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Simultaneous determination of *3-nitro* tyrosine, *o*-, *m*-, and *p*-tyrosine in urine samples by liquid chromatography–ultraviolet absorbance detection with pre-column cloud point extraction

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Abstract

Stable 3-nitro tyrosine (3-NO_2-Tyr), *o*-, *m*-, and *p*-tyrosine isomers induced by oxidation of tyrosine residues in protein were considered important biomarkers for the existence of toxic oxidizing agents peroxynitrite ($ONOO^-$) and $OH^•$, which could lead to such diseases as acute lung injury, neurodegenerative disorders, atherosclerosis, cancers and many other diseases. Therefore, development of an accurate, simple and sensitive method to simultaneously detect *o*-, *m*-, and *p*-tyrosine and 3-NO_2-Tyr is necessary. Fluorescence detection is highly sensitive to *o*-, *m*-, and *p*-tyrosine, but it cannot be used to detect 3-NO_2-Tyr, due to the strong fluorescence-quenching characteristic of the NO_2 group. In this study, we developed a highly sensitive reversed HPLC–UV method, combined with pre-column cloud point extraction (CPE), to simultaneously determine *o*-, *m*-, and *p*-tyrosine and 3-NO_2-Tyr. The procedure included derivatization of a sample with 6-aminoquinolyl-*N*-hydroxy-succinimidyl carbomate (AccQ) at 0.20 mol/l borate buffer (pH 8.80) for 30 min at 70 °C, and pre-concentration with surfactant cloud point extraction. The surfactant-rich phase was then diluted with deionized water and injected directly into the to HPLC column for analysis. A C_{18} column (3.9 mm i.d. × 300 mm) was used for gradient elution separation at 25 °C and the detection wavelength was at 254 nm. Nineteen general amino acids showed no interference. The detection limits of *p*-, *o*-, *m*-Tyr and 3-NO_2-Tyr were between 5 and 15 nmol/l. The linear range was from 0.05 to ∼100 μmol/l.

Keywords: Tyrosine; 3-Nitrotyrosine

1. Introduction

Nitric oxide (NO or $NO^•$) is a ubiquitous gaseous free radical which is important in regulating numerous biological processes [1–4]. A NO group can react with superoxide anions to yield toxic oxidizing agents called reactive nitrogen species (RNS). For example, peroxynitrite ($ONOO^-$) [5–7] reacts with tyrosine residues in proteins to form 3-nitro-tyrosine (3-NO_2-Tyr) [8], which results in such diseases as acute lung injury, neurodegeneration, atherosclerosis and some cancers. Since peroxynitrite has a very short half-life at physiological pH, the stable 3-nitro-tyrosine (3-NO_2-Tyr), induced by oxidation of tyrosine residues in protein, was considered an important biomarker of RNS production in various tissues. In addition, the levels of *ortho*- and *meta*-tyrosine have been reported to be endogenous biomarkers of oxidative damage by oxygen radicals $OH^•$ [9–11]. Therefore, the development of an accurate, simple and sensitive method to simultaneously detect *ortho*-, *meta*-, *para*-tyrosine (*o*-, *m*- and *p*-Tyr) and 3-NO_2-Tyr is necessary. In the past, several analytical methods were developed to measure them, including GC [8,12,13], GC–MS [14–20], HPLC with electrochemical detection (HPLC–ECD) [14–21], HPLC–UV [8,22], and HPLC with fluorescence detection [23–25]. However, these methods cannot be used to simultaneously determine all of these biomarkers. GC–MS and HPLC–ECD are the most sensitive methods for assessing these compounds. However, high cost is associated with GC–MS because tedious sample pretreatment is required, and electrode poisoning is often encountered in ECD caused by other biological compounds in the sample which may result in loss of detection sensitivity. Even though a fluorescence detector can be used to detect the other three tyrosine isomers, it cannot be directly used to detect of 3-NO_2-Tyr

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due to the strong fluorescence-quenching characteristic of the nitro-group. UV detection is the commonly used technique for HPLC and is sufficiently sensitive to determine 3-NO_2-Tyr in vitro; generally, however, it is not sensitive enough to use routine quantitative analysis of 3-NO_2-Tyr in vivo [26].

