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Metabolic study of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone to the enantiomers of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in vitro in human bronchial epithelial cells using chiral capillary electrophoresis

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ABSTRACT

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) with one chiral center at the carbinol is a major metabolite of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). As tobacco specific N-nitrosamines (TSNAs), NNK and NNAL are the most pulmonary carcinogens in tobacco products and smoke. In this paper, a chiral CE method modified with highly sulfated β-cyclodextrin (S-β-CD) was developed to investigate the stereoselective formation of NNAL from NNK in vitro in normal human bronchial epithelial (NHBE) cells. Combined with solid phase extraction (SPE) of the cell samples, NNK and NNAL enantiomers were baseline separated under the proposed CE conditions, with satisfactory recoveries (72.5–113% for NNK and (±)-NNAL) and low limits of detection (LOD, 2.5–3 μg/mL for NNK and (±)-NNAL). The cytotoxicity of NNK in NHBE cells was investigated through the cell counting kit (CCK) assay and proved to be highly dependent on the NNK's concentration. The metabolic results obtained from CE analysis demonstrated that NNK was preferentially metabolized to (+)-NNAL through carbonyl reduction. Meanwhile, the ratio of [(+)-NNAL]/[(−)-NNAL] was independent of NHBE cells' incubation time with NNK, but could be changed according to the original incubation concentration of NNK. This chiral CE method could be useful for the study on toxicology and metabolic transformations of related TSNAs.

1. Introduction

4-Methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK, see the structure in Fig. 1), one of the seven tobacco specific N-nitrosamines (TSNAs), is the most pulmonary carcinogen in tobacco products and smoke [1]. It is presumed to be responsible for lung cancer and related carcinogenesis in smokers [2–4]. 4-Methylnitrosamino-1-(3-pyridyl)-1-butanol (NNAL, see the structure in Fig. 1), a major metabolite of NNK, has the similar tumorigenicity to NNK. There is a chiral center at the carbinol in NNAL, which will lead to the existence of enantiomers (+)-NNAL and (−)-NNAL with different toxicity and pharmacokinetics in vivo [5]. Hence, it is of great interest to resolve the NNAL enantiomers to better access their carcinogenic potential in vivo.

In the past few years, the stereoselectivity of carbonyl reduction of NNK to NNAL has been investigated in human's or mice's bile, plasma and tissues using LC and GC methods with chiral columns [6–9] or usual LC methods through derivatization with chiral reagents [10,11]. However, in these methods, several drawbacks existed such as lack of general applicability [12], high expense, complicated sample handling procedures, and time-consuming.

CE modified with chiral selectors, which emphasizes on the high flexibility, the wide applicability and the great economy [13,14], has become prevalent in separating enantiomers [12]. McCorquodale et al. [15] had used hydroxypropyl-β-cyclodextrin (HP-β-CD) to separate N′-nitrosonornicotine (NNN) enantiomers, one of TSNAs. The key factor in the chiral CE is the selection of chiral selectors. Until now cyclodextrins (CDs), crown ethers, macrocyclic antibiotics, proteins, carbohydrates, chiral ion-paring reagents, chiral surfactants and ligand-exchanges have been used as the chiral selectors [16,17]. Among those chiral selectors, CDs are the most favorite. Moreover, the CD derivatives like the charged CDs showed some advantages over neutral CDs. They could not only increase the solubility in water [18] because of the addition of hydrophilic groups but also improve the resolutions caused by the electrostatic forces [19].

In our lab, CE–MS [20] and cation-selective exhaustive injection (CSEI)-sweeping-MEKC [21] methods had been used to detect and

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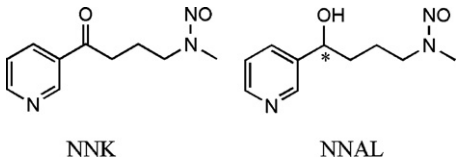

NNK NNAL

Fig. 1. The structures of NNK and NNAL. *The chiral center.

concentrate TSNAs, respectively. Besides, LC–MS method [22] was utilized to investigate the metabolism of TSNAs in the rabbit. In this study, using highly sulfated β-CD (S-β-CD) as the chiral selector, an efficient CE method was developed to separate the NNAL enantiomers for the first time. Normal human bronchial epithelial (NHBE) cells were chosen as the research model since NNK impaired the pulmonary epithelial cells [23]. The cytotoxicity of NNK in NHBE cells was also assessed and the metabolism of NNK to NNAL enantiomers in vitro in NHBE cells was investigated using the developed chiral CE method.

