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LC–MS-Based Direct Quantification of MicroRNAs in Rat Blood

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ABSTRACT: MicroRNA (miRNA) has recently garnered significant research attention, owing to its potential as a diagnostic biomarker and therapeutic target. Liquid chromatography–mass spectrometry (LC–MS) offers accurate quantification, multiplexing capacity, and high compatibility with various matrices. These advantages establish it as a preferred technique for detecting miRNA in biological samples. In this study, we presented an LC–MS method for directly quantifying seven miRNAs (rno-miR-150, 146a, 21, 155, 223, 181a, and 125a) associated with immune and inflammatory responses in rat whole blood. To ensure miRNA stability in the samples and efficiently purify target analytes, we compared Trizol- and proteinase K-based extraction methods, and the Trizol extraction proved to be superior in terms of analytical sensitivity and convenience. Chromatographic separation was carried out using an oligonucleotide C18 column with a mobile phase composed of *N*-butyldimethylamine, 1,1,1,3,3,3-hexafluoro-2-propanol, and methanol. For MS detection, we performed high-resolution full scan analysis using an orbitrap mass analyzer with negative electrospray ionization. The established method was validated by assessing its selectivity, linearity, limit of quantification, accuracy, precision, recovery, matrix effect, carry-over, and stability. The proposed assay was then applied to simultaneously monitor target miRNAs in lipopolysaccharide-treated rats. Although potentially less sensitive than conventional methods, such as qPCR and microarray, this direct-detection-based LC-MS method can accurately and precisely quantify miRNA. Given these promising results, this method could be effectively deployed in various miRNA-related applications.

INTRODUCTION

MicroRNA (miRNA) is a small, noncoding RNA molecule, typically comprising approximately 21–25 nucleotides, that plays a crucial role in regulating gene expression.[1] Although miRNAs are transcribed from DNA, they do not encode proteins directly. They bind to complementary sequences on target mRNA molecules, inhibiting their translation into proteins. This mechanism allows miRNAs to regulate target gene expression, impacting various biological processes, including development, cell differentiation, metabolism, and immune response.[2,3] Furthermore, miRNAs have been associated with various diseases, including inflammatory diseases, cancer, and neurological disorders. This has prompted investigations into their potential as therapeutic targets and diagnostic biomarkers.[4]

The two primary methods for quantitatively analyzing miRNAs are quantitative polymerase chain reaction (qPCR) and microarray.[5] qPCR, a widely used technique for miRNA quantification, offers high sensitivity and specificity, enabling the detection of even low levels of miRNAs in a sample. However, qPCR requires a specific primer design, which can be time-consuming and may necessitate optimization for different miRNAs. Moreover, it can be susceptible to variations in amplification efficiency or nonspecific amplification, which may affect the accuracy of quantification.[6] Microarray enables the simultaneous measurement of the expression of hundreds of miRNAs, providing a comprehensive view of miRNA expression in a sample. Nonetheless, its limited dynamic range constrains its ability to accurately detect miRNAs at significantly low or high levels. Additionally, it can be prone to cross-hybridization and batch effects, introducing potential variability into the results.[7]

Recently, mass spectrometry (MS) has been applied for determining miRNA. MS is an effective technique for quantifying various molecules, from chemicals to macromolecules, including proteins and lipids. Compared to conventional methods, MS offers accurate quantification, high compatibility with diverse biological matrices, and multiplexing capacity.[8] These advantages are further enhanced when MS is coupled with separation tools like liquid chromatography (LC) and capillary electrophoresis (CE).[5] LC is a versatile analytical tool used for separating a wide variety of biomolecules, ranging from polar to nonpolar compounds. As miRNA is a highly charged molecule, ion-pair reversed-phase liquid chromatography (IP-RP-LC) and hydro-

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philic interaction liquid chromatography (HILIC) have been employed for its precise separation. CE, an effective technique for analyzing polar substances, has been gradually gaining popularity in the separation of miRNA and other oligonucleotides due to its advantages in terms of efficiency and requirement of smaller sample amounts.[5,9] However, analyzing miRNA using MS is challenging due to its large molecular size and distribution of multiply charged molecular ions, which can lead to lower ionization efficiency.[8] To address this issue, researchers have explored alternative methods to enhance MS detection sensitivity. Xu et al. suggested a method utilizing peptides as reporters to improve sensitivity.[10−15] Shi et al. integrated MS detection with a duplex specific nuclease (DSN)-based signal amplification strategy, allowing target miRNAs to recycle and produce large amounts of complementary DNA fragments.[16] Certain methods employed nucleobases as reporters for miRNA analysis, leveraging rolling circle amplification or DSN-assisted amplification.[17,18] Despite these indirect methods using peptides, DNA fragments, and nucleobases compensating for the low detection sensitivity, they were considerably complicated and time-consuming for practical use. Additionally, multiplex miRNA analysis with these approaches proved to be cumbersome and laborious.

