Video Article

Integration of Miniaturized Solid Phase Extraction and LC-MS/MS Detection of 3-Nitrotyrosine in Human Urine for Clinical Applications

Xiaoguang Sunny Li[1], Shu Li[1], Melissa Ahrens[2], Gottfried Kellermann[1,2]

[1]Pharmasan Labs, Inc.

[2]NeuroScience, Inc.

Correspondence to: Xiaoguang Sunny Li at xiaoguang.li@pharmasan.com

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Abstract

Free 3-nitrotyrosine (3-NT) has been extensively used as a possible biomarker for oxidative stress. Increased levels of 3-NT have been reported in a wide variety of pathological conditions. However, existing methods lack the sufficient sensitivity and/or specificity necessary to measure the low endogenous level of 3-NT reliably and are too cumbersome for clinical applications. Hence, analytical improvement is urgently needed to accurately quantify the levels of 3-NT and verify the role of 3-NT in pathological conditions. This protocol presents the development of a novel liquid chromatography tandem mass spectrometry (LC-MS/MS) detection combined with a miniaturized solid phase extraction (SPE) for the rapid and accurate measurement of 3-NT in human urine as a non-invasive biomarker for oxidative stress. SPE using a 96-well plate markedly simplified the process by combining sample cleanup and analyte enrichment without tedious derivatization and evaporation steps, reducing solvent consumption, waste disposal, risk of contamination and overall processing time. The employment of 25 mM ammonium acetate (NH_4OAc) at pH 9 as the SPE elution solution substantially enhanced the selectivity. Mass spectrometry signal response was improved through adjustment of the multiple reaction monitoring (MRM) transitions. Use of 0.01% HCOOH as additive on a pentafluorophenyl (PFP) column (150 mm x 2.1 mm, 3 µm) improved signal response another 2.5-fold and shortened the overall run time to 7 min. A lower limit of quantitation (LLOQ) of 10 pg/mL (0.044 nM) was achieved, representing a significant sensitivity improvement over the reported assays. This simplified, rapid, selective and sensitive method allows two plates of urine samples (n = 192) to be processed in a 24 h time-period. Considering the markedly improved analytical performance, and non-invasive and inexpensive urine sampling, the proposed assay is beneficial for pre-clinical and clinical studies.

Video Link

The video component of this article can be found at https://www.jove.com/video/55778/

Introduction

The effects of oxidative stress on clinical presentation have been thrust into the forefront in recent years[1]. One of the biomarkers being explored is 3-nitrotyrosine (3-NT), an end stable product formed when reactive nitrogen species (RNS) interact with tyrosine, a catecholamine neurotransmitter precursor. While 3-NT may have clinical value as a biomarker for RNS *in vivo,* the substantial changes of the properties and functions of tyrosine may adversely affect corresponding proteins and cellular functions[1,2]. Emerging research has suggested that 3-NT may play an important role in inflammatory conditions[3], neurodegenerative disorders[4,5], cardiovascular disease[6] and diabetes[7] as well as conditions related to oxidative stress. However, these observations are based on results from methodologies lacking in sensitivity and/or selectivity[8,9,10,11]. The enormous 3-NT concentration ranges for the biological samples previously reported in the literature reveal that serious analytical problems are associated with these assays and technical improvement is needed to accurately quantify the levels of 3-NT and verify its role in the pathology of these conditions.

The quantitation of free 3-NT in biological matrices presents a special challenge to man and instrument[8,9,10,11]. First, the trace level of endogenous 3-NT demands an ultra-sensitive detection; second, the existence of numerous structurally similar analogues, especially tyrosine, which is present in vast excess, requires a high degree of selectivity; third, the artefactual formation of 3-NT by tyrosine nitration with ubiquitous nitrate and nitrite requires special consideration during sample preparation to avoid false overestimation of 3-NT.