This paper, for the first time, presents a highly sensitive reversed HPLC–UV method by combining pre-column cloud point extraction (CPE) with pre-concentration to simultaneously determine *o*-, *m*-, and *p*-tyrosine, and 3-NO_2-Tyr. CPE, which has been reported by several authors [27–31], is briefly defined as surfactants which exhibit turbidity when a concentration of salt is added to the solution. As the turbid solution is centrifuged, two phases are formed, and hydrophobic molecules from the original aqueous solution can be extracted into the surfactant-rich phase. In our study, samples were derivatized with 6-aminoquinolyl-*N*-hydroxy-succinimidyl carbomate (AccQ) in a borate buffer (0.20 mol/l, pH 8.80) for 30 min at 70 °C, and pre-concentrated with a surfactant, TX-114. The surfactant-rich phase was then diluted with a small volume of deionized water and injected directly into the HPLC column for analysis. Separation was performed in an id 3.9 mm × 300 mm, 5 μm, C_{18} column at 254, nm with a gradient elution of two mobile phases: acetonitrile and 0.15 mol/l sodium acetate solution (pH 6.35). The detection sensitivity had improved about 10-fold when compared with the HPLC–UV methods without pre-column cloud point extraction. This also makes the detection sensitivity of *o*-, *m*-, and *p*-tyrosine comparable with that of the fluorescence detection method.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were of analytical reagent grade (unless stated otherwise) and were used without further purification. The following chemicals were purchased from Sigma (St. Louis, MO, USA): DL-*ortho*-tyrosine, DL-*meta*-tyrosine, DL-*para*-tyrosine, L-*3-nitro* tyrosine and 19 amino acid (AA) standards, DL-alanine (Ala), DL-arginine HCl (Arg), DL-asparagine (Asn), DL-cysteine (Cys), DL-glutamic acid (Glu), DL-glutamine (Gln), DL-glycine (Gly), DL-histidine HCl (His), DL-isoleucine (Ile), DL-leucine (Leu), DL-lysine HCl (Lys), DL-methionine (Met), DL-asparatate (Asp), DL-phenylalanine (Phe), DL-proline (Pro), DL-serine (Ser), DL-threonine (Thr), DL-trptophan (Trp), DL-valine (Val), ammonium sulfate, sodium acetate, TX-114, polyvinylpyrrolidone (PVP), poly (ethylene oxide) (PEO, MV = 600,000 and 8,000,000), hydroxyproyl-β-cyclodextrin, methoxypolyethylene glycol 2000, polyoxyethylene sorbitan mono-oleate (Tween 80), polyethylene glycol 8000, polyoxyethylene 23-lauryl ether (Brij35), poly (vinyl alcohol), poly(ethylene glycol), TX-100, TX-114, hydroxypropylmethylcellulose (HPMC, centipoises: 5, 50, 100, 4000, 5000, 80,000, 90,000, 100,000, 150,000, 370,000), polyethylene glycol mono-4-nonylphenyl ether (PONPE 7.5) and sodium dodecyl sulfate (SDS).

A 6-Aminoquinolyl-hydroxysuccinimidyl cabomate reagent kit (named Waters AccQ• Fluor Reagent Kit), including 0.2 mol/l borate buffer pH 8.80, was purchased from Waters Corporation (Milford, MA, USA). Triethylamine (TEA), 0.1% (w/v) phenol, acetic acid and acetonitrile and HCl were purchased from Fisher Scientific (Pittsburgh, PA, USA). Dimethyloctadecylchlorosilane was from Fluka Chemika.

A 1.0 mmol/l stock solution of each AA standard was prepared with deionized water and stored at 4 °C before use. The working solution was a 1:100 dilution of the stock solution with deionized water. A 10% TX-114 solution (w/v) was prepared with deionized water. A 10.0 mmol/l of AccQ solution was prepared with acetonitrile by following the kit instruction sheet, and a 1.0 mmol/l working solution of AccQ was prepared by dilution with acetonitrile. A 0.15 mol/l acetate buffer of the HPLC mobile phase A was prepared by dissolving 11.50 g of sodium acetate in 1 l deionized water, to which 0.33 g TEA had been added. This solution's pH was adjusted to 6.35 with acetic acid. Mobile phase B was acetonitrile only. 3.0 mol/l ammonium sulfate was prepared with deionized water and used as the reagent to make TX-114 form micelle in the cloud point extraction pre-concentration.

2.2. Apparatus

Separation was carried out at 25 °C using a Model 1090, Hewlett-Packard Series II HPLC, with a UV detection of 254 nm wavelength and equipped with an id 3.9 mm × 300 mm, 5 μm, Spherical C_{18} column (Waters, Milford, MA, USA). It was operated at a flow rate of 1.0 ml/min with a gradient program of double mobile phase A (acetate buffer) and B (acetonitrile). The following gradient was used for the optimum resolution of *o*-, *m*-, *p*-, 3-nitro-tyrosine and other amino acids: 16% B isocratic (for 15 min) followed by a linear increase to 20% B (over 10 min) and then by another linear increase to 60% B (over 5 min). A 60% mobile phase B, continued to increase to 100% B over the next 5 min. The column was equilibrated under the initial conditions for 15 min prior to the next injection. Data were collected and processed by a HP chemstation, version 8.03, and centrifugation was done by an Eppendorf Centrifuge 5403 (Hamburg, Germany). A Speed-Vac concentrator system (SVC 100H, Savant Instruments Inc., USA), was used for evaporation which derivatization was done in an Imperial V laboratory oven (Terra Universal Inc., USA).