Direct analysis using MS holds the potential to address these issues. However, there have been limited studies of direct miRNA quantification, particularly from in vivo samples. Studzinska et al. analyzed four different miRNAs and one modified miRNA in human serum using LC−MS/MS and LC−quadrupole−time-of-flight (Q−TOF) MS, and liquid−liquid extraction (LLE) combined with solid-phase extraction (SPE) was implemented to determine the target analytes.[19] Basiri et al. quantified miR-451 in human and rat plasma using LC−Q−TOF MS, incorporating proteinase K digestion and a biotinylated capture probe for selective extraction.[20] Recent research has also revealed that post-transcriptional modifications at the 3′ end of miRNA play an important role in its stability and activity.[21−23] MS-based direct and label-free analysis can provide not only quantitative but also qualitative information, such as miRNA modifications. For example, Khan et al. successfully detected iso-miR-16−5p (miR-16−5p with 3′-uridylation) and miR-21−5p in human serum from a patient with chronic lymphocytic leukemia using CE−Q−TOF MS.[24] Hence, there is a need to further research the direct detection of miRNA, which offers substantial potential benefits such as efficient sample preparation, multiplexability, and qualitative capability.

In this study, we developed an analytical method using LC−MS for determining seven miRNAs (rno-miR-150, 146a, 21, 155, 223, 181a, and 125a) in rat whole blood. These miRNAs are associated with anti-inflammatory and immune-modulatory activities.[25−29] We aimed to directly and simultaneously analyze miRNAs using LC−MS, while streamlining the sample preparation process. To achieve this goal, we developed and validated an MS-based label-free assay for miRNAs using Trizol extraction rather than the established indirect quantitation methods. The method validation was performed for linearity, limit of quantification (LOQ), accuracy, precision, recovery, matrix effect, and stability. Furthermore, we applied the method to actual samples in which immune responses were induced to assess its feasibility and practicality and successfully detected miRNAs in the samples.

RESULTS AND DISCUSSION

Optimization of LC−MS Conditions. miRNA possesses a highly negatively charged phosphate backbone, hindering analysis using reversed-phase LC (RP-LC). To overcome this issue, ion-pairing reagents that can interact with oppositely charged analytes and the stationary phase are commonly employed as mobile phase additives in LC separation.[30] The combination of trimethylamine (TEA) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) is the most frequently used reagent for this purpose.[31−33] *N*-Butyldimethylamine (DMBA) has also been reported in the literature and studies related to the chromatographic separation of miRNA and oligonucleotides.[19,34,35] Based on these data, TEA and DMBA were evaluated. Both amines demonstrated favorable outcomes with negligible differences in the elution order, peak shape, and resolution. However, DMBA offered superior analytical sensitivity (Figures S1 and S2). Therefore, DMBA was selected for further experiments, with its concentration being optimized for detection sensitivity at concentrations of 5, 10, 15, and 20 mM. Peak area and signal-to-noise (S/N) ratios were used as indicators to assess analytical sensitivity. The values of these parameters for seven analytes increased as DMBA concentration rose to 15 mM but declined thereafter (Figure 1). Higher DMBA concentrations can increase the conductivity of spray solution in the electrospray ionization (ESI) process, resulting in smaller droplet size and improved ionization efficiency.[36] However, above 15 mM, ionization suppression seemed to predominantly impact the detection sensitivity. Consequently, 15 mM was determined as the optimal DMBA concentration. The influence of HFIP concentration on sensitivity was also examined at 50, 100, 200, and 300 mM. As depicted in Figure 2, the detection sensitivity improved up to 100 mM, but adverse effects were observed beyond this concentration. HFIP serves as a

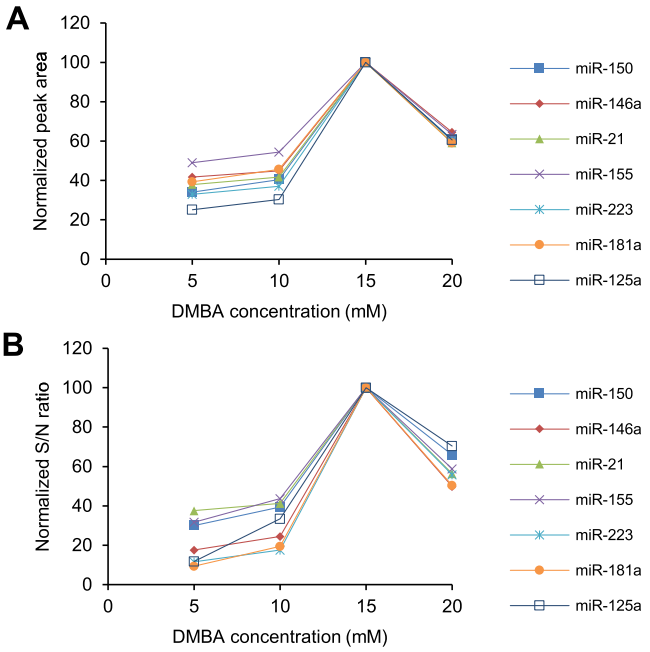

Figure 1. Effect of the *N*-butyldimethylamine (DMBA) concentration on detection sensitivity. Peak areas (A) and signal-to-noise (S/N) ratios (B) were normalized relative to the corresponding results obtained from 15 mM DMBA.