Among a wide variety of methodologies employed to measure 3-NT, MS/MS has been considered the gold standard method due to its superior sensitivity and selectivity[11,12,13,14]. Gas chromatography (GC) coupled MS/MS offers the best sensitivity, however, the indispensable sample derivatization steps are too tedious and time-consuming to be efficient for clinical utility[15,16]. LC-MS/MS does not require complex sample derivatization, making it the more promising option. Nonetheless, there are several obstacles to overcome such as the sensitivity of LC-MS/MS methods reported in the literature needs to improve for the measurement of low abundant 3-NT[7,17,18] and the relatively long turnaround time must be shortened for high-throughput applications[12,13,17,19].

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Additionally, when considering clinical applications, the biological matrix used plays a significant role. It should be easy and inexpensive to obtain and non-invasive if possible[20,21,22]. Plasma, the traditionally used sample in the literature, is not a clinically desirable matrix, so a methodology utilizing urine which is non-invasive and cost-effective, was sought.

Several attempts to develop reliable and specific LC-MS/MS methodologies have been made using urine[9,10,11]. However, they have all fallen short of being either selective, reliable or efficient enough for clinical use. The effectiveness of the predominant SPE using traditional reversed-phase cartridge (C18 type) as sample cleanup for the 3-NT analysis has been questioned and a sequential SPE of strong cation exchange (SCX) and reversed phase C18-OH has been proposed[6,7,19]. One recently developed LC-MS/MS method utilized a multi-step purification process of manual C18 SPE, preparative high pressure liquid chromatography (HPLC), and online SPE for analysis of 3-NT[23]. Although this method was sensitive enough for clinical purposes, with an LLOQ of 0.041 nM, the cleanup process was intensive and tedious and required 3 mL of urine, limiting its feasibility for high-throughput. A molecularly imprinted polymer was employed as the SPE sorbent to improve the efficiency of the cleanup process[14], but the resulting LLOQ (0.7 µg/mL) was not low enough for clinical specimens. Another method required two-dimensional (2D) LC-MS/MS and immunoaffinity chromatography for sample cleanup in order to achieve a limit of detection (LOD) of 0.022 nM[24]. While all these methods have made advancements in the assessment of 3-NT, none have achieved the sensitivity, reliability, and efficiency necessary for clinical applications.

In order to investigate the pathology of free 3-NT and its role as a biomarker of oxidative stress in clinical settings, we have developed a methodology that is simple, efficient, accurate and precise, enabling for high-throughput clinical applications[25]. A miniaturized mixed-mode cation exchange (MCX) 96-well extraction microplate was implemented to achieve simple and effective sample cleanup and enrichment of 3-NT in a single extraction bypassing the drawbacks seen in the existing methods that require derivatization, evaporation and 2D-LC. Liquid chromatography with 0.01% HCOOH as an additive in mobile phase offered an enhanced signal response with a rapid cycle time. Selectivity was further improved through application of a mild NH_4OAc elution solution for selective elution of 3-NT, and use of MRM transition for both 3-NT and the internal standard (IS). The matrix effect was compensated for by using a reduced amount of a preferred ^{13}C-labeled isotopic IS for quantification. With the advent of this methodology, researchers and clinicians will be able to verify the role of 3-NT in clinical conditions and further explore the impact of oxidative stress.

Protocol

All studies involving human urine samples were conducted adherence to the procedure approved by Pharmasan/Neuroscience Institutional Review Board (IRB).

1. Urine Sample Collection and Creatinine (Cr) Determination

1. Collect 5 mL of the next morning urine samples after ca. 10 h overnight fasting in a 5 mL transport tube A containing 250 µL of 3 N HCl as preservative and store at -20 °C until use.
2. Thaw and vortex 5 mL transport urine A tube and centrifuge in a centrifuge (*e.g.* Sorvall) (2297 x g, 10 min).
3. Aliquot 1 mL of urine twice from the 5 mL transport tube A.
4. Determine Cr by a urinary creatinine method[26].