2.3. Preparation of urine samples

Procedures for preparing urine samples to determine tyrosine isomers and 3-nitro-tyrosine were similar to those of published methods, with some modifications [32,33]. Urine

was collected from healthy human volunteers over a 24 h period and kept at 4 °C before analysis. No one was taking any medicine and all were eating a vegetarian diet, with smoked food excluded. Ten milliliter acetonitrile were added to 10 ml of the urine sample (with and/or without tyrosine isomers and 3-nitro-tyrosine standards) and the solutions were immediately placed in an ice bath for 30 min. Precipitate was obtained after centrifugation at 3800 × *g*/4 °C for 10 min. The supernatant was separated from the precipitate and kept for later use. The precipitate was placed in a 15 ml ampoule tube, purged with argon gas, sealed, and hydrolyzed for 24 h in a 110 °C oven by adding 1.0 ml 6.0 mol/l HCl containing 1.0% phenol. After acid hydrolysis was completed, the aliquot of hydrolysis solution was mixed with the aliquot of supernatant obtained from the centrifugation procedure, described above, and evaporated at 80 °C. by the concentrator system until dry. The resulting sample was dissolved with 1.0 ml deionized water and the total contents of the tyrosine isomers and 3-nitro-tyrosine were determined.

2.4. Derivatization procedures

One milliliter of the analyte solution was put into a 1.5 ml centrifuge tube, followed by the addition of 70 μl of 0.2 mol/l borate buffer (pH 8.80) and 40 μl of 1.0 mmol/l AccQ reagent. The derivatization vial was capped with a silicone-lined septum, and the contents was mixed immediately for several seconds, with the aid of a vortex mixer. It was then heated in an oven for 30 min at 70 °C. The derivatization mechanism of AccQ with tyrosine isomers and 3-NO_2-Tyr was similar to those of amines, as shown in Fig. 1 [34].

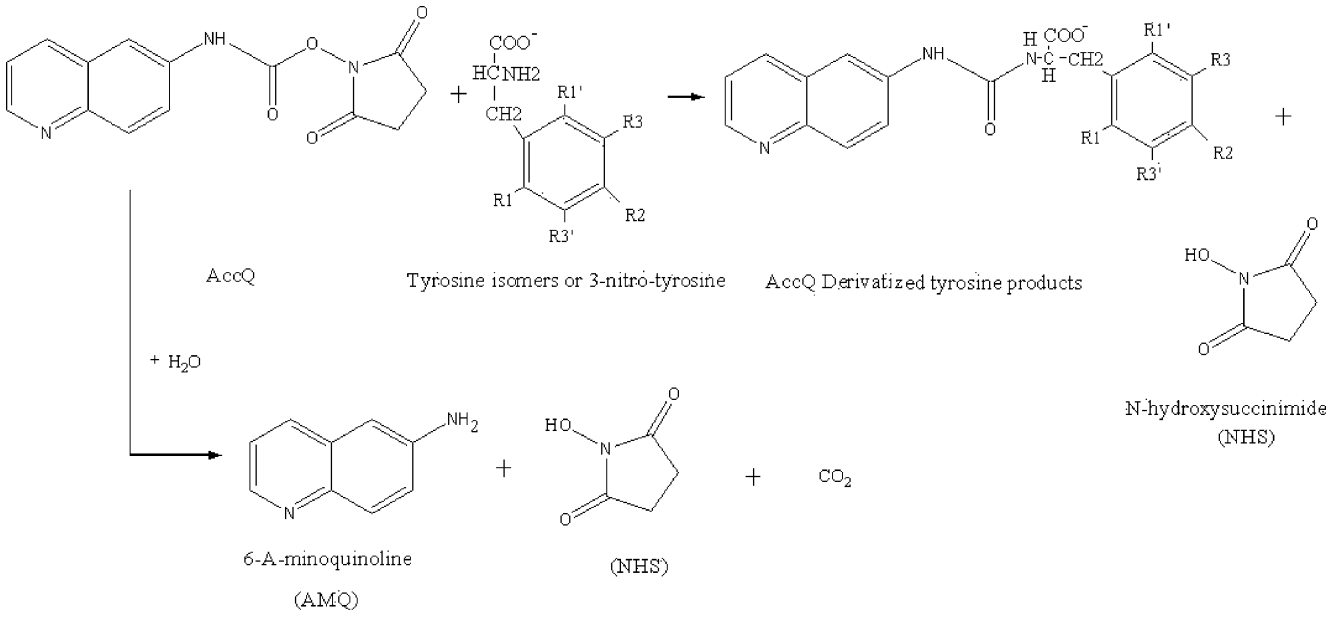
2.5. Pre-concentration of derived products with TX-114

Two hundred microliter of 10% TX-114 (w/v) and 300 μl of 3.0 mol/l of ammonium sulfate were added to the above derivatization tube containing the derived products. After the cloud point formed, it was centrifuged at 6000 rpm at 20 °C for 10 min. The supernatant aqueous phase was discarded. One hundred microliter of water was added to 100 μl of the surfactant-rich phase to dissolve it. This brought the final sample volume to 200 μl. The solution was mixed with a vortex for 2 min and then 20 μl of the solution was injected directly into the HPLC column for analysis.