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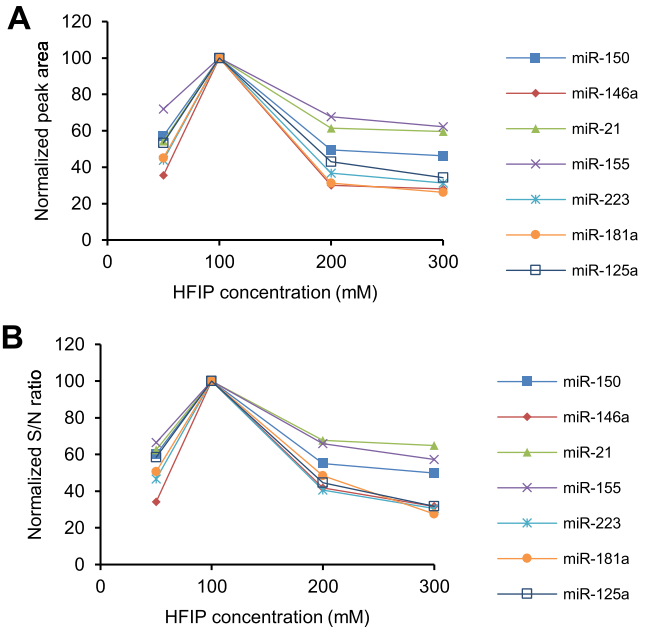

Figure 2. Effect of the 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) concentration on detection sensitivity. Peak areas (A) and signal-to-noise (S/N) ratios (B) were normalized relative to the corresponding results obtained from 100 mM HFIP.

counteranion to amines, adjusting the pH of the mobile phase. Unlike other anions, such as acetate and carboxylate, HFIP has a low boiling point (58 °C), facilitating its evaporation at the droplet surface during the electrospray process. After evaporation, the increased surface pH aids in the miRNA-DMBA ion-pair dissociation, enhancing ionization efficiency.[33,37] The ionic additive in the mobile phase requires a balance between its effectiveness and its ionization suppression. Concentrations exceeding 100 mM led to ionization suppression becoming the dominant effect. Therefore, 100 mM was determined as the optimal HFIP concentration. Moreover, the retention time was extended with higher concentrations of DMBA and HFIP. To maintain consistent retention times, all experiments were conducted with minor modifications to the gradient profile. The resulting chromatograms and gradient conditions are depicted in Figures S3 and S4. Due to the insolubility of HFIP in acetonitrile, the choice of organic solvent was limited to methanol.

For high-resolution MS analysis, we compared the full scan mode and the selected ion monitoring mode. Although both modes exhibited similar sensitivity, the full scan mode, which enables profiling of ions within a specific range, was selected as the detection method. ESI with a negative ion mode was employed for sensitive miRNA quantification. The major ions of miRNA were detected in the m/z 600−2000 range, with charge states of −4 to −9. As shown in Figures S5−S11, all target miRNAs displayed the most intense signal at the charge state of −4 ($[M - 4H]^{4-}$). Consequently, the $[M - 4H]^{4-}$ ion was selected as the target for the seven miRNAs, and their theoretical monoisotopic masses are detailed in Table 1. With a resolution of 70,000, we confirmed that the mass error of the observed ions fell within the range of 0.5−1.9 ppm. The ESI parameters, including sheath gas flow rate, aux gas flow rate, spray voltage, capillary temperature, and aux gas heater temperature, were optimized to maximize the signal response. The results are provided in the Materials and Methods section.

Development of Sample Preparation. Developing an accurate bioanalytical method using LC−MS requires samples fortified with the target analyte. Particularly, miRNA degradation in biological samples is critical, as miRNA is significantly less stable than DNA and other oligonucleotides. Extracting spiked miRNA from rat whole blood using conventional solvent extraction with a phenol/chloroform/isoamyl alcohol mixture was challenging. To address this issue, we examined two methods for sample preparation: Trizol- and proteinase K-based extraction. Trizol extraction was conducted according to the manufacturer's guidelines and previous reports, and as far as we know, this is the first case of using Trizol reagent for MS direct miRNA quantification.[38] In the Trizol protocol, fortified samples, including calibration standards and quality control samples, were obtained by spiking miRNA working solution into blank samples pretreated with Trizol reagent at a volumetric ratio of 1:4 (sample/Trizol). Proteinase K was implemented based on the work of Basifi et al.[20] For the fortified samples prepared using the proteinase K method, the blank sample was digested with proteinase K, and the miRNA working solution was spiked into the digested sample. This was followed by LLE using a phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) mixture combined with SPE to extract and purify the target analytes. Both methods successfully recovered all analytes in the final extract, demonstrating satisfactory linearity, accuracy, and precision (Table 2). However, Trizol extraction (LOQ 6.25 nM) exhibited superior detection sensitivity over the proteinase K method (LOQ 20 nM). Moreover, the Trizol method proved to be more efficient and practical considering the total time required, labor, and technical complexity. The proteinase K digestion necessitated additional cleanup due to the high salt and detergent concentrations in the enzyme reaction solution, potentially impairing MS performance. Consequently, Trizol-based extraction was selected as the optimal method for the quantification of miRNAs in rat blood samples.