2. Preparation of Standard, IS and Quality Control (QC) Samples

1. Prepare a stock solution of 3-NT at 100 µg/mL in water with 0.01% HCOOH mobile phase A (MA) and store at -20 °C.
2. Make a 3-NT working standard solution at concentration of 100 ng/mL by diluting the 100 µg/mL 3-NT stock solution with MA.
3. Generate standards ranging from 5 to 2500 pg/mL by dilution of the 100 ng/mL working standard with 0.15 M HCl acidified blank urine free of 3-NT along with blank and double blank samples.
4. Prepare a stock solution of IS $^{13}C_9$-3-NT at 100 µg/mL in water with MA and store at -20 °C.
5. Make an IS working solution at 500 pg/mL by dilution of the 100 µg/mL $^{13}C_9$-3-NT IS stock solution with MA.
6. Establish five levels of QC samples covering the LLOQ, low, medium and high levels (*i.e.*, 10, 25, 100, 500 and 1,250 pg/mL), by dilution of 3-NT working standard in the acidified blank urine.

3. Solid Phase Extraction Procedure

1. Thaw and vortex urine samples, standards and QC samples.
2. Add urine samples, standards and QC samples (250 µL each) to 32 wells of a clean 2 mL 96-well collection plate.
3. Introduce the 500 pg/mL IS working solution (50 µL) to each well except double blank sample well. Add 0.01% HCOOH (50 µL) to the double blank sample well.
4. Add LC-MS/MS water with 0.1% HCOOH (250 µL).
5. Mix the above mixture with an 8-channel pipette three times.
6. Cover the plate until SPE loading.
7. Place an MCX 96-well extraction plate and a collection reservoir on a positive pressure processor.
8. Condition the extraction plate with flowing MeOH (200 µL) through the sorbent.
9. Equilibrate by flowing water with 2% HCOOH (200 µL) through the sorbent.
10. Load the entire volume of each of the pre-mixed samples onto the pre-conditioned extraction plate carefully with an 8-channel pipette.
11. Set low positive pressure (*e.g.*, 3 psi) on the positive pressure processor to allow the mixture to flow through the sorbent slowly, adjust the pressure if needed.
12. Wash the wells by flowing water with 2% HCOOH (200 µL) through the sorbent.
13. Wash the wells by flowing methanol (200 µL) through the sorbent.

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14. Wash the wells by flowing water (200 µL) through the sorbent.
15. Dry the wells completely with high positive pressure setting (*e.g.*, 40 psi) on the positive pressure processor.
16. Replace the reservoir with a clean 2 mL 96-well collection plate.
17. Apply 25 mM NH_4OAc at pH 9 (50 µL) to elute the retained analyte and IS from the extraction plate.
18. Pipette LC-MS water with 5% HCOOH (50 µL) to neutralize the eluate.
19. Mix with the 8-channel pipette three times and submit to LC-MS/MS station for analysis.

4. LC-MS/MS Analysis

1. Accurately measure 2,000 mL of LC-MS water with a graduated cylinder and transfer it into a 2 L bottle.
2. Pipette 200 µL pure HCOOH into the above bottle containing LC-MS water.
3. Mix thoroughly and label as mobile phase A (MA). Include initials, preparation date and expiration date.
4. Take a 2 L bottle of LC-MS methanol and label as mobile phase B (MB) with starting date and expiration date.
5. Set temperature of auto-sampler to 4 °C.
6. Place the collection plate with prepared samples into the autosampler.
7. Place a PFP column (150 mm x 2.1 mm i.d., 3 µm) and guard column in the oven.
8. Set the oven temperature to 30 °C.
9. Equilibrate 10 min using the acquisition method with the LC gradient elution as shown in **Table 1**.

Time (min)	Module	Events	Parameter
0	Pumps	Pump B Conc.	5
0.5	Pumps	Pump B Conc.	20
1	Pumps	Pump B Conc.	50
3	Pumps	Pump B Conc.	80
4	Pumps	Pump B Conc.	90
4.01	Pumps	Pump B Conc.	95
5.5	Pumps	Pump B Conc.	95
5.6	Pumps	Pump B Conc.	5
7	Controller	Stop	

Table 1: Liquid Chromatography Gradient Elution Conditions

10. Create a batch list including standards, QC and urine samples.
11. Start the batch by injection of the prepared samples (12 µL).