3. Results and discussion

3.1. Optimization of derivatization condition

AccQ is widely used in the determination of amino acids and primary and secondary amines because of its high sensitivity (with a detection limit obtainable at the fmol level) and rapid reaction speed. This reaction speed between the amino groups of AAs and AccQ, is generally faster than that of other AA derivatization reagents because of its it $t_{1/2}$ value ($t_{1/2} \ll 1$ s), such as quinine sulfate [35,36],



(1) p-Tyr: R2=OH, R1 (or R1′), R3 (or R3′) = H
(2) m-Tyr: R3 (or R3′) = OH, R1 (or R1′), R2 = H
(3) o-Tyr: R1 (orR1′) = OH, R2, R3 (or R3′) = H
(4) 3-NO2-Tyr: R3 (or R3′) = NO2, R1 (or R1′ , R2) = OH

Fig. 1. Scheme of derivatization of AccQ with amino acids or with primary and secondary amines.

1-methoxycarbo-nylindolizine-3,5-dicarbaldehyde (IDA) [37,38], naphthalene-2,3-dicarboxaldehyde (NDA) [39,40], *o*-phthalaldehyde (OPA) [41–43], and fluoresceinisothiocyanate isomer I (FITC) [44]. Of course, the derivatization efficiency of AccQ was affected by many factors, including the pH of the deriving system, temperature, concentration of AccQ and reaction time. In our experiment, the derivatization was carried out according to the procedure prescribed by the Waters AccQ-Fluor Reagent Kit, with a slight modification. The optimal pH value of derivatization was found to be 8.80, and a significant decrease in the signal response was observed at a pH lower than 7.00. A concentration of 1.0 mmol/l of AccQ was used instead of the suggested 10 mmol/l because a higher concentration of AccQ (10 mmol/l) could cause a higher NHS peak background. To get the highest signal response, the derivatization temperature and time were optimized at 70 °C and 30 min, respectively.

3.2. Comparison of preconcentration efficiency of different surfactants and polymers

A polymer facilitated liquid–liquid extraction, in which two liquid phases are formed at room temperature in the presence of a suitable salt, is called an aqueous biphasic system (ABS) [45–49]. When a surfactant is used to replace a polymer, it is called a cloud point extraction [50–53], since the aqueous solution of the surfactant micellar system becomes turbid, either when a suitable concentration of salt is added to the solution at room temperature or within a narrow high temperature range (referred to as their cloud point temperature). Two separate phases can be obtained after the solution is centrifuged, and the hydrophobic components are concentrated into a polymer-rich, or surfactant-rich, phase. The extraction efficiency can be quantitatively evaluated by fraction extracted (*E*):

$$E = \frac{Q_A}{Q_{A'}}$$

where Q_A represents the number of moles of the analyte in the surfactant-rich phase and Q_A' corresponds to the number of initial moles [50]. *E* value is in direct proportion to the recovery of surfactants used. It can achieve the maximum at a favorable surfactant percentage. In our experiment, 24 polymers, or surfactants, were evaluated for cloud point pre-concentrations at room temperature under optimal polymer or surfactant percentage conditions. Table 1 shows the *E* value for these 12 polymers and surfactants that were used in the extraction of *p*-Tyr, *m*-Tyr, *o*-Tyr and 3-NO_2-Tyr, under optimal experimental conditions. TX-114 had the largest *E* value and was selected for use in our experiment.

3.3. Pre-concentration of tyrosine isomers with TX-114

TX-114 is an amphiphilic molecule and, therefore, has one hydrophilic head and hydrophobic tail. It exists in monomer form at a low concentration or in an aqueous solution, but after a suitable concentration of salt is added, or the solution is heated, TX-114 molecules will form aggregates. In these aggregates (which contain between 60 and 100 monomers) the hydrophobic groups face the center and the hydrophilic groups extend outward to the aqueous solution. The hydrophobic sample molecules in the aqueous solution are enclosed in the central hydrophobic area, allowing the pre-concentration to take place. At the same time, the samples can be pre-concentrated since sample molecules are transferred from a larger volume of the aqueous phase into a smaller volume of the surfactant-rich phase. Fig. 2 shows the chromatograms of tyrosine isomers without TX-114 pre-concentration (Fig. 2a) and with TX-114 pre-concentration extraction (Fig. 2b). We can see that the peaks of hydrophilic molecules before 5 min of retention time in Fig. 2b became smaller after pre-concentration with TX-114, compared to those in Fig. 2a. This means that most of the hydrophilic impurities in the samples remained in the aqueous solution, and did not interfere with