2. Experimental

2.1. Reagents and materials

NHBE cells were purchased from Shanghai Touching Living Things Information Technology Co., Ltd (Shanghai, China). Roswell Park Memorial Institute (RPMI)-1640 medium and trypsin were obtained from Beijing Four-Ring Sunny Bioscience Co., Ltd (Beijing, China). Neonatal bovine serum was purchased from Sijiqing Biological Engineering Materials Co., Ltd (Hangzhou, China). Mixture of penicillin (10,000 U/mL) and streptomycin (10,000 μg/mL) was obtained from Beijing Botena Bioscience Co., Ltd (Beijing, China). Cell counting kit-8 (CCK-8) was purchased from Nanjing Keygen Biotech. Co. Ltd (Nanjing, China). NNK and racemic NNAL were obtained from Toronto Research Chemicals (North York, Canada). Methanol with chromatographic grade was purchased from Dima Technology (Lake Forest, CA, USA). Purified water was obtained from Hangzhou Wahaha Group Co. (Hangzhou, China). HP-β-CD was purchased from Acros Organics (97%, NJ, USA). Both carboxymethyl β-CD (CM-β-CD) and sulfated β-CD (S-β-CD) were from Fluka (Buchs, Switzerland). Ammonium formate was obtained from Beijing Xudong Chemical Factory (Beijing, China). Formic acid was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Apparatus

CE experiments were performed on an Agilent 3D CE system equipped with air-cooling and a diode array detector (Agilent, Palo Alto, CA, USA). Detection wavelength was set at 240 nm. An untreated fused-silica capillary (Xinnuo, Handan, China) of 58.5 cm × 50 μm i.d. (50 cm effective length) was utilized. Each new capillary was preconditioned sequentially with 1 M NaOH (15 min) and pure water (20 min). Between consecutive analyses, the capillary was flushed with 0.1 M NaOH (2 min), pure water (3 min) and background electrolytes (BGE, 3 min).