5. Peak Identification, Integration and Data Process

1. Control data acquisition and processing using the software.
2. Identify and integrate 3-NT and IS peaks for all the samples.
3. Establish a standard curve with the range of 10-2,500 pg/mL for 3-NT quantitation by linear regression of the peak area ratio of 3-NT and IS versus the nominal 3-NT concentration with a weighting factor of 1/x.
4. Quantify all the samples using the standard curve.
5. Determine if the QC samples fall in the established range.
6. Convert the detected concentrations of urine samples to final results in the unit of nM or nmol/mmol Cr.

Representative Results

Figure 1 illustrates that 3-NT is completely chromatographically separated from other structurally similar tyrosine analogues under the optimized LC condition, which eliminates the co-eluting interferences due to these vastly excessive compounds and consequently enhances the degree of assay selectivity. In addition, the gradient elution with 0.01% HCOOH as additive in MA and methanol at a flow rate of 0.45 mL/min allows rapid elution of 3-NT (*i.e.*, 3 min with a turnaround time of 7 min).

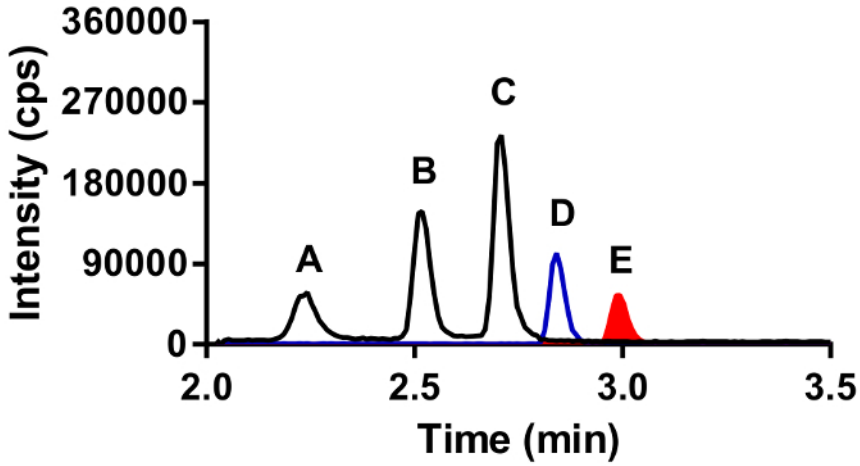

Figure 1: Baseline LC Chromatographic Separation of 3-NT from Other Tyrosine Analogues in a Standard Solution. (**A**) *p*-Tyrosine; (**B**) *m*-Tyrosine; (**C**) *o*-Tyrosine; (**D**) Cl-Tyrosine; (**E**) 3-NT. Please click here to view a larger version of this figure.

Figure 2 shows that no 3-NT signal is observed in the double blank sample, indicating no formation of artefactual 3-NT during the entire process. **Figure 3** illustrates representative MRM chromatograms of 3-NT and IS for a healthy individual. As can be seen, no interfering signals are observed at the retention times of 3-NT and IS. Furthermore, less than ± 6% difference in 3-NT between the non-spiked and spiked pooled urine samples with nitrite (50 µM), nitrate (50 µM) and tyrosine (50 mg/L)[25] was observed, which further lends support to the specificity of the assay.

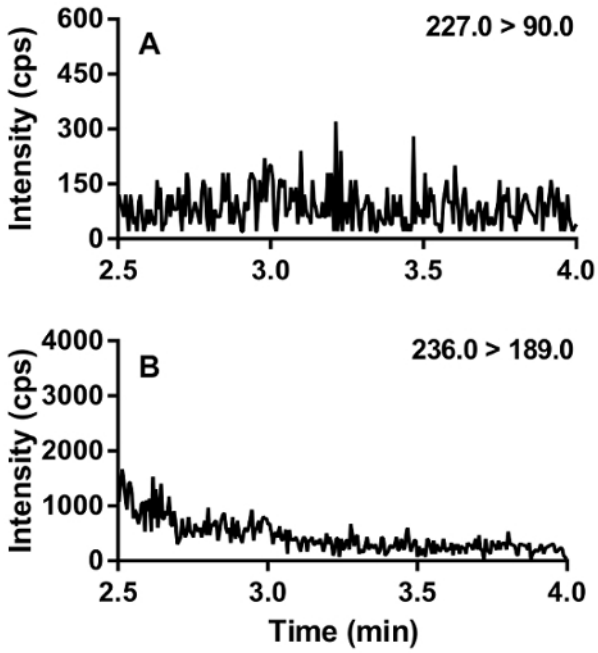

Figure 2: MRM chromatograms of 3-NT and IS in a Representative Double Blank Sample. (**A**) 3-NT MRM quantifier 227.0 >90.0; (**B**) IS MRM quantifier 236.0 >189.0. Please click here to view a larger version of this figure.

