

Nota de aplicación

## Extraction and Analysis of Orthocetamol, Metacetamol and Paracetamol in Human Plasma Using Oasis™ PRiME HLB and Waters ACQUITY™ Biphenyl RP Column with MaxPeak™ Technology

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Kenneth Berthelette, Chris Collins, Thomas H. Walter

Waters Corporation, United States

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### Abstract

The positional isomers orthocetamol, metacetamol and paracetamol were analyzed using a Waters ACQUITY Biphenyl RP Column with MaxPeak Technology. Subsequently, a suitable solid-phase extraction (SPE) method for extracting these compounds from pooled human plasma was created using the Oasis PRiME HLB 2-step protocol. Baseline separation of the three structurally similar compounds combined with high recovery indicates that this workflow would be suitable for monitoring these compounds in a bioanalytical setting.

### Benefits

- Baseline separation of acetaminophen and its ortho- and meta- positional isomers
- >90% recovery of all three analytes from spiked human plasma

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## Introduction

Structurally similar components can be challenging to separate in reversed-phase liquid chromatography (RPLC) due to their comparable chemical properties. Positional isomers present an even tougher challenge as not only may they have similar chemical properties affecting their retention and elution, but they also have the same mass to charge ratio ( $m/z$ ), making them difficult to distinguish by mass spectrometry (MS). To identify isobaric compounds, individual standards are often analyzed to establish identifications by retention time as well as  $m/z$ . This methodology requires not only a robust method which achieves baseline separation of the isomers, but also a stationary phase that produces reproducible results from batch to batch. In this application note, three positional isomers were analyzed. Orthocetamol, metacetamol and paracetamol were analyzed as neat standards and subsequently spiked into pooled human plasma. The plasma was then subjected to SPE to remove phospholipids and other matrix components which may affect the analysis or the lifetime of the column. SPE was performed using an Oasis PRiME HLB 96-well plate on a semi-automated positive pressure manifold using a 2-step protocol to minimize sample preparation time.<sup>1</sup>

ACQUITY BEH™ Biphenyl Column was used for this application for important reasons. First, these columns employ the highly reproducible ethylene-bridged hybrid (BEH) particle, which not only is stable across a wide range of mobile phase pHs, but also produces excellent batch to batch results as seen in the literature.<sup>2,3</sup> Second, this column uses MaxPeak Premier High-Performance Surface (HPS) technology, which mitigates interactions between analytes and the column hardware.<sup>4</sup> This technology also reduces variability caused by differences in the metal hardware.<sup>5</sup> Lastly, the biphenyl bonded ligand has been shown to improve separations of isomers in biological assays.<sup>6,7</sup> Using this column should provide the best separation possible for these compounds, while ensuring that the method is developed on a robust and reproducible stationary phase that can be used for the lifetime of the assay.

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## Experimental

### Sample Description

A single stock solution was created with 1 mg/mL of each of the three compounds; paracetamol metacetamol and orthocetamol. Pre-spike plasma was spiked with stock solution to a concentration of 80 µg/mL. Pre-spike plasma, post-spike plasma, and non-matrix post spike were diluted 1:1 with 4% phosphoric acid in water and

vortexed to mix. Samples were then passed through an Oasis PRiME HLB 96-well plate (p/n: [186008054 < https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/186008054-oasis-prime-hlb-96-well-plate-30-mg-sorbent-per-well-1-pk.html>](https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/186008054-oasis-prime-hlb-96-well-plate-30-mg-sorbent-per-well-1-pk.html) ) using a 2-step protocol on a semi-automated positive pressure manifold. Post-spike plasma and non-matrix post-spike sample were then spiked to a concentration of 40 µg/mL. Lastly, all samples were diluted with water for analysis. The final analyte concentration was 20 µg/mL for each isomer.

## LC Conditions

LC system:	ACQUITY UPLC™ H-Class Plus with CM and CM Aux, PDA Detector and ACQUITY QDa™ Mass Detector
Detection:	SIR (single ion recording) 152 <i>m/z</i>
Column:	Waters Acquity Biphenyl RP Column with MaxPeak Technology, 2.1 x 50 mm, 1.7 µm (p/n: 186011744)
Column temperature:	30 °C
Sample temperature:	10 °C
Injection volume:	1.0 µL
Flow rate:	0.4 mL/min
Mobile phase A:	Water
Mobile phase B:	Methanol
Mobile phase D:	2% formic acid in water

Gradient conditions: Constant 5% D to maintain mobile phase additive concentration; initial mobile phase conditions 5% B; linear ramp to 95% B in 5.00 minutes; hold for 1 minute; return to initial conditions and re-equilibrate for 2.00 minutes; total run time 8.00 minutes.