Method Validation. Complete validation results are summarized in Table 3, with detailed information in the Supporting Information (Tables S1−S4). While the FDA and EMA guidelines for LC−MS bioanalytical validation primarily

Table 1. Sequence and Chemical Properties of the Target miRNAs

analyte	sequence	selected ion	theoretical m/z value	detected m/z value	mass error (ppm)
miR-150	UCUCCCAACCCUUGUACCAGUG	$[M - 4H]^{4-}$	1737.21598	1737.46952	1.9
miR-146a	UGAGAACUGAAUUCCAUGGGUU	$[M - 4H]^{4-}$	1779.47221	1779.72544	1.7
miR-21	UAGCUUAUCAGACUGAUGUUGA	$[M - 4H]^{4-}$	1769.71668	1769.96926	1.3
miR-155	UUAAUGCUAAUUGUGAUAGGGGU	$[M - 4H]^{4-}$	1866.22608	1866.47894	1.4
miR-223	CGUGUAUUUGACAAGCUGAGUUG	$[M - 4H]^{4-}$	1859.97727	1860.22901	0.8
miR-181a	AACAUUCAACGCUGUCGGUGAGU	$[M - 4H]^{4-}$	1855.48653	1855.73838	0.8
miR-125a	UCCCUGAGACCCUUUAACCUGUGA	$[M - 4H]^{4-}$	1905.98698	1906.23820	0.2

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Table 2. Quantitative Performance of Trizol- and Proteinase K-Based Extraction

method	test item	miR-150	miR-146a	miR-21	miR-155	miR-223	miR-181a	miR-125a
trizol	LOQ (nM)	6.25	6.25	6.25	6.25	6.25	6.25	6.25
	range (nM)	6.25−500	6.25−500	6.25−500	6.25−500	6.25−500	6.25−500	6.25−500
	R^2	0.9954	0.9977	0.9939	0.9975	0.9982	0.9994	0.9968
	accuracy (%)	90.9−101.4	95.7−105.2	90.3−100.2	96.0−104.5	98.4−110.9	91.2−104.1	87.1−96.5
	precision (RSD, %)	≤5.5	≤8.3	≤6.9	≤6.3	≤10.1	≤7.0	≤8.0
proteinase K	LOQ (nM)	20	20	20	20	20	20	20
	range (nM)	20−500	20−500	20−500	20−500	20−500	20−500	20−500
	R^2	0.9971	0.9982	0.9943	0.9946	0.9988	0.9951	0.9990
	accuracy (%)	91.9−99.4	92.4−109.3	94.9−108.1	101.4−108.2	93.5−107.6	92.8−102.6	91.1−105.8
	precision (RSD, %)	≤7.2	≤10.6	≤8.7	≤11.9	≤4.2	≤8.7	≤8.8

Table 3. Summary of the Validation Results

	linearity			accuracy (%)		precision (RSD, %)			
analyte	range (nM)	R^2	LOQ (nM)	within-run (n = 5)	between-run (n = 15)	within-run (n = 5)	between-run (n = 15)	recovery[a] (%, n = 3)	matrix effect[a] (%, n = 3)
miR-150	6.25−500	0.9959	6.25	91.6−107.0	90.8−106.2	2.1−13.9	2.0−8.9	89.4. 82.2	100.5, 102.6
miR-146a	6.25−500	0.9960	6.25	87.4−106.7	91.4−106.9	7.6−10.0	6.1−11.0	92.8, 84.4	101.8, 104.0
miR-21	6.25−500	0.9961	6.25	92.0−103.9	92.8−106.8	4.9−9.0	4.0−8.0	91.0, 82.7	98.3, 104.3
miR-155	6.25−500	0.9971	6.25	89.3−108.7	94.8−106.8	2.2−4.9	3.3−7.6	85.9, 91.8	97.8, 102.4
miR-223	6.25−500	0.9960	6.25	92.2−103.1	93.7−106.9	3.5−7.1	4.1−6.2	90.7, 83.9	102.6, 96.3
miR-181a	6.25−500	0.9963	6.25	94.5−105.6	93.1−105.0	3.1−5.1	3.2−7.8	87.9, 84.6	100.1, 101.7
miR-125a	6.25−500	0.9986	6.25	92.2−105.3	92.2−104.4	2.1−5.5	4.1−8.3	91.6, 84.6	99.3, 100.2

[a]Data are expressed as mean obtained from low and high QCs (18.75 and 400 nM), respectively.

