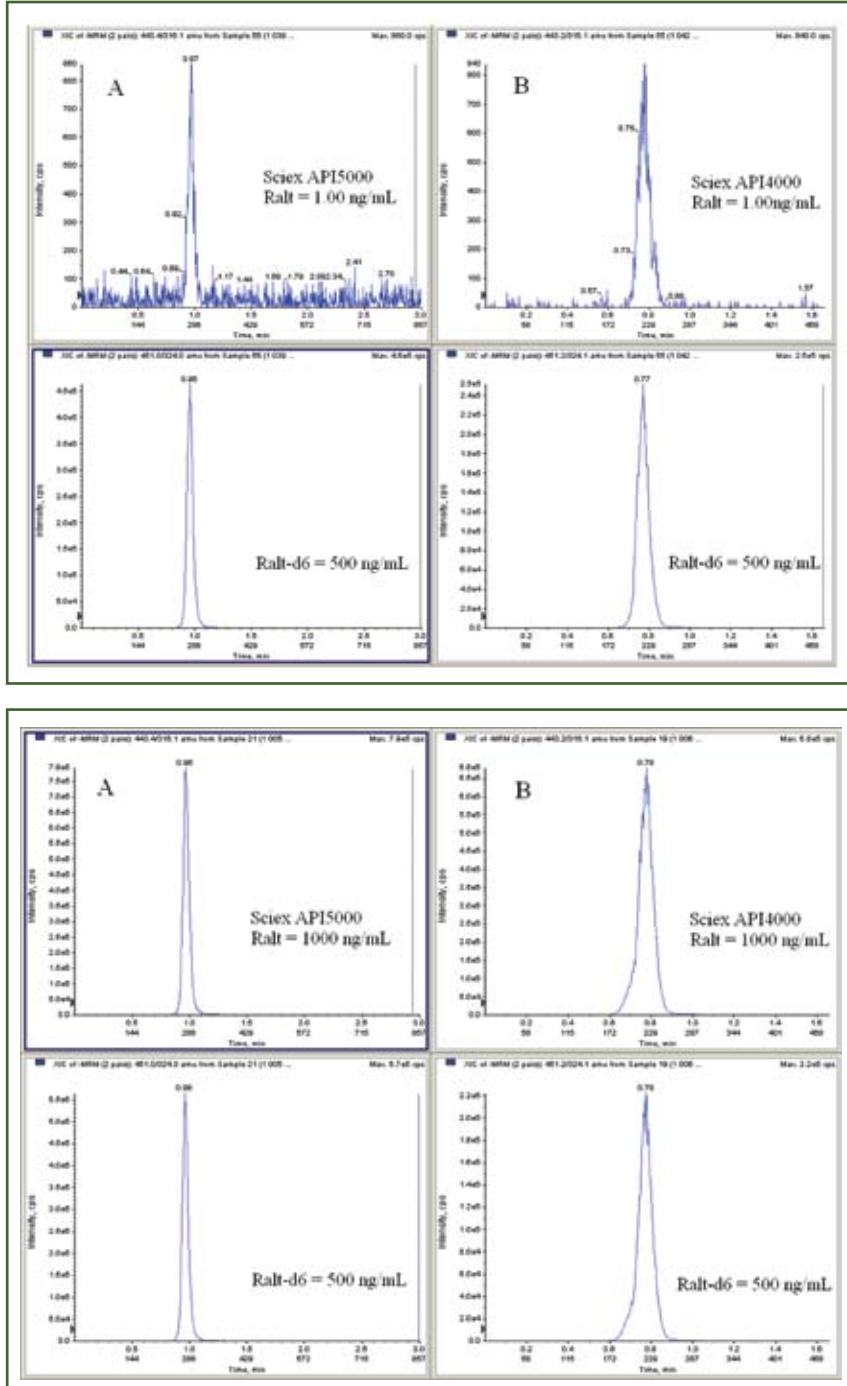
- Any assay developed on Symbiosis online SPE would be as an “elute-and-shoot” method. Drying down is neither necessary nor possible. As a result, the elution solvent needs to be compatible with the HPLC column and its corresponding mobile phase.
- In the “elute-and-shoot” mode, no sample extract is collected. Reinjection reproducibility needs to be demonstrated as diluted matrix stability, in which the stability of matrix samples diluted with buffer prior to extraction is tested. Compounds that are unstable in plasma would pose a challenge for diluted matrix stability.
- Relative extraction recovery is tested by collecting the mobile phase after extraction but prior to mass spec. Then neat sample and the correspondence compensation solvent are spiked and analyzed for recovery data. This extra step is only needed for assay validation, but it is not applicable in sample analysis.

