

Hybridization LC-MS/MS: A Selective Solution for PMO and PPMO Bioanalysis

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BACKGROUND

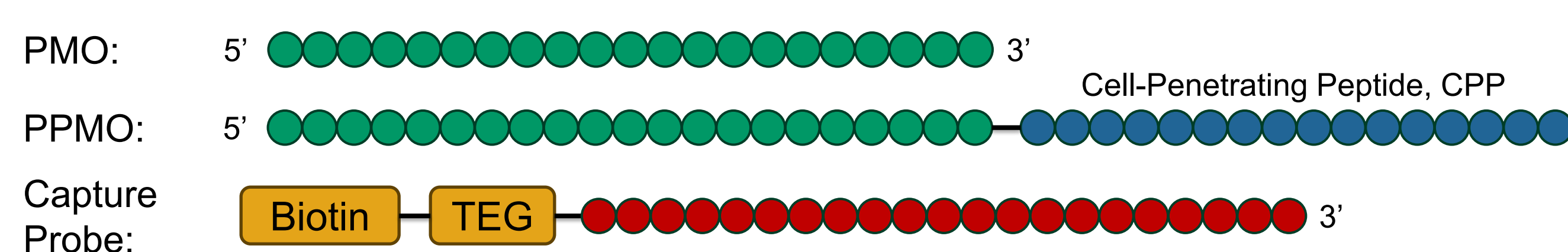
Phosphorodiamidate morpholino oligomers (PMOs) and peptide-conjugated PMOs (PPMOs) are emerging therapeutic modalities that present increasing challenges in bioanalysis due to their structural complexity and the presence of closely related species. Accurate quantitation in biological matrices, therefore, requires highly selective analytical approaches.

A hybridization LC-MS/MS method was developed for the selective and sensitive quantitation of PMO and PPMO in rabbit plasma. The workflow utilizes biotinylated capture probes and magnetic beads to selectively isolate target oligonucleotides prior to LC-MS/MS analysis. Key parameters, including probe chemistry, capture buffer composition, and probe-to-beads ratio, were systematically optimized to improve extraction efficiency and overall assay performance.

OBJECTIVE

- Develop LC-MS/MS conditions for the analysis of a 21-mer PMO and its peptide-conjugated form (PPMO) (Figure 1A).
- Optimize hybridization extraction parameters to enhance selectivity and recovery.
- Establish a robust and selective method for the simultaneous quantitation of PMO and PPMO in rabbit plasma.