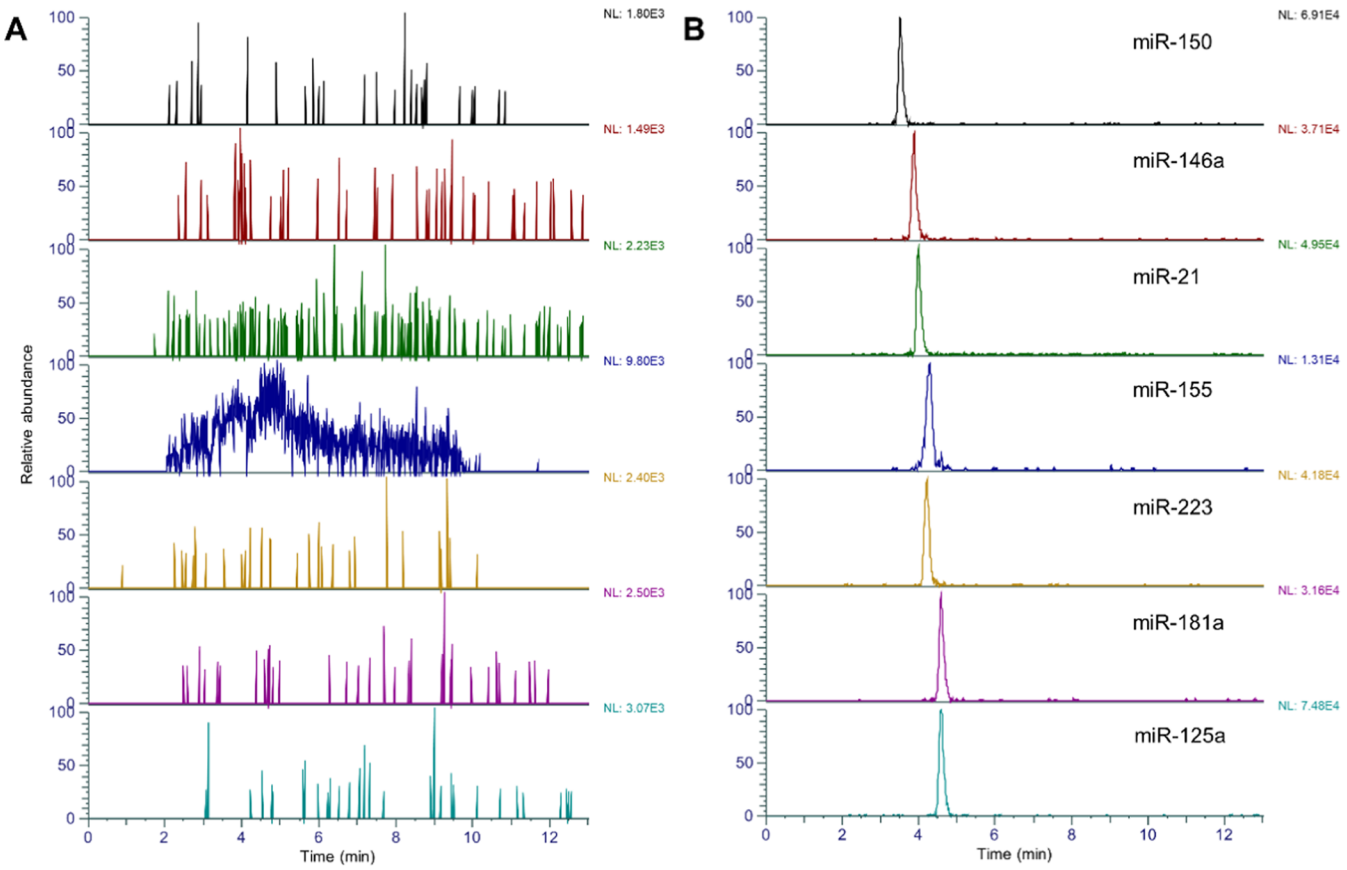

Figure 3. Liquid chromatography−mass spectrometry (LC−MS) chromatograms of seven miRNAs in (A) a representative blank sample and (B) a blank sample spiked with target miRNAs at the limit of quantification (LOQ, 6.25 nM).

focus on chemical and biological drugs, specific guidelines for miRNAs are not available.[39,40] Nevertheless, the validation results were evaluated according to the existing guidelines, due to the absence of other alternatives. With a mass accuracy

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Table 4. Monitoring Results of Target miRNAs in Lipopolysaccharide-Treated and Control Rats

				concentration (nM)						
group	dose (mg/kg)	collection time (h)	animal no.	miR-150	miR-146a	miR-21	miR-155	miR-223	miR-181a	miR-125a
control	0	6	1	ND[a]	ND	ND	ND	ND	ND	ND
			2	ND	ND	ND	ND	ND	ND	ND
			3	ND	ND	ND	ND	ND	ND	ND
T1	5	6	1	ND	ND	ND	ND	ND	ND	ND
			2	8.51	ND	18.89	13.35	10.57	ND	14.20
			3	ND	ND	13.84	ND	ND	ND	ND
T2	5	24	1	ND	ND	10.03	ND	ND	ND	ND
			2	ND	ND	8.27	ND	ND	ND	ND
			3	ND	ND	14.00	ND	ND	ND	ND
T3	10	6	1	ND	ND	15.59	ND	ND	ND	10.32
T4	10	24	1	ND	ND	9.84	ND	ND	ND	ND

[a]ND: Not detected.