FIGURE 2: Calibration curves for (A) offline SPE and (B) Symbiosis Online SPE Method (Linear  $1/x^2$ ).



## Limitation of the Symbiosis Online SPE Method continued

FIGURE 3: Representative LC-MS/MS chromatograms of LLOQ at 1.00 ng/mL (Top) and ULOQ at 1000 ng/mL (Bottom) for Offline SPE (A) and Symbiosis Online SPE (B) Method.



## Limitation of the Symbiosis Online SPE Method continued

FIGURE 4: Accuracy for Raltegravir in Quality Control Samples with Offline SPE and Symbiosis Online SPE Method (n=18).

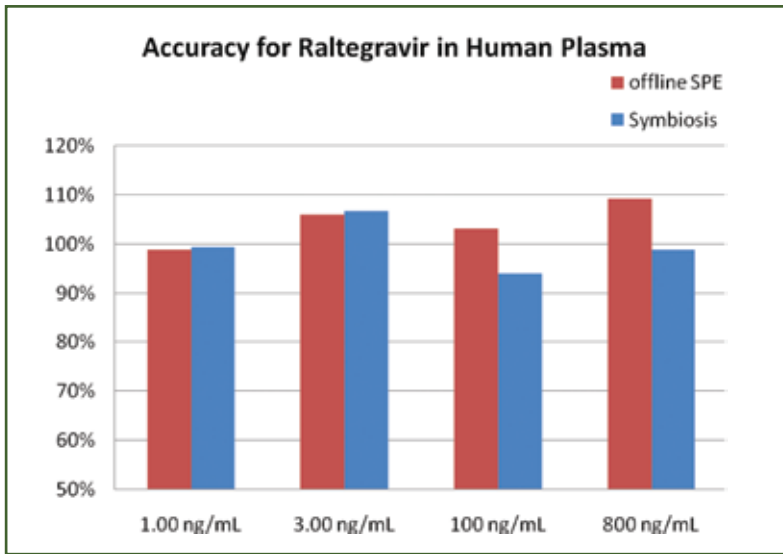
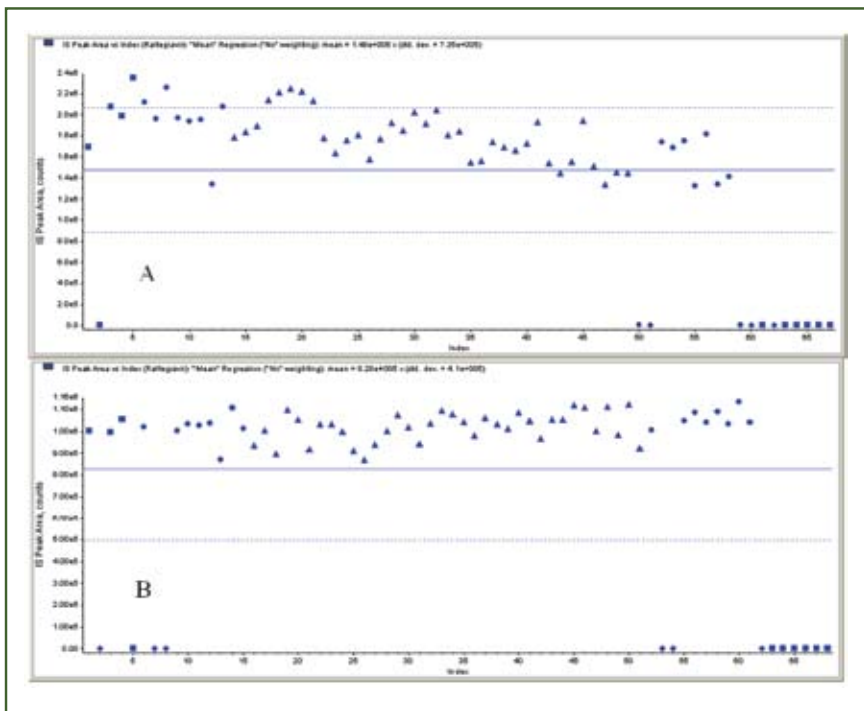
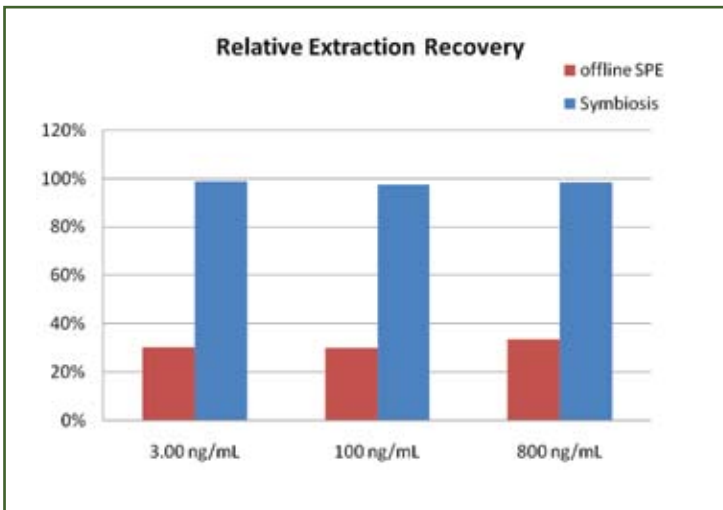