(A) Structures of PMO, PPMO and Capture Probe



(B) Hybridization LC-MS/MS Workflow

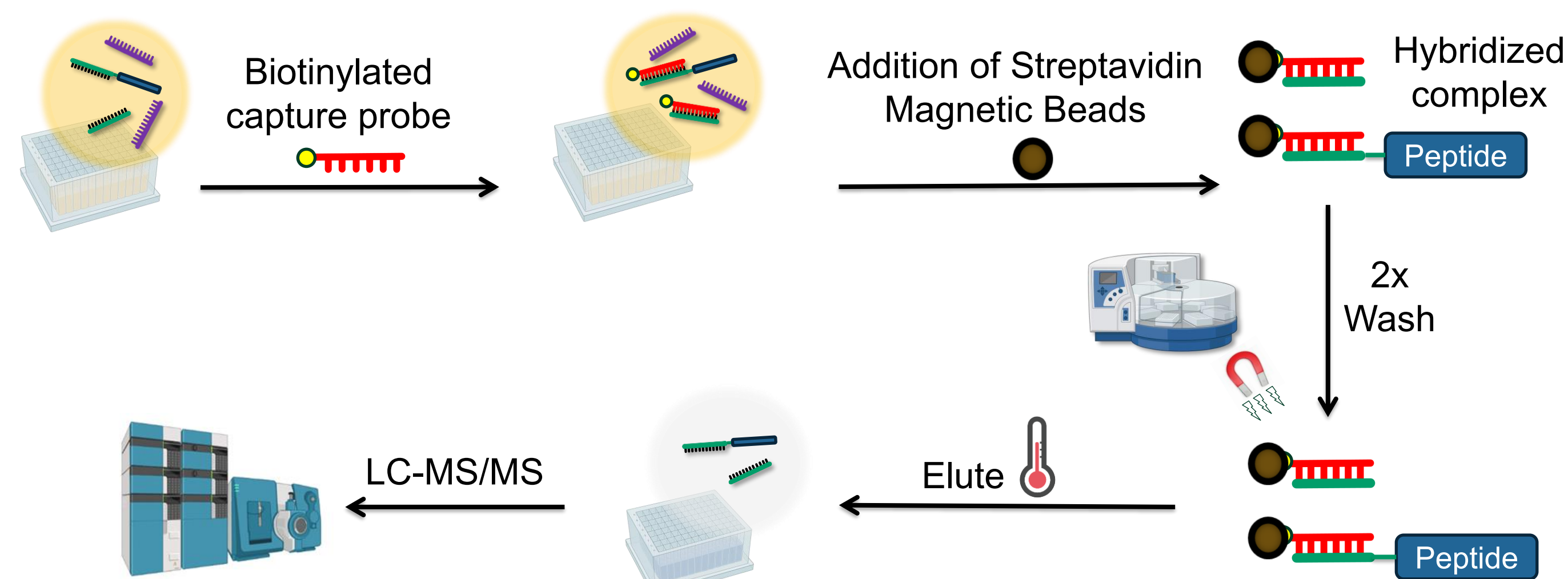


Figure 1. Structures of PMO, PPMO and Capture Probe (A) and Schematic of Hybridization LC-MS/MS Workflow (B).

METHOD

Hybridization Extraction

Rabbit plasma samples (50 µL) were subjected to hybridization extraction using a biotinylated capture probe. Following hybridization, probe-analyte complexes were captured with streptavidin magnetic beads, washed on a KingFisher processor to remove matrix interferences, and thermally eluted into an organic solution. An analogue internal standard was added following extraction to the eluate prior to LC-MS/MS analysis (Figure 1B).

LC-MS/MS Analysis

Chromatographic separation was performed on a Shimadzu Nexera X2 UHPLC system, with detection by a SCIEX 6500+ triple quadrupole mass spectrometer operated in positive ESI MRM mode. Detailed conditions are summarized in Table 1.

Table 1. LC-MS/MS Conditions for the Analysis of PMO and PPMO

Parameter	Description
Analytical Column	Waters Acquity Premier BEH Shield RP18 column, 50 x 2.1 mm, 1.7 µm
Column Temperature	70 °C
Mobile Phase A	0.2% Trifluoroacetic Acid (TFA), 10 mM Ammonium Bicarbonate in H ₂ O
Mobile Phase B	Acetonitrile:H ₂ O 90:10% v/v
Flow Rate	0.50 mL/min
Post-column addition	400 mM Ammonium Bicarbonate, 0.15 mL/min
MRM Transitions	PMO 796.2 / 136.0
	PPMO 709.7 / 136.0
	Internal Standard 922.1 / 136.0

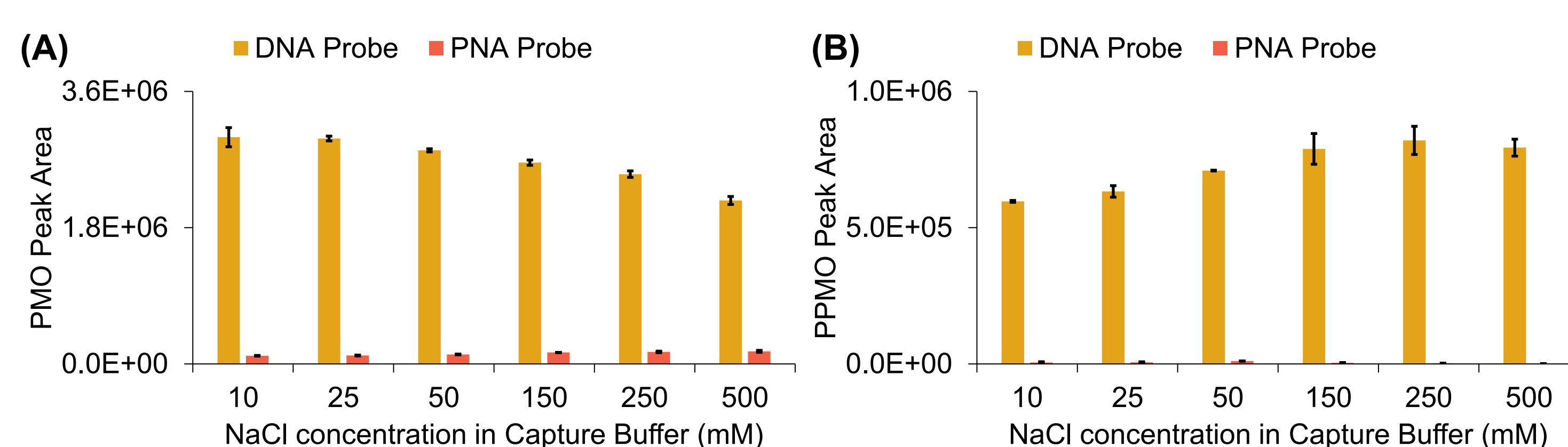


Figure 2. Comparison of DNA and PNA capture probes for hybridization extraction of PMO (A) and PPMO (B).