Table 5. Comparison of the Proposed Method with Other Mass Spectrometry (MS)-Based Methods for In Vivo miRNA Detection

type	target matrix	sample preparation	instrument	target analyte	LOQ	refs
indirect	human breast cells and tissue	DNA-peptide probe (tryptic digestion)	LC−MS/MS	miR-21	1 pM	10
	human breast cells and tissue	DNA-peptide probe (tryptic digestion)	LC−MS/MS	miR-21, let-7a, miR-200c, miR-125a, miR-15b	1 pM	11
	human breast cells and tissue	DNA-peptide probe (photocleavable)	LC−MS/MS	miR-21, miR-125a, miR-200c	1 pM	35
	human serum	DNA-peptide probe (tryptic digestion)	LC−MS/MS	miR-224	5 pM	15
	human breast cells and tissue	DNA-peptide probe (tryptic digestion)	LC−MS/MS	miR-21	5 pM	12
	human breast cells and tissue	DNA-peptide dendrimer probe (tryptic digestion)	LC−MS/MS	miR-21	0.2 pM	14
direct	human serum	LLE + SPE	LC−MS/MS	miR-17, miR-29, miR-92, miR-191, miR-17-A[a]	49−63 nM	19
	human serum	RNA isolation kit	CE−Q−TOF MS	miR-21, miR-16, miR-223, miR-150	5 nM (LOD[b])	24
	human serum	online SPE	CE−MS	miR-21, let-7g	25 nM	42
	rat whole blood	Trizol extraction	LC−Q−orbitrap MS	mir-150, miR-146a, miR-21, miR-155, miR-223, miR-181a, miR-125a	6.25 nM	this work

[a]miR-17-A is a modified oligonucleotide of miR-17 with phosphorothioate backbone. [b]LOD: Limit of detection.

below 5 ppm, no interference was observed around the peaks of target analytes in the blank sample, and they could be analyzed within 5 min (Figure 3). The correlation coefficients of linear calibration curves with $1/x$ weighting exceeded 0.99 for all miRNAs. When each calibration standard was backcalculated on the curves, the calculated values fell within 20% of the nominal concentration at the LOQ and 15% of the nominal concentrations at other levels. The LOQ was established as the lowest point of the calibration curve, consistently at 6.25 nM for all analytes. Accuracy results for the within-run and between-run assays were 87.4−108.7 and 90.8−106.9%, respectively. The precision results were determined as relative standard deviation (RSD) values of less than 13.9 and 11.0% for within-run and between-run assays, respectively. The recovery for the seven miRNAs was 82.2−92.8%, and their respective RSD values were below 14.0%. The matrix effect was 96.3−104.3%, indicating that the biological matrix minimally impacted the target analytes, with an RSD not exceeding 11.4%. There was no carry-over observed for any miRNAs. The stability results were as follows: The short-term stability over 45 h was 93.2−104.0% at room temperature (RT), 85.3−106.9% at 4 °C, and 88.8−104.8% at −20 °C. The long-term stability, evaluated over 30 days at −80 °C, showed a stability range of 87.4−108.5%.

Application to In Vivo Study. The developed method was utilized to analyze blood samples collected from eight lipopolysaccharide (LPS)-treated rats and three control rats with the results summarized in Table 4. LPS, a well-known immunostimulatory component produced by Gram-negative bacteria, was introduced to induce immune response in rats. It could potentially impact miRNAs expression, and the blood concentration of the target miRNAs associated with immune and inflammatory responses was measured over time.[41] miRNAs were detected in seven samples: miR-21 in seven samples (8.27−18.89 nM); miR-125a in two samples (10.32 and 14.20 nM); and miR-150, 155, and 223 in one sample (8.51, 13.35, and 10.57 nM, respectively). These results demonstrated that the established method can quantify the target miRNAs in rat whole blood. Based on the limited data from the detection of miR-21 and miR-125a, it appears that a higher concentration of miRNAs could be detected in the blood of LPS-treated rats at 6 h post-treatment than at 24 h. The miRNA detection did not significantly vary between rats administered with 5 and 10 mg/kg. However, caution is advised when generalizing these findings to all populations given that no detectable miRNAs were found in four subjects. Despite the detection of miRNAs in some cases, the variety identified was limited. This implies a need for a lower LOQ to

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perform a more exhaustive analysis of miRNA in rat whole blood.

Comparison with Other Methods. The key features of this study are summarized and compared with those of previous reports in Table 5. All of these studies employed MS-based analysis to determine miRNA from in vivo matrices. Unlike other studies that utilized extraction methods, including LLE, SPE, and RNA isolation kit for direct miRNA detection, our approach uniquely employed Trizol extraction.[19,20,42] While indirect methods have focused only on the analysis of miRNAs with specific sequences using capture probes, our method could simultaneously determine multiple miRNAs regardless of their sequences. Furthermore, the sensitivity of our method matched or surpassed those of other direct methods. LOQs can vary across different matrices, and whole blood is relatively complicated as a biological sample compared to serum. It is noteworthy that the analytical sensitivity remained comparable to that of other direct methods, even when applied to such a complex matrix. Despite having lower analytical sensitivity, direct methods, including ours, offer simplified sample preparation and the capability to concurrently detect multiple miRNAs, maintaining their appeal despite limitations.