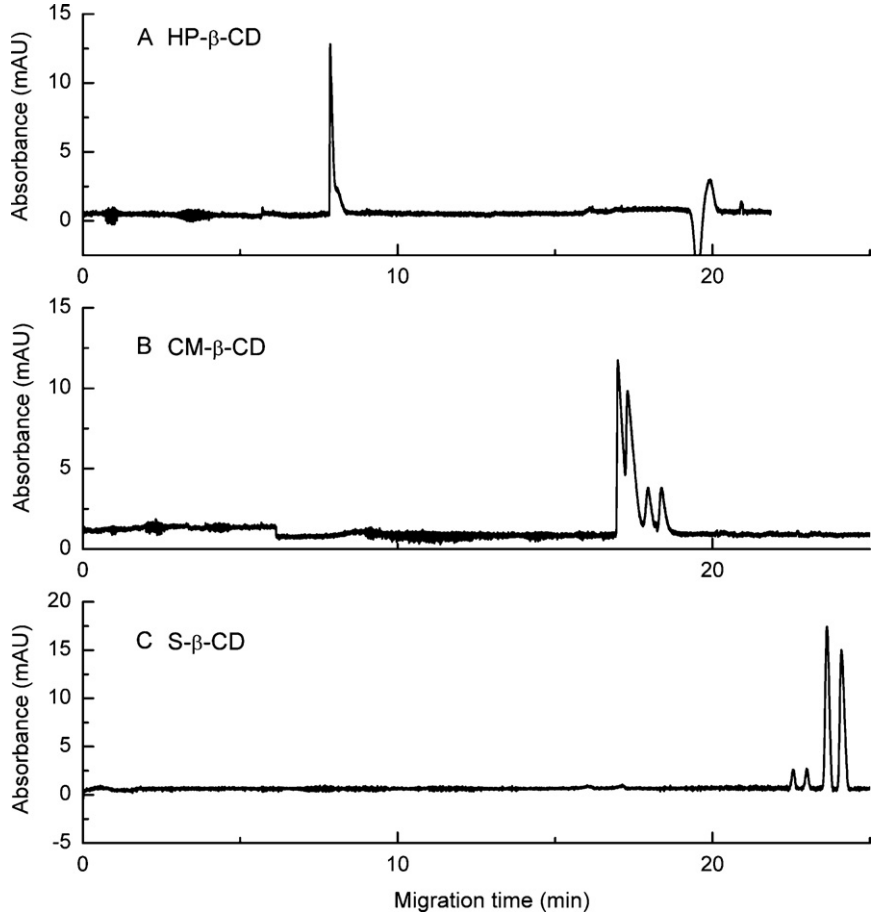

Fig. 2. Electropherograms of NNAL enantiomers using HP-β-CD (A), CM-β-CD (B) and S-β-CD (C) as the chiral selectors. Other conditions: running buffer, 50 mM ammonium formate at pH 2.5 with 2.6% (w/v) HP-β-CD (A), 3.9% (w/v) CM-β-CD (B) and 0.9% (w/v) S-β-CD (C); sample concentration, 100 μg/mL in pure water; hydrodynamic injection, 50 mbar for 30 s; separation voltage, 20 kV; capillary temperature, 20 °C; detection wavelength, 240 nm.

Table 1
Method validation for the determination of (+)/(−)-NNAL and NNK in NHBE cell samples using the chiral capillary electrophoresis.

	Regression equation[a]	Correlation coefficient (r)	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
(+)-NNAL	$y = 1.53x - 0.40$	0.991	5.0–100.0	2.5	5.0
(−)-NNAL	$y = 1.55x - 0.43$	0.994	5.0–100.0	2.5	5.0
NNK	$y = 0.85x + 0.83$	0.993	6.6–212.0	3.0	6.6

[a] x and y mean log[concentrations of determinants] and log[peak areas], respectively.

LC experiments were performed on an Agilent 1100 system (Agilent, Palo Alto, CA, USA) equipped with an autosampler and a diode array detector, on-line coupled to an Agilent Trap XCT ion trap.

Circular dichroism spectra were recorded on a J-810 spectrometer (Jasco, Japan). The temperature was mediated with a PolyScience programmable temperature controller. The light pathlength of the quartz cells used were 0.1 mm.

2.3. Preparation of NNAL enantiomers

Preparative separation of NNAL enantiomers was achieved on a chiral column with SINU-PC chiral separation phase [24,25] (CSP, 250 × 4.6 mm, i.d. 5 μm, serial number: SPCKM542-2203001, Singapore) at 30 °C, with the mobile phase of water–methanol (90:10, v/v) at a flow rate of 1 mL/min. The assignment of peaks in the HPLC chromatogram to NNAL enantiomers was confirmed using circular dichroism and mass spectra. The fractions of (*E*)/(*Z*)-(+)-NNAL and (*E*)/(*Z*)-(−)-NNAL were collected manually and used in the following CE analysis.

2.4. Cell culture

NHBE cells were cultured in RPMI-1640 medium with 10% (v/v) neonatal bovine serum, 1% (v/v) mixtures of penicillin and streptomycin at 37 °C in an atmosphere of 5% CO_2. When a monolayer was formed, the cells were harvested using 0.25% trypsin, spun at 110 × *g* for 5 min, and then resuspended in fresh medium.

2.5. Cell counting kit assay

To evaluate NNK's role in NHBE cell viability, CCK assay was used. NHBE cells (200 μL/well) were seeded at a density of 10^5 cells/mL in a 96-well microplate and treated with NNK of different concentrations: 0, 12.5, 25, 50, 100 and 200 μg/mL, respectively. After 24 h incubation in the 5% CO_2 atmosphere at 37 °C, 20 μL WST-8 solution were added and the cells were incubated for another 4 h. Then the absorbance was measured at 450 nm using a microplate reader (Hangxin ZS-2SWK). The relative cell viability (%) was defined as (the average absorbance of the drug group/the average absorbance of the control group) × 100.