Table 1
Comparison of extraction efficiency of 12 polymers and surfactants

Polymer or surfactant	Favorable initial concentration (%)	E value (%)			
		o-Tyr	*m*-Tyr	*p*-Tyr	3-NO_2-Tyr
HPMC-5	10	12.14	13.56	11.87	15.87
HPMC-50	5	24.45	25.98	23.45	26.32
HPLC-100	5	24.56	25.32	24.98	26.31
HPMC-4000	5	25.76	25.31	26.42	27.17
HPMC-50,000	5	27.64	28.75	26.45	28.34
HPMC-80,000	5	34.79	36.86	34.39	39.54
HPMC-90,000	5	36.99	36.15	37.38	38.54
HPMC-100,000	2	34.34	34.79	35.96	34.76
HPMC-300,000	2	21.75	27.35	15.52	25.42
Poly(vilyl)alcohol	20	44.56	46.61	43.75	45.81
PONPE 7.5	20	75.65	78.15	79.6	77.85
TX-114	10	93.25	96.27	91.65	97.19

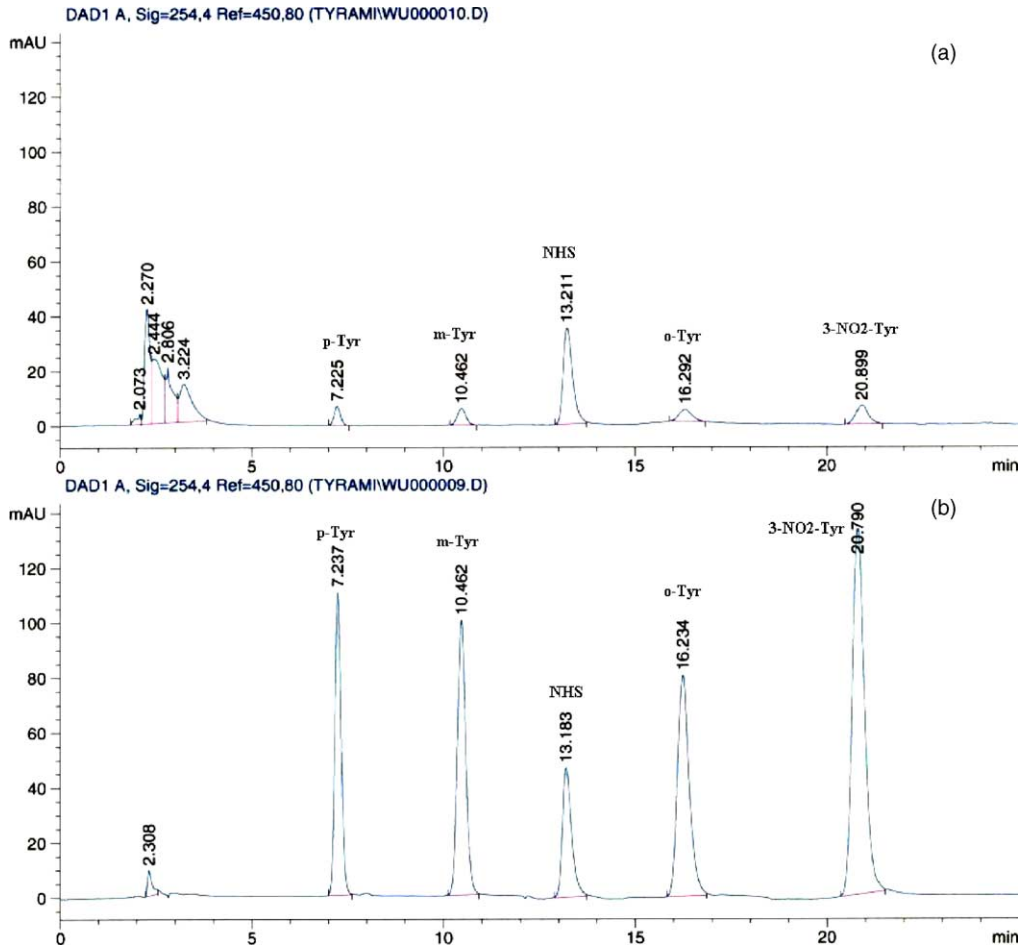


Fig. 2. Chromatograms of HPLC separations of tyrosine isomers and 3-NO_2-tyr without TX-114 pre-concentration (a) and with TX-114 pre-concentration (b). Concentrations of each standard: *p*-Tyr: 100 μmol/l; *m*-Tyr: 100 μmol/l; *o*-Tyr: 200 μmol/l; 3-NO_2-Tyr: 200 μmol/l. Separation conditions: an i.d. 3.9 mm × 300 mm, 5 μm, C_{18} column was used for the separation at 25 °C. Flow rate was 1.0 ml/min with a gradient program of a mobile phase A (0.15 mol/l) acetate buffer, 0.33 g/l TEA, pH = 6.35) and B (acetonitrile). The detailed gradient program was shown in the Section 2. Detection wavelength was 254 nm.

the tyrosine separation. In addition, the peaks of tyrosine isomers and 3-NO_2-tyrosine-derived products increased nearly 10 times. This was due to the hydrophobic nature of *N*-hydroxysuccinimide (NHS), which made it easily dissolve in hydrophobic solvents, but not in water. After derivatization, the hydrophobicity of the AccQ-derived products of tyrosine isomers and 3-NO_2-tyrosine increased; these products were easily extracted into the TX-114 phase. However, the retention times of the tyrosine isomers and 3-NO_2-tyrosine did not change, which shows that TX-144 did not interfere with the detection of the tyrosine isomers.