## MS Conditions

MS system: ACQUITY QDa Mass Detector

Function: SIR 152 *m/z*

ESI polarity: Positive

Capillary voltage (kV): 1.5

Cone voltage (V): 15

Scan rate (pts/sec): 5

## Data Management

Chromatography software: Empower™ Chromatography Data System (CDS)

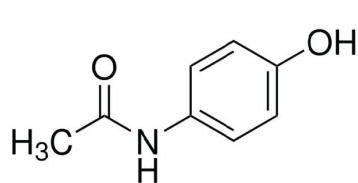
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## Results and Discussion

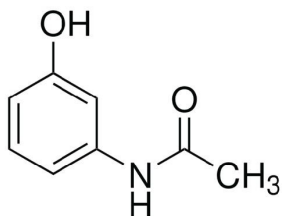
The three positional isomers whose structures are shown in Figure 1 were analyzed using an ACQUITY BEH Biphenyl Column. Acetaminophen (paracetamol), and its ortho and meta positional isomers were selected as model compounds for this work. Once a suitable separation was achieved, the compounds were spiked into

pooled human plasma.

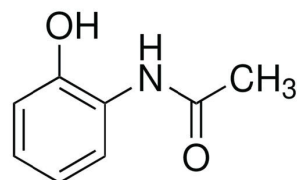
### Chemical Structures



1. Paracetamol  
(Acetaminophen)



2. Metacetamol



3. Orthocetamol

Figure 1. Chemical structures of the positional isomers tested.

Plasma samples and non-matrix samples (neat solutions) were processed using an Oasis PRiME HLB 30 mg plate and the 2-step protocol. Oasis PRiME HLB \ SPE devices are designed to remove phospholipids from biological fluids to reduce matrix effects and extend column lifetime.<sup>8</sup> The 2-step protocol is shown in Figure 2.

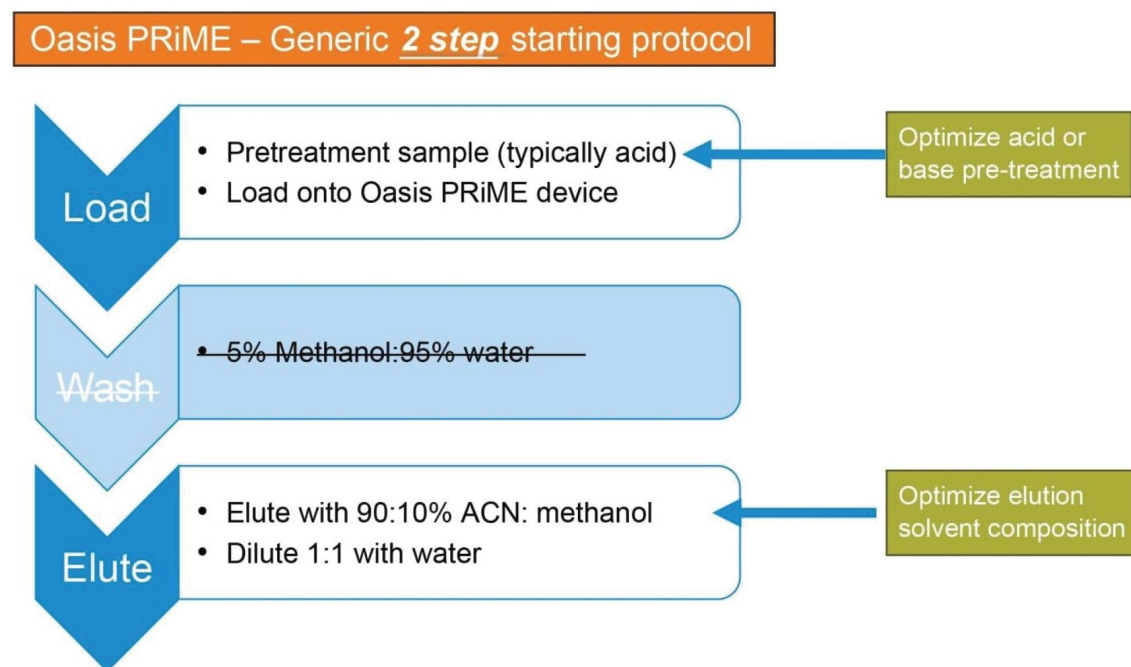


Figure 2. Graphical representation of the generic 2-step protocol for Oasis PRiME HLB SPE.