2.6. Cell sample preparation

In this experiment, the cell samples were lysed with sonication and then centrifuged at 990 × *g* for 5 min. In addition, solid phase extraction (SPE) was used to handle the supernatant. After preconditioning of the Cleanert ODS–SPE cartridge obtained from Agela (Beijing, China) with 1 mL methanol and 1 mL water, the supernatant (300 μL) was loaded and washed with 1 mL water and 1 mL methanol/water (5:95, v/v) sequentially. Then the eluents were obtained with 0.6 mL methanol containing 1% formic acid (v/v). Finally, the extracts were evaporated to dryness in the vacuum oven and redissolved in purified water (300 μL) before CE analysis.

3. Results and discussion

3.1. Separation of NNAL enantiomers

The selection of chiral selectors is a key factor in the chiral CE. Several CD derivatives had been investigated in our experiments, including HP-β-CD, CM-β-CD and S-β-CD (shown in Fig. 2). Different orientations of the –N=O bond and the unshared electron pair in NNK and NNAL would result in (*E*)/(*Z*) isomers. As seen in Fig. 2, separation of NNAL enantiomers could not be achieved with HP-β-CD as the chiral selector. Instead, using CM-β-CD and S-β-CD, both of the enantiomers and the (*E*)/(*Z*) isomers could be potentially separated. 0.9% (w/v) S-β-CD in the buffer would be the best choice based on the following considerations: First, complexation, dipole–dipole interaction and hydrogen bond were the three of the multiple elements included which would affect the chiral recognition [26]. Besides, when the charged CDs were used, electrostatic force would become another significant factor to facilitate the separation [19]. At pH 2.5, NNAL was positively charged, resulting in the strong interaction with negatively charged CDs. Therefore, compared with HP-β-CD, CM-β-CD and S-β-CD could provide a better separation of NNAL enantiomers. Second, the substitution degrees of hydroxyl groups in CM-β-CD and S-β-CD were 3 and 7–11, respectively, hence, S-β-CD had more negative charges and stronger interaction with NNAL isomers at pH 2.8 than CM-β-CD did, leading to a better chiral recognition. Third, with the increase of S-β-CD concentration in the BGE, the resolution of NNAL isomers was improved. However, the higher concentration of S-β-CD would

Table 2
Recoveries of (+)/(−)-NNAL and NNK and reproducibilities of the chiral CE method for analysis of (+)/(−)-NNAL and NNK at different concentrations in cell samples.

TSNAs	Concentration (μg/mL)	Recovery[a] (%, $n = 3$)	RSD (%, $n = 5$)	
			Migration time	Peak area
(+)-NNAL	7.5	104 ± 7.8	2.0	4.2
	25	90.2 ± 8.0	0.9	1.2
	50	90.3 ± 2.3	0.8	2.7
(−)-NNAL	7.5	113 ± 8.7	2.0	5.6
	25	93.9 ± 8.9	0.9	1.2
	50	89.1 ± 4.4	0.7	5.1
NNK	13.25	72.5 ± 4.5	3.6	2.6
	53	92.7 ± 6.6	2.7	3.4
	106	101 ± 4.3	3.1	1.9

[a] Data are expressed as mean ± SD, $n = 3$.