RESULTS AND DISCUSSION

Hybridization Extraction Optimization

To establish an efficient hybridization-extraction workflow, key parameters affecting analyte recovery were evaluated, beginning with capture probe chemistry.

DNA probes provided significantly higher signal intensity than peptide nucleic acid (PNA) probes across all tested NaCl concentrations, indicating superior hybridization efficiency and recovery (Figure 2). In contrast, PNA probes showed minimal response and were not suitable for this application.

Optimal performance was achieved at a moderate ionic strength, balancing efficient hybridization and reduced non-specific interactions. DNA probes were therefore selected for subsequent method development.

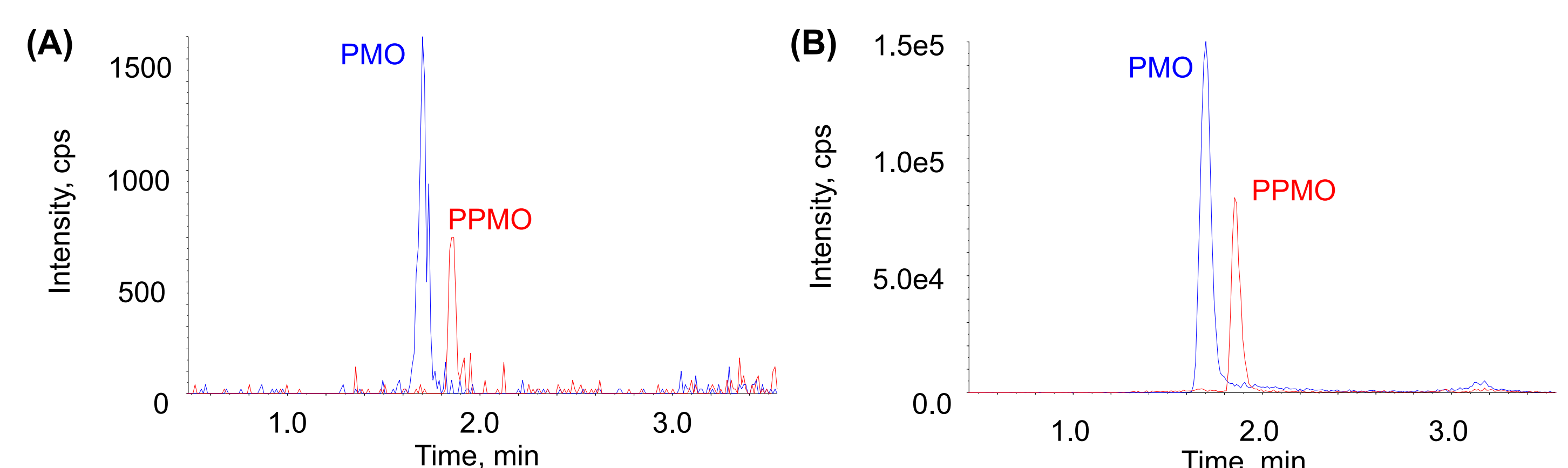


Figure 3. Post-column addition improves signal intensity by mitigating TFA-induced ion suppression: without (A) and with (B) post-column addition.

LC-MS/MS Establishment

To achieve adequate chromatographic performance for PMO and PPMO, the effect of TFA in the mobile phase was first evaluated. The inclusion of TFA significantly improved peak shape and chromatographic resolution; however, it also resulted in substantial ionization suppression, leading to reduced MS sensitivity. Post-column addition of ammonium bicarbonate was introduced to mitigate TFA-induced suppression, resulting in a marked increase in signal intensity (Figure 3). Under optimized conditions, PMO, PPMO, and internal standard were well resolved with sharp peak shapes and consistent retention times, enabling accurate and reliable quantitation (Figure 4A).