The 2-step protocol, which includes load and elute steps, was specifically developed to reduce sample preparation time without sacrificing recovery and is suitable as a first step in most bioanalytical workflows to extract analytes of interest by hydrophobic interactions. Prior to loading the samples onto the SPE device, a pre-treatment step is employed to ensure the analytes are in a neutral charge state. This improves retention on the SPE sorbent while allowing polar interferences, like salts, to pass through during the load step. Depending on the nature of the samples, a wash step may be needed to improve the SPE results. However, for initial scouting, the 2-step protocol gives adequate removal of matrix interferences, providing a good basis for further development work. The elute step uses a high organic content solvent to elute the analytes from the device. Due to the proprietary design of the Oasis PRiME HLB sorbent, phospholipids are retained even under high organic conditions, eliminating them from the final eluate, producing a cleaner sample. A final dilution with water is recommended to ensure that the elution solvent does not affect the chromatographic separation.

The three analytes of interest were easily detected by mass spectrometry using SIR at 152  $m/z$  in pre-spike plasma after cleanup, with good MS signal using an ACQUITY QDa Mass Detector. A blank plasma sample was also processed using the 2-step protocol to determine if any of the analytes are endogenous in the plasma.

Figure 3 shows representative chromatograms of the pre-spike and blank plasma samples.

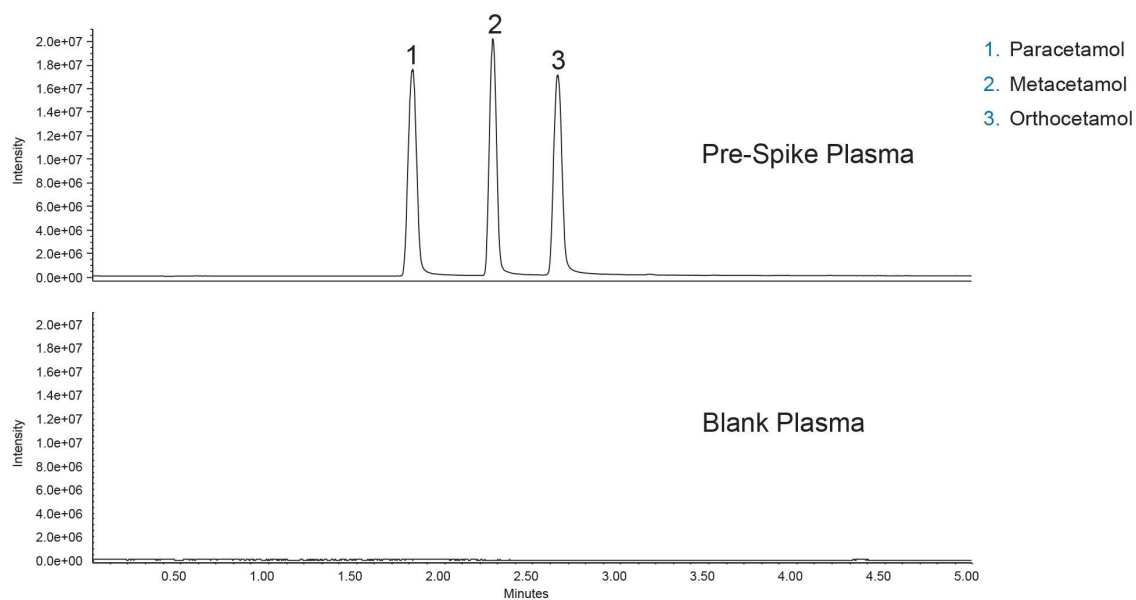
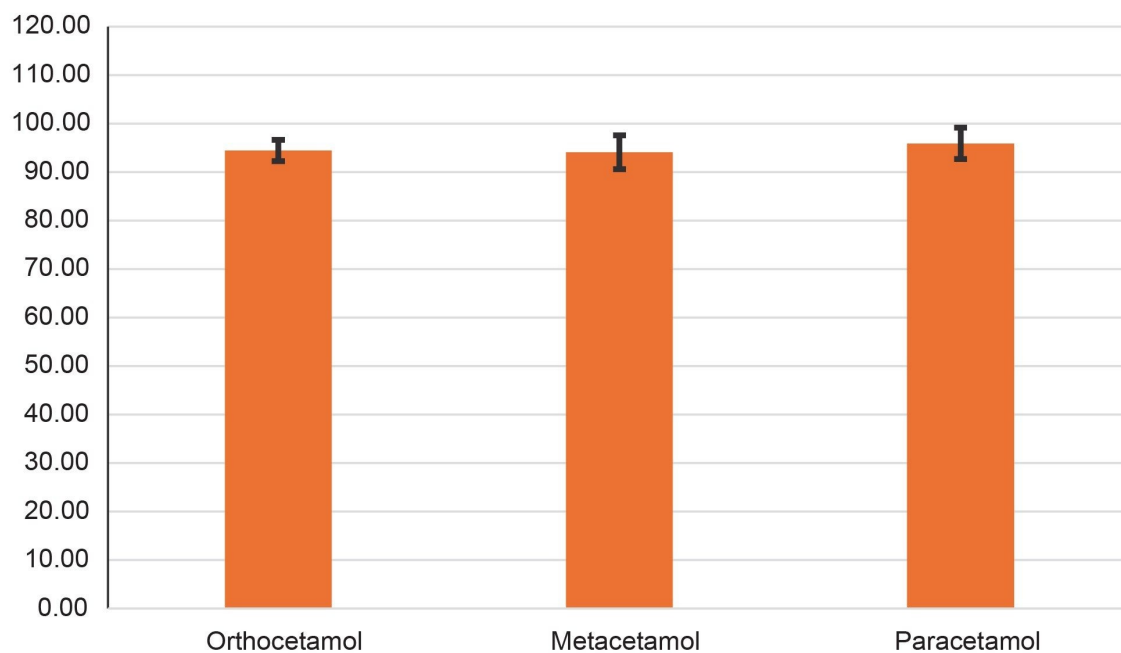