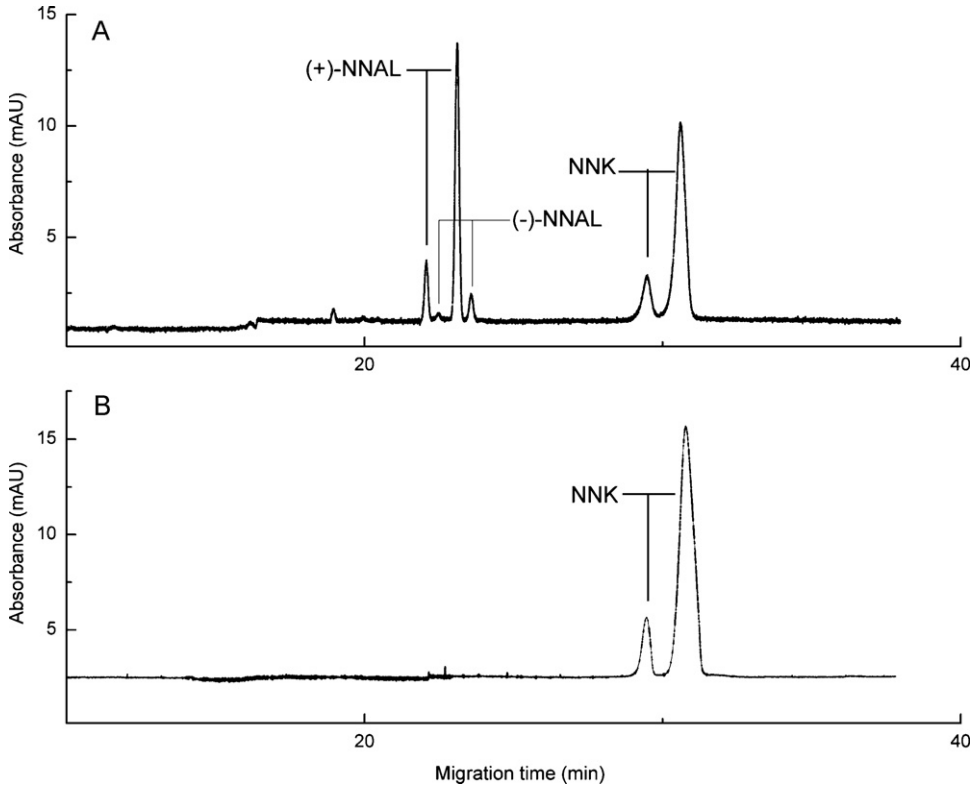

Fig. 3. Electropherograms of the cell culture medium incubated with NNK with cells (A) and without cells (B) for 30 h. Separation conditions were the same as those in Fig. 2C.

lead to a higher complexation degree between S-β-CD and NNAL enantiomers, resulting in the prolonged migration time.

Capillary temperature could also greatly influence the chiral separation in CE analysis. It emphasized the influences on the affinity of the enantiomers to CDs, the viscosity and the pH of the BGE [27]. In this study, temperatures ranging from 15 to 30 °C were investigated (data not shown). Owing to the high viscosity of BGE at low temperature, the migration time would be increased. However, with the capillary temperature elevated, the chiral resolution and the enantioselectivity would be decreased because of the relatively unstable complexation [28,29]. Taking these two aspects into consideration, 20 °C was the optimum temperature.

In CE system, buffer type determined the pH range, which could alter the charge state of the NNAL enantiomers, influencing the interaction between the NNAL enantiomers and CD derivatives, and also has a great influence on the EOF. In this experiment, 50 mM ammonium formate at pH 2.8 was chosen as the buffer system, which could give a better separation.

3.2. Method validation

In this study, NNK and NNAL enantiomers in NHBE cell matrix were subjected to SPE procedures followed by the chiral CE method. Since (*E*)-NNAL and (*Z*)-NNAL were interconvertible [6,11], the quantitation of (*E*)/(*Z*)-NNAL would not be discussed. In the following research, the total amount of (+)-NNAL was considered to be the total amount of (*E*)-(+)-NNAL and (*Z*)-(+)-NNAL, and it is the same to (−)-NNAL as well as NNK.

The linearity of this method was evaluated from 5 to 100 μg/mL for both (+)-NNAL and (−)-NNAL and from 6.6 to 212.0 μg/mL for NNK. The calibration curve was obtained through plotting the peak area to the concentration for each analyte. From the data listed in Table 1, the correlation coefficients (*r*) for (+)-NNAL, (−)-NNAL and NNK were 0.991, 0.994 and 0.993, respectively. Furthermore, the limits of detection (LOD), defined as a signal-to-noise ratio of 3, were 2.5, 2.5 and 3.0 μg/mL for (+)-NNAL, (−)-NNAL and NNK, respectively.

Sample handling efficiency was evaluated in terms of recoveries [30]. In this study, the recovery test was investigated using the blank cell samples spiked with NNK and NNAL at three different concentrations. And the recovery was defined as the ratio of the experimental concentrations of determinants spiked before and after sample handling. As shown in Table 2, the results demonstrated that the average recoveries of (+)-NNAL, (−)-NNAL and NNK at three different concentrations were in the ranges of 90.2 ± 8.0–$104 \pm 7.8\%$, 89.1 ± 4.4–$113 \pm 8.7\%$ and 72.5 ± 4.5–$101 \pm 4.3\%$, respectively, indicating the effectiveness of the sample preparation method.

Reproducibility was assessed to evaluate the precision and the feasibility of this chiral CE method. The RSDs ($n = 5$) of peak areas and migration times of (+)-NNAL, (−)-NNAL and NNK were examined at different concentration levels in detail (Table 1), showing that the RSDs of migration time and peak area in each case were less than 4% and 6%, respectively.