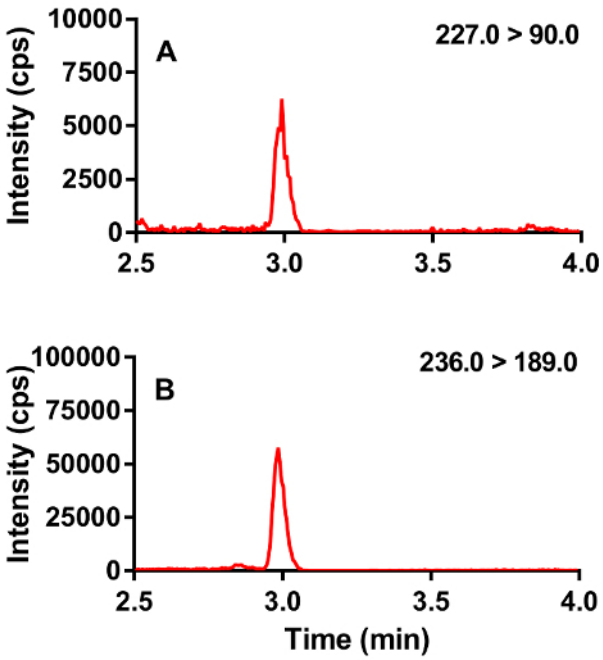

Figure 3: MRM Chromatograms of 3-NT and IS in a Representative Urine Sample of Healthy People. (**A**) 3-NT MRM quantifier 227.0 >90.0; (**B**) IS MRM quantifier 236.0 >189.0. Please click here to view a larger version of this figure.

The standard curve was established by extraction of acidified blank urine spiked with 3-NT in the range of 10-2,500 pg/mL by plotting the peak area ratio of 3-NT and IS versus the nominal concentration of 3-NT with a linear fitting of 1/x weighting. A representative standard curve is demonstrated in **Figure 4**. The LOD, defined as the lowest concentration with a signal-to-noise ratio greater than three, was determined to be 2 pg/mL (0.0088 nM). The LLOQ was determined to be 10 pg/mL (0.044 nM) by definition as the lowest concentration to be measured within ± 20% of imprecision and accuracy with the signal-to-noise ratio greater than ten.

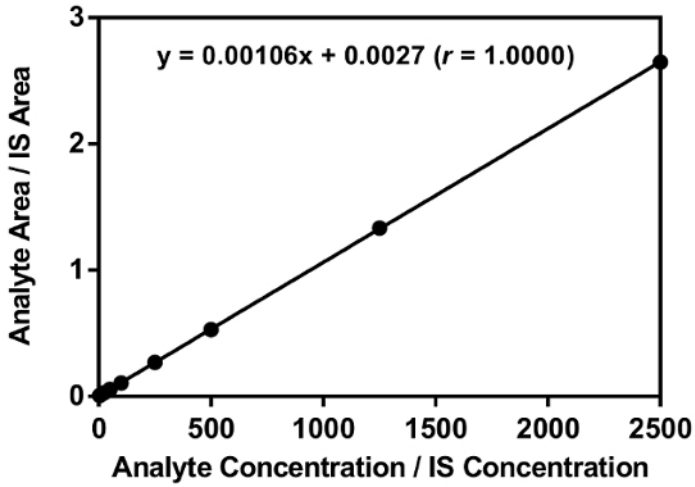

Figure 4: A Representative 3-NT Standard Curve in the Range of 10-2,500 pg/mL. Please click here to view a larger version of this figure.