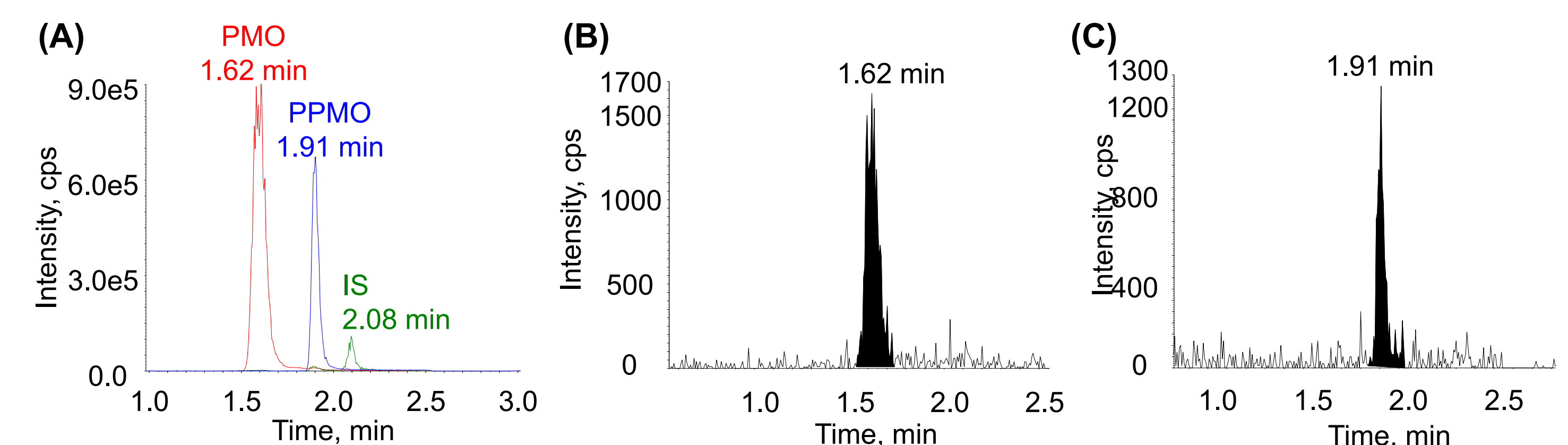


Figure 4. Representative chromatograms showing separation of PMO, PPMO, and IS (A), PMO at LLOQ (2.00 ng/mL) (B), and (C) PPMO at LLOQ (5.00 ng/mL).

Method Qualification

The method was successfully qualified, meeting acceptance criteria for accuracy, precision, and sensitivity across the calibration range for PMO (2.00 – 1000 ng/mL) and PPMO (5.00 – 2500 ng/mL) in rabbit plasma (Tables 2-3, Figure 4B-C).

Table 2. Inter-Run Precision and Accuracy (3 Runs, 12 Replicates/QC Level)

Parameters	PMO			
	QC LOQ (2.00 ng/mL)	Low QC (6.00 ng/mL)	Mid QC (500 ng/mL)	High QC (750 ng/mL)
%Nominal	99.3	97.7	101.5	100.2
%C.V.	5.9	4.7	5.1	3.2
Parameters	PPMO			
	QC LOQ (6.00 ng/mL)	Low QC (15.00 ng/mL)	Mid QC (1250 ng/mL)	High QC (1875 ng/mL)
%Nominal	102.3	103.5	103.5	97.9
%C.V.	8.6	6.7	7.5	6.3

Table 3. Matrix Effect of PMO and PPMO in Rabbit Plasma

Parameters	PMO		PPMO	
	Low QC (6.00 ng/mL)	High QC (750 ng/mL)	Low QC (15.0 ng/mL)	High QC (1875 ng/mL)
Donor #1	5.76 ± 0.48	793 ± 8	13.7 ± 0.7	1781 ± 144
Donor #2	6.42 ± 0.18	829 ± 9	13.1 ± 0.3	1772 ± 41
Donor #3	6.21 ± 0.20	817 ± 33	13.9 ± 0.9	1785 ± 124
Donor #4	6.18 ± 0.09	840 ± 21	14.9 ± 0.8	1784 ± 143
Donor #5	6.08 ± 0.76	820 ± 16	14.8 ± 0.7	1856 ± 153
Hemolyzed Plasma	6.39 ± 0.18	848 ± 22	14.4 ± 1.3	1727 ± 144

CONCLUSION

A robust hybridization LC-MS/MS method was developed and qualified for the simultaneous quantification of PMO and PPMO in rabbit plasma. The method provides efficient extraction, improved chromatographic performance, and enhanced sensitivity, supporting reliable bioanalysis of PMO-based therapeutics.