CONCLUSIONS

An LC−MS-based analytical method was developed to directly quantify seven miRNAs (miR-150, 146a, 21, 155, 223, 181a, and 125a) in rat whole blood. To address miRNA degradation in blood samples, we compared two sample preparation methods, Trizol- and proteinase K-based extraction, with the aim of efficient miRNA extraction. The Trizol extraction outperformed the proteinase K method in analytical sensitivity and efficiency. This marks the first application of the Trizol protocol for LC−MS quantification. IP-RP-LC was utilized to effectively separate highly polar miRNAs before MS analysis. The concentrations of DMBA and HFIP were optimized to enhance the sensitivity to ESI-MS detection. Full scan high-resolution accurate mass detection with an orbitrap mass analyzer enabled target miRNA identification within a linear range of 6.25−500 nM and an LOQ of 6.25 nM. The developed method was validated for selectivity, LOQ, linearity, accuracy, precision, recovery, matrix effect, carry-over, and stability. Subsequently, we applied the proposed method to monitor samples from 11 rats and successfully detected target miRNAs in blood samples from seven LPS-treated rats. Recently, miRNAs have emerged as valuable biomarkers for various diseases. Exploring miRNA-based gene expression regulation may unveil novel therapeutic approaches. The present study holds promise for accurately and precisely quantifying diverse miRNAs, extending the potential applications of the developed method in related research.

MATERIALS AND METHODS

Chemicals and Reagents. miRNAs, synthesized with 5′ phosphate groups and purified using high-performance liquid chromatography (HPLC), were procured from Bioneer (Daejeon, Republic of Korea), and their sequences and chemical properties are given in Table 1. Trizol reagent and RNase-free water for sample preparation were obtained from Invitrogen (Waltham, MA). The chloroform/isoamyl alcohol (24:1, v/v) mixture, absolute ethanol, *E. coli* O111:B4 lipopolysaccharide (LPS), and RNaseZAP were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade isopropanol and methanol were obtained from Burdick & Jackson (Muskegon, MI). HFIP was purchased from Thermo Fisher Scientific (Fair Lawn, NJ), and DMBA was obtained from TCI (Tokyo, Japan). Normal saline was purchased from Dae Han Pharm (Seoul, Republic of Korea). Deionized water for the mobile phase was obtained using a Millipore Elix and Milli-Q Biocel system (Millipore, Milford, MA). All other chemicals were of analytical grade.

Preparation of Standards and Other Solutions. Stock solutions for each of the seven miRNAs were prepared at a concentration of 100 μM in RNase-free water. The working solutions were prepared by serially diluting and mixing each stock solution with RNase-free water. All standard solutions were stored at −20 °C until use. Calibration standard and quality control (QC) samples were prepared by adding 800 μL of Trizol reagent to 180 μL of a blank blood sample, followed by mixing. Subsequently, 20 μL of the working solution was spiked into the mixture. All blank samples used for method development and validation were treated with Trizol reagent and stored at −80 °C until use.

Sample Collection. Male Sprague−Dawley rats (8 weeks old; weighing 253.4−280.6 g; n = 11) were purchased from Orient Bio (Seongnam, Republic of Korea). All rats were acclimated for 7 days and were kept in groups of four per cage under regulated environmental conditions, including temperature (23 ± 3 °C), humidity (30−70%), illumination (150−230 lx), ventilation (10−20 times/h), and a regular 12 h light/12 h dark cycle. The rats were randomly selected to receive intraperitoneal injections of either the control substance (normal saline) or LPS at doses of 5 and 10 mg/kg (Table 5). After inducing anesthesia with isoflurane, whole blood samples were collected from the abdominal artery at 6 and 24 h postdose and stored in K_2-EDTA tubes (BD Vacutainer, Franklin Lakes, NJ). For each sample, 200 μL of blood was transferred into a microtube containing 800 μL of the Trizol reagent, and the processed samples were stored at −80 °C until use. All sampling equipment was treated with RNaseZAP before sample collection. The animal experiment protocol was approved by the Institutional Animal Care and Use Committee (IACUC) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) in the Korea Institute of Toxicology. All procedures were performed in compliance with the Animal Welfare Act and Guide for the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research (ILAR).