The pre-concentration factors (Fc) for all of the sample components in our study were calculated according to reference [50]. The Fc values for *o*-, *m*-, and *p*-tyrosine isomers and 3-NO_2-tyrosine were 9.17, 9.33, 9.63, and 9.72, respectively (the phase ratio of aqueous phase versus surfactant-rich phase equaled to 10), which greatly enhanced the detection sensitivity of each sample component.

3.4. Effect of TEA concentration on the separation of tyrosine isomers and 3-nitro tyrosine

TEA concentrations in the mobile phase had a more significant effect on the separation of tyrosine isomers as compared to other variables. Different TEA concentrations were examined while other components and the pH of the mobile phase were maintained at an optimal condition. From Fig. 3a, we can see that the peaks of *m*-tyrosine and NHS were overlapped in the absence of TEA. By increasing its concentration in the acetate elution buffer, better peak shapes and a satisfactory separation of tyrosine isomers and 3-NO_2-tyrosine were obtained, as shown in Fig. 3b and c. Furthermore, the retention time improved and was shorter.

3.5. Investigation of interference from Co-existing amino acids

Free amino acids contained in biological samples were a potential source of interference with the determination

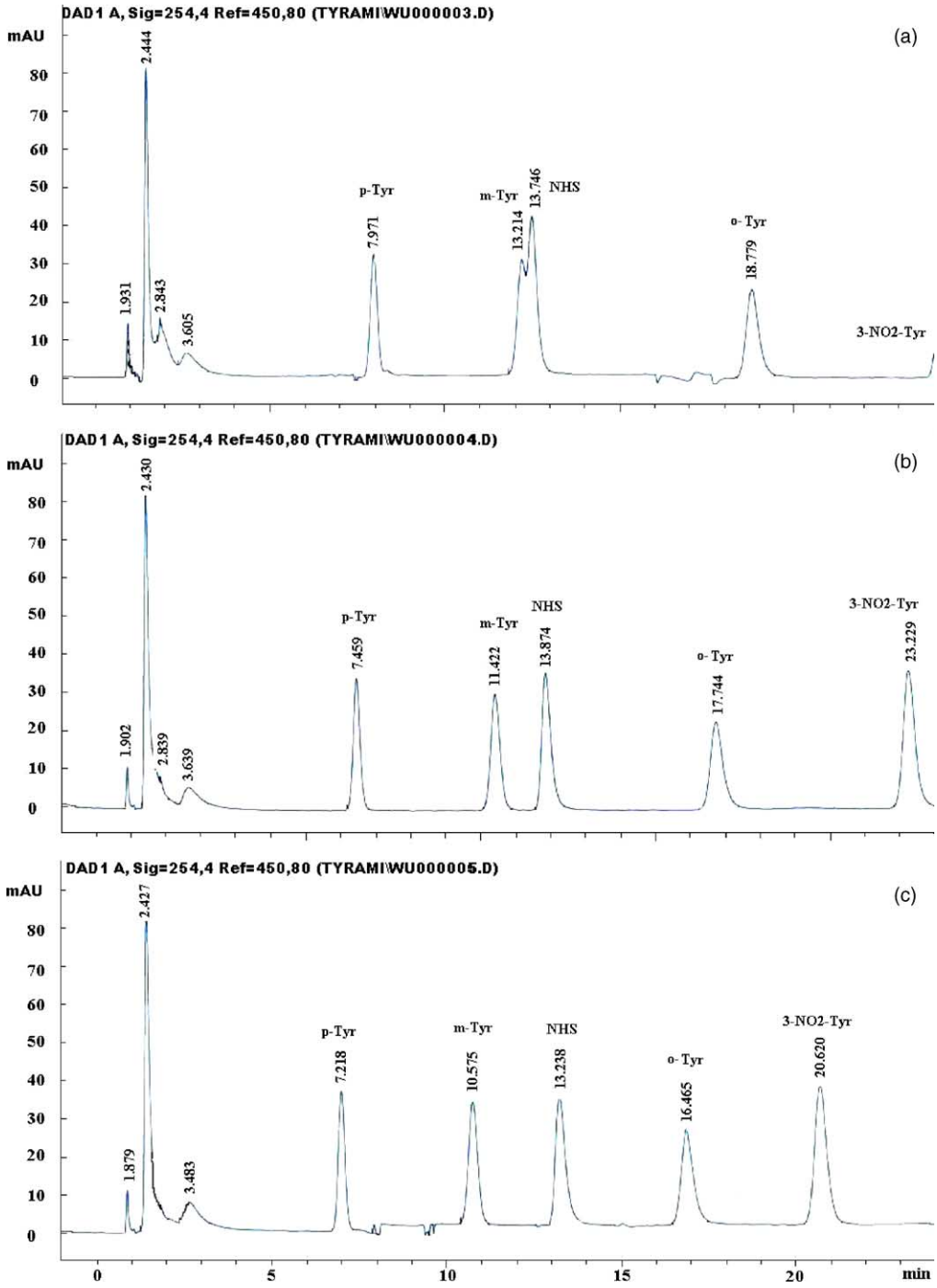


Fig. 3. Chromatograms of the separations of tyrosine isomers and 3-NO_2-Tyr under different TEA concentrations. (a) 0.0 mg/ml TEA; (b) 0.15 mg/ml TEA; (c) 0.33 mg/ml TEA. After TEA was added, the pH was re-adjusted to pH 6.35. Peak identification and other experimental conditions were the same as those in Fig. 2.

of tyrosine isomers and 3-NO_2-tyrosine. Since only L-form amino acids exist in the human body, we initially used both the L-form and racemic mixture form of standard amino acids to determine whether there was a difference in the UV intensity and other factors. Since we did not observe any differences between them, we decided to use the racemic mixture of amino acids and tyrosine isomers instead of the L-form (except that 3-NO_2-tyrosine was the L-form). L-3-NO_2-tyrosine was of special concern because it was the major biomarker of oxidative damage contributed by nitric oxide to proteins [54,55]. Nineteen co-existing amino acids (except tyrosine) were added to the solution of tyrosine isomers and 3-nitro-tyrosine standard solution in order to determine the possible interferences. Under an optimized

Table 2
Retention times of 19 amino acids for tyrosine isomers and 3-NO_2-tyrosine at optimized separation conditions. Column: C_{18} column, 5 μm (i.d. 3.9 mm × 300 mm)