FIGURE 5: Raltegravir IS response of offline method (A) and Symbiosis Online Method (B) for validation run.



## Limitation of the Symbiosis Online SPE Method continued

FIGURE 6: Accuracy for Raltegravir in Quality Control Samples with Offline SPE and Symbiosis Online SPE Method (n=18).



**TABLE 1:**  
**Accuracy and Precision of Calibration Standards for Raltegravir with Offline SPE (Top) and Symbiosis Online SPE (Bottom) Method (ng/mL)**

Nominal concentration	1.00	2.00	10.0	50.0	200	600	900	1000
Precision (%)	2.3	2.1	6.9	3.3	4.0	7.0	4.0	7.1
Accuracy (%)	96.3	107	99.0	99.4	100	99.7	100	97.7
Precision (%)	2.6	4.4	0.8	2.0	1.1	1.2	1.6	0.9
Accuracy (%)	97.3	105	103	94.6	93.0	95.3	96.4	99.0
n	6	6	6	6	6	6	6	6

## Limitation of the Symbiosis Online SPE Method continued

**TABLE 2:**  
**Precision for Raltegravir in Quality Control Samples with Offline SPE (Top) and Symbiosis Online SPE (Bottom) Method (n=18).**

Nominal concentration	LLOQ 1.00	Low 3.00	Medium 100	High 800
Between Run Precision (%)	5.1	2.6	4.6	4.1
Within Run Precision (%)	3.8	3.9	2.9	3.3
Total Variation (%)	6.4	4.7	5.4	5.2
Between Run Precision (%)	0.0	2.1	1.4	1.7
Within Run Precision (%)	3.5	3.2	2.3	2.2
Total Variation (%)	3.4	3.8	2.7	2.8
n	18	18	18	18
Number of Runs	3	3	3	3

## Conclusion

- Two robust methods, offline SPE and Symbiosis online SPE were developed and GLP validated for raltegravir with a range of 1.00 ng/mL – 1000 ng/mL.
- The online method has a short cycle time of 100 seconds with no analytical column needed. HySphere C18HD 7µm cartridge was validated to reuse for five times to further reduce cost.
- Sample goes through online SPE process before it reaches LC gradient. With the same volume for elution solvent, increasing the injection volume will increase sensitivity but has no negative impact on chromatography.
- A mixture of TEA and EDTA is the key to eliminate system and SPE cartridge carryover for online method.

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