Discussion

Substantial variations in concentrations previously reported in the literature for the endogenous free 3-NT in human urine samples reveal methodological problems associated with available assays[8,9,10,11]. Accurate determination of the low basal level of 3-NT in human urine remains a challenging task that requires special precautions for sample preparation and LC-MS/MS analysis. This protocol outlines a novel SPE procedure combined with a selective LC-MS/MS detection that allows the specific and sensitive determination of urinary 3-NT with high throughput.

Careful selection of MRM parameters improved selectivity for the detection of 3-NT. The MRM transition m/z 236.0 >96.0 of IS for 3-NT in plasma[27] caused severe contamination for urine samples. A cleaner, 9-fold increase in signal response was achieved with MRM transition m/z

236.0 >189.0. MRM transition m/z 227.0 >90.0 was selected for 3-NT quantification due to severe interference using the most intensive transition m/z 227.0 >181.0. The detailed MRM transitions and compound parameters are summarized in **Table 2**.

Analyte	MRM transition (m/z)	DP (V)	EP (V)	CE (eV)	CXP (V)
3-NT (quantifier)	227.0 > 90.0	50	10	38	13
3-NT (qualifier)	227.0 > 103.9	50	10	45	16
$^{13}C_9$-NT (IS)	236.0 > 189.0	50	10	21	14
[a] Ion spray voltage: 2200 V; Temperature: 600 °C; Curtain gas: 35; CAD gas: 9; Nebulizer gas (GS1): 50; Heater gas (GS2): 55.					

Table 2: Optimized MRM Conditions.

The traditional C18-type column commonly used for LC chromatographic separation of 3-NT in the literature[12,13,17,19] typically required a long turnaround time for reducing interference, making it non-conducive to high throughput. In this protocol, a PFP column was employed for optimization of LC chromatographic separation of 3-NT based on its enhanced retention of polar compounds[22,27,28]. The concentration of mobile phase additives has an important influence on the signal intensity[25,28]. HCOOH at 0.01% was found to be the optimal additive in MA as it resulted in a 2.5-fold signal gain over 0.1% concentration, and further decreases in concentration reduced signal response. A gradient elution profile using 0.01% HCOOH in water (MA) and methanol (MB) was established with a flow rate of 0.45 mL/min, achieving an optimal separation and fast elution of 3-NT within 3 min with a total run time of only 7 min, allowing significantly faster analysis of 3-NT in complicated biological matrices than reported in the literature[12,13,17,19].

To address the ineffectiveness of the traditional reversed-phase cartridge (C18 type) predominantly used for SPE of biological samples[6,7], we recently developed an LC-MS/MS method to determine 3-NT in human plasma by SPE on a single dual-functional 96-well MCX plate[27], which resulted in improvements in SPE efficiency and selectivity. However, a distinct drawback of all the SPE approaches in the literature is laborious and risk-associated evaporation and reconstitution steps. Additionally, the plasma method required an extra pre-washing of the extraction plate to eliminate contamination from the sorbent. In this protocol, these drawbacks have been eliminated by a tailored SPE with the use of a miniaturized 96-well microplate. The selection of elution solution was found to be crucial for the extraction efficiency. Substantial interferences were observed in urine samples by applying the common NH_4OH elution solution with varied composition of MeOH. It was hypothesized that interfering substances were co-eluted with the analyte due to the strong elution power of the methanolic NH_4OH elution solution, consequently resulting in the low selectivity. To improve the selectivity, it was necessary to identify a solution that would be both strong enough to elute 3-NT and weak enough not to cause the elution of interfering compounds. After detailed investigation of less basic NH_4OAc at different pH and concentrations, a 25 mM NH_4OAc solution with pH 9 was found to be optimal as elution solution. With the optimized elution solution, the issue of interfering compounds was resolved and a 40% gain in sensitivity was achieved compared to the regular methanolic NH_4OH elution solution.