Amino acids	Retention time (min)	Amino acids	Retention time (min)
Ala	3.978	Leu	16.998
Arg	5.088	Lys	4.194
Asn	3.013	Met	9.788
Asp	2.779	Phe	21.831
Cys	5.962	Pro	4.146
Gln	5.255	Ser	3.099
Glu	2.798	Thr	3.604
Gly	3.443	Trp	4.582
His	4.383	Val	7.978
Ile	14.841		

Separation was carried out at 25 °C, with a flow rate of 1.0 ml/min, by using a gradient program of double mobile phase A (acetate buffer, pH 6.35) and B (acetonitrile). The optimum gradient program for best resolution of *o*-, *m*-, *p*-, 3-nitro-tyrosine and other amino acids was: 16% B isocratic, for 15 min, followed by a linear increase to 20% B, over 10 min, and then by another linear increase to 60% B, over 5 min, and a 60% mobile phase B, continued to increase to 100% B over the next 5 min. Other experimental conditions were the same as those of the apparatus section.

gradient elution, the acids did not interfere with the targeted analytes. The results are shown in Table 2.

3.6. Elution order, linearity, detection limits and reproducibility

Representative separations of *p*-Tyr, *m*-Tyr, *o*-Tyr, 3-NO_2-Tyr and NHS are shown in Fig. 2 to demonstrate the excellent separations that can be obtained in less than 30 min under optimum conditions. Their retention times were in the following order: *p*-Tyr < *m*-Tyr < NHS < *o*-Tyr < 3-NO_2-Tyr. This elution order can be explained in terms of the polarity of the derived products, which depends on either the position of the hydroxyl group (–OH) or the nitro group (NO_2) on the benzene ring, or on the 3-dimensional structure of the molecule. The *para*-, *meta*-, or *ortho*-position hydroxyl groups on the benzene ring had a significant effect on the distribution of electron density on the benzene ring because of the strong electron-donating characteristic of the hydroxyl group. This increased the polarity of the derived products and resulted in shorter retention times for *p*-Tyr, *m*-Tyr, and *o*-Tyr. Conversely, in the case of 3-NO_2-Tyr, the negative charges in the aromatic ring transferred to the nitro group because it was a strong electron-withdrawing group. This caused an increase in the polarity symmetry of the 3-dimensional molecule and, therefore, resulted in a longer retention time for 3-NO_2-Tyr.

Table 4
Recovery and content of tyrosine isomers and 3-NO_2-tyrosine in healthy human urine samples ($n = 6$)

Tyrosines	Added standards	Recovery (±R.S.D.) (%)	Final tyrosine contents (nmol per day)
p-Tyr	200 nmol	94.7 ± 1.9	827.7 ± 7.9
m-Tyr	150 pmol	85.3 ± 6.3	0.4 ± 0.3
o-Tyr	150 pmol	91.4 ± 3.7	0.6 ± 0.2
3-NO_2-Tyr	20 nmol	89.1 ± 7.1	61.3 ± 3.4

The experimental conditions were the same as those in Table 2.