Figure 3. LC-MS chromatograms of SIR 152  $m/z$  of a pre-spike plasma and blank plasma sample after processing through an Oasis PRiME HLB plate (see Figure 1 for peak identifications).

The BEH Biphenyl Column provided baseline resolution of the positional isomers, with sharp symmetrical peaks. The blank plasma sample shows none of the analytes of interest, indicating that the spiked amounts should be accurate. The selective nature of SIR eliminates background noise and improves overall sensitivity. The presence of endogenous compounds would have altered the final concentrations and could have affected recovery or matrix effect calculations. However, as the blank plasma showed no compounds present, the spiked amount should be the correct concentration in the samples. Calculations of % recovery and matrix effects showed great recovery with minimal matrix effects (see Figure 4 and Figure 5).



*Figure 4. % Recovery for each analyte spiked into pooled human plasma. Replicate samples were analyzed (n=4) to ensure accuracy of the data. Error bars show plus or minus one standard deviation of recovery for each analyte.*

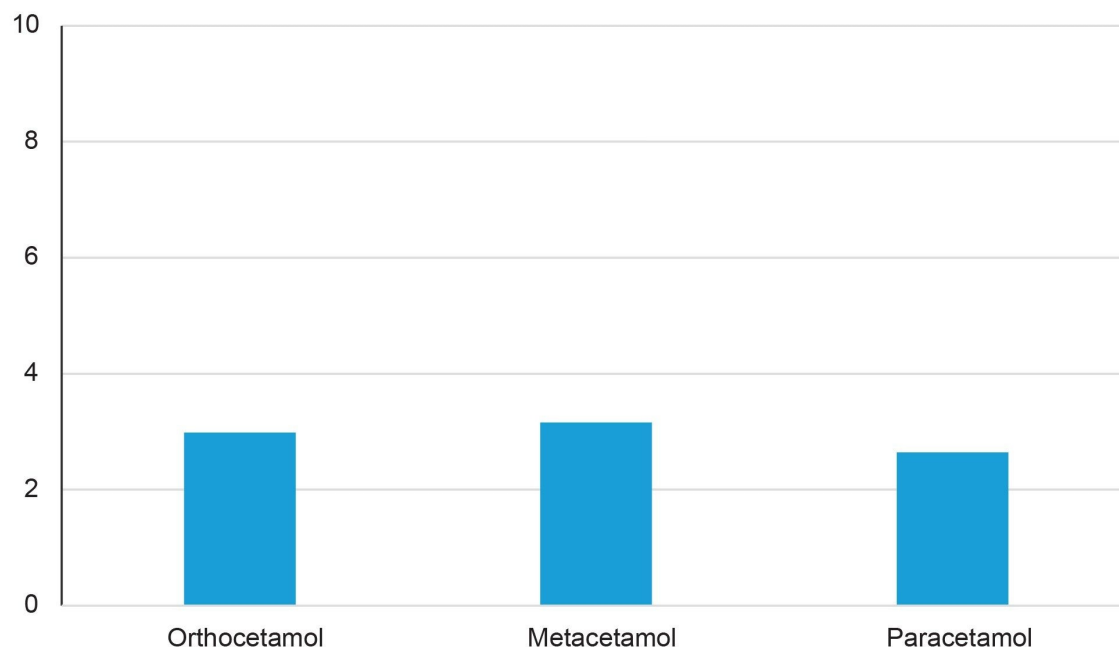


Figure 5. Matrix effect for each analyte spiked into pooled human plasma. Replicate samples were analyzed ( $n=4$ ) to ensure accuracy of the data.

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## Conclusion

With high recoveries, low matrix effects, baseline separation and good peak shape for all analytes, the use of an Oasis PRiME HLB SPE 96-well plate and an ACQUITY Premier BEH Biphenyl Column yielded a method for analyzing the positional isomers orthocetamol, metacetamol and paracetamol in human plasma.

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