Sample Preparation. 800 μL of Trizol reagent was added to 200 μL of rat whole blood in a microtube, followed by 30 s of agitation. 200 μL of chloroform-isoamyl alcohol mixture (24:1, v/v) was added, and the tube was agitated for 1 min, followed by incubation for 2 min at 4 °C. After centrifugation at 13,000 rpm for 10 min at 4 °C, approximately 550−600 μL of the total supernatant was carefully transferred to a new microtube. 600 μL of cold isopropanol was added, and the tube was inverted 10 times. After incubation for 60 min at −20 °C, the mixture was centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was carefully removed to avoid disturbing the gel-like pellet at the bottom of the tube. Subsequently, 1 mL of cold-75% ethanol was added, and the tube was tapped. After centrifugation at 13,000 rpm for 10 min at 4 °C, the supernatant was again carefully discarded. The RNA pellet was air-dried for 15 min at RT. The dried pellet was then dissolved

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in 50 μL of RNase-free water and incubated for 15 min at 50 °C. 5 μL of the residue was injected into the LC−MS system. All equipment and working surfaces were meticulously cleaned and decontaminated with RNaseZAP before sample preparation.

LC−MS Analysis. LC−MS analysis was performed using a Vanquish ultra high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific) coupled with a Q-Exactive Plus hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific). The LC separation was conducted on an Acquity UPLC Oligonucleotide BEH C18 column (2.1 mm × 50 mm, 1.6 μm particle size, 130 Å pore size) obtained from Waters (Milford, MA), at a column temperature of 50 °C. The mobile phase comprised 15 mM DMBA and 100 mM HFIP in water (solvent A) and methanol (solvent B). The analytes were eluted with the following gradient profiles: initially at 17% B (0−0.2 min), increasing to 21% B (0.2−8 min), then decreasing back to 17% B (8−8.1 min), and reconditioning at 17% B for 4.9 min. The flow rate was 0.3 mL/min, and the injection volume was 5 μL. MS detection was conducted in full scan mode, with the following parameters: negative polarity; resolution at 70,000; automatic gain control (AGC) target at 3×10^6; maximum injection time (IT) of 100 ms; and a scan range of m/z 1500−2500. Ionization was conducted with a heated electrospray ionization-II (HESI-II) source, utilizing high-purity nitrogen as the nebulizing and desolvating gas. The optimized ESI parameters were: Sheath gas flow rate of 45 arbitrary units (AU), aux gas flow rate of 10 AU, sweep gas flow rate of 1 AU, spray voltage of 3.5 kV, capillary temperature of 325 °C, S-lens RF level of 55 AU, and aux gas heater temperature of 350 °C. Data acquisition and processing were performed using Xcalibur (version 4.5.747.0), FreeStyle (version 1.8.63.0), and TraceFinder (version 5.1, build 203) software from Thermo Fisher Scientific.

Method Validation. The developed method was validated for selectivity, linearity, LOQ, precision, accuracy, recovery, matrix effect, carry-over, and stability to ensure quantitative reliability. Since miRNAs are endogenous compounds, procuring a blank matrix can be challenging. Therefore, we utilized blood samples, wherein the target miRNAs were undetected as the blank matrix. Selectivity was assessed by comparing LC−MS chromatograms from three distinct blank samples against a blank sample fortified with the seven miRNAs. For assessing linearity, calibration curves were constructed by plotting peak areas of the target analytes against their corresponding concentrations, employing least-squares regression. Calibration standards were prepared at seven concentrations ranging from 6.25 to 500 nM. LOQ was determined as the concentration at which an S/N ratio was greater than 10. Accuracy and precision (within-run and between-run) were assessed at four concentrations: 6.25, 18.75, 100, and 400 nM, corresponding to LOQ, low, medium, and high QCs, respectively. Accuracy was determined as the percentage of the measured concentration compared to the corresponding theoretical concentration, and precision was expressed as the RSD of the accuracy results. Five and three replicate analyses were performed for the within-run and between-run assays, respectively. The recovery and matrix effect were evaluated using low and high QCs (18.75 and 400 nM, respectively) with three replicates each. The recovery was determined by comparing the peak area of an analyte-spiked blank sample before and after extraction. The matrix effect was quantified as the percentage of the peak area of a blank sample spiked with the analyte after extraction compared to the peak area of the corresponding standard solution. Carry-over was examined by analyzing a blank sample following upper LOQ (ULOQ, 500 nM) analysis. The short-term (45 h; RT, 4 °C, and −20 °C) and long-term stability (30 days; − 80 °C) of the samples was assessed using low and high QCs (18.75 and 400 nM, respectively) with three replicates each.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06045.

LC−MS chromatograms of target miRNAs obtained using TEA and DMBA (Figure S1); effect of mobile phase amine additives on detection sensitivity (Figure S2); LC−MS chromatograms of target miRNAs at different DMBA concentrations (Figure S3); LC−MS chromatograms of target miRNAs at different HFIP concentrations (Figure S4); MS spectrum of miR-150 (Figure S5); MS spectrum of miR-146a (Figure S6); MS spectrum of miR-21 (Figure S7); MS spectrum of miR-155 (Figure S8); MS spectrum of miR-223 (Figure S9); MS spectrum of miR-181a (Figure S10); MS spectrum of miR-125a (Figure S11); accuracy and precision results of target miRNAs (Table S1); recovery results of target miRNAs (Table S2); matrix effect results of target miRNAs (Table S3); stability results of target miRNAs (Table S4); and sample preparation using proteinase K (PDF)

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Author Contributions

All authors contributed to the writing of the manuscript and have approved the final version.

Notes

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