The linearity, reproducibility, and detection limits are listed in Table 3. Reproducibility was expressed as a relative standard deviation (R.S.D.), and was calculated for 0.1 mmol/l of tyrosine isomers and 3-NO_2-tyrosine standard solutions ($n = 15$). In general, the R.S.D. values were between 3.05 and 5.25%. The linearity of the method was determined by using standard mixtures within a concentration range of 0.05–100 μmol/l, and correlation coefficients ranging between 0.982 and 0.998. Detection limits for the tyrosine isomers and 3-NO_2-tyrosine were obtained from a signal-to-noise ratio (S/N) of 3, with a range of between 100 and 300 fmol at 20 μl injection volume (i.e. 5–15 nmol/l).

3.7. Applications

Urine samples were collected over a 24 h period and were pretreated according to procedures described in the Section 2. The peaks were first identified by adding standards to the samples and detected by using the standard addition method under optimal HPLC separation conditions. A representative chromatogram of the separation and detection of *o*-, *m*-, *p*-Tyr and 3-NO_2-Tyr in human urine samples is shown in Fig. 4. We can clearly see that all of these compounds can be identified and quantified simultaneously. The standard recoveries and total amount of tyrosine isomers and 3-NO_2-Tyr contents (free and protein-bound) are shown in Table 4. The 3-NO_2-Tyr contents correspond to the values that are reported by using the GC method [12].

Table 3
Linearity, reproducibility and detection limits of tyrosine isomers and 3-NO_2-tyrosine

Tyrosine	RT	R.S.D.	Linearity			Detection limits (fmol) (S/N = 3) (injection = 20 μl)
			Slope	Intercept	Correlation coefficient (%)	
p-Tyr	7.22 ± 0.05	3.05 ± 0.13	11.25 ± 0.04	116.82 ± 0.03	98.2 ± 0.4	100
m-Tyr	10.46 ± 0.08	4.13 ± 0.24	10.10 ± 0.05	105.72 ± 0.04	99.7 ± 2.3	100
o-Tyr	16.29 ± 0.10	5.25 ± 0.17	7.76 ± 0.03	342.71 ± 0.09	99.8 ± 1.7	200
3-NO_2-Tyr	20.85 ± 0.04	4.87 ± 0.05	13.39 ± 0.07	251.42 ± 0.10	98.9 ± 0.6	300

The calculations were based on data obtained from 15 consecutive injections under optimal HPLC conditions, as stated in Table 2. RT: retention time; R.S.D.: relative standard deviation; S/N: signal-to-noise ratio.

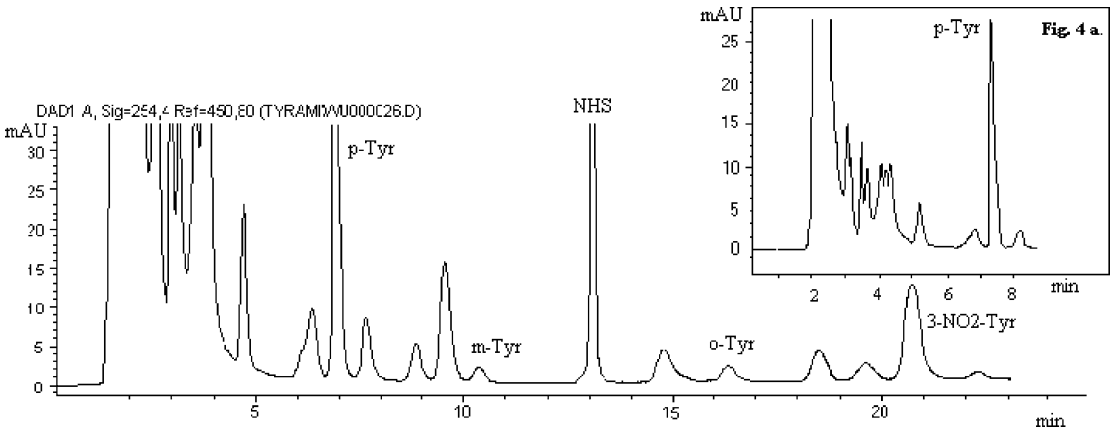


Fig. 4. A representative chromatogram of the separation of tyrosine isomers and 3-NO_2-Tyr under optimal conditions. The experimental conditions were the same as those in Fig. 2. (a) The *p*-Tyr peak after three times further than the dilution of the urine sample for *p*-Tyr quantitation. Each peak was identified by using the standard addition method and was labeled in the chromatogram.

4. Conclusions

A reverse phase HPLC method combined with the off-line derivatization/cloud point extraction method has been successfully applied for simultaneous determination of *p*-Tyr, *m*-Tyr, *o*-Tyr, and 3-NO_2-Tyr with UV detection. AccQ was used as the off-line derivatization reagent at pH 8.80 in an alkaline buffer, while cloud point extraction was used to improve determination sensitivity. The combination of these two procedures contributed to the high sensitivity of this method. The sensitivity was significantly improved in comparison to that of previously developed HPLC methods that used direct UV detection and even in comparison to fluorescence detection methods. This technique can be conveniently used for direct assessment of 3-NO_2-Tyr in biological samples for clinical purposes.

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