Table 3 provides a detailed summary of the analytical performance of this protocol[25] compared with other available methods for the determination of the free 3-NT in biological matrices. This protocol offers several distinct advantages over previously reported assays. First, by employing the 96-well extraction microplate, a single step for sample cleanup and analyte enrichment was achieved, avoiding the 1 - 5 cycles of evaporation and reconstitution typically required in the 3-NT methods involving SPE. Second, the solvent usage per sample for SPE was drastically reduced, from 5.5 - 118 mL to only 1.1 mL, representing a 5–107 times reduction in solvent and waste disposal. Third, the LC turnaround time per sample was decreased 2-7 fold compared with other assays and was 30% faster than our previous plasma method. Fourth, a 10-3,000 fold lower amount of the preferable ^{13}C-labeled IS was required for compensation of matrix effect. Lastly, this assay represents a significant sensitivity improvement over other conventional SPE-based LC-MS/MS methods for the quantitation of urinary 3-NT.

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Sample preparation	Analytical method	Matrix	LOD (nM)	LOQ (nM)	LC run (min)	Evap[c]	Sol[d] (mL)	IS (ng)		Ref.
SPE(C18) +filtration	LC-MS/ MS	Plasma	0.034	0.112	20	1	13	Analog[f]	2	[6]
SPE (MCX plate)	LC-MS/ MS	Plasma	0.0088	0.022	10	1	5.6	$^{13}C_9$-NT	0.25	[27]
PPT+SPE (MCX)+der[a]	HPLC-UV	Serum	NA[b]	100	40	1	7.3	NA[b]	NA[b]	[12]
HPLC+der[a]	GC-MS/ MS	Urine	0.004	0.125	NA[b]	5	NA[b]	d_3-NT	4.6	[16]
PPT+SPE (amino)+der[a]	LC-MS/ MS	Cat urine	NA[b]	14.5	40	3	23	d_3-NT	75	[17]
PPT+hydrolysis+SPE (SCX-C18)	LC-MS/ MS	Urine (protein)	400	NA	50	2	118	d_3-NT	2	[19]
IA-2D LC[e]	LC-MS/ MS	Urine	0.022	NA	14	NA[b]	NA[b]	$^{13}C_9$-NT	0.85	[24]
SPE (C18)+hydrolysis	HPLC-ECD	Urine (total)	NA[b]	4	40	2	5.5	NA[b]	NA[b]	[13]
SPE (C18)+Prep LC[g] +online SPE	LC-MS/ MS	Urine	0.0088	0.041	30	2	38	d_3-NT	5	[23]
SPE (MIP)	HPLC-UV	Spiked Urine	700	NA	20	1	18	NA[b]	NA[b]	[14]
SPE (MCX µElution)	**LC-MS/ MS**	**Urine**	**0.0088**	**0.044**	**7**	**NO**	**1.1**	**$^{13}C_9$-NT**	**0.03**	**This work**

[a]der: derivatization; [b]NA: not available; [c]Evap: Evaporation; [d]Sol: Solvent per sample; [e] IA-2D LC: immunoaffinity chromatography and two-dimensional LC; [f]Analog: o-Methyl-Tyrosine; [g]Prep LC: preparative HPLC purification.

Table 3: Comparison of the Analytical Performance of this Protocol with Existing Assays for the Detection of 3-NT in Biological Matrices

The analytical and clinical validity of the proposed assay was further assessed through the determination of the reference interval for urinary 3-NT established from the authentic urine samples of 82 healthy people[25]. The improved simplicity and throughput method allows two plates of urine samples (n = 192) to be processed and analyzed in a 24 h time period. The developed method utilizing the non-invasive urine sampling, being simple, rapid, sensitive and selective, is expected to be a powerful tool in verifying the role of 3-NT in clinical conditions and further exploring the impact of oxidative stress. The critical steps of the protocol include SPE on an MCX microplate using a mild NH4OAc as elution buffer, LC separation on a PFP column with 0.01% HCOOH as an additive and MRM selection for 3-NT quantification. Future application of the method is for quantifying 3-NT concentrations in patients with pathological conditions such as inflammatory and neurodegenerative disorders, *etc.* The potential pitfalls of the proposed protocol for clinical applications remain to be addressed.

Disclosures

The authors declare that they have no conflict of interest.

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