3.3. Cytotoxicity of NNK in NHBE cells

NNK is a significantly strong carcinogen which could probably induce the lung cancer or the oral cavity cancer in those who are associated with tobacco products [1,5]. To explore the cytotoxicity of NNK in NHBE cells, CCK-8 assay was used to determine the viability of NHBE cells incubated with NNK of different concentrations for 24 h, ranging from 12.5 to 200 μg/mL. As expected, the incubation with NNK could lead to the decrease of the NHBE cell viability. With NNK at 200 μg/mL, the relative cell viability was decreased to 68%, indicating that the NNK could suppress the cell proliferation but the inhibition effect was not quite significant.

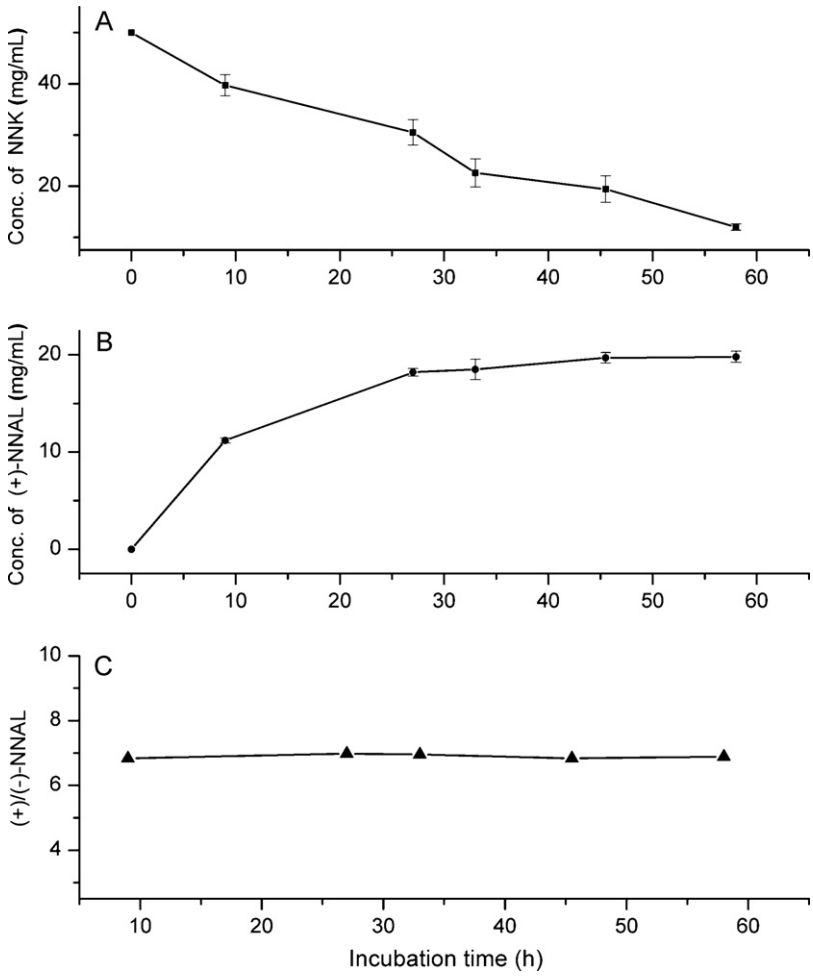

Fig. 4. Metabolic tendencies of NNK (A) and its metabolite (+)-NNAL (B) as well as the variation tendency of the ratio of [(+)-NNAL] and [(−)-NNAL] (C) in NHBE cells.

3.4. Metabolism of NNK to enantiomers of NNAL

NNK can be enantioselectively metabolized to (+)-NNAL or (−)-NNAL through the carbonyl reduction [6,7]. In addition, (+)-NNAL and (−)-NNAL demonstrate different pharmacokinetics [5]. The enantioselectivity of NNK reduction to NNAL in vitro in NHBE cells was investigated using the proposed CE method and the results are shown in Fig. 3. The enantioselective reduction of NNK to NNAL was investigated for different incubation time from 10 to 58.5 h at different incubation concentrations of NNK, namely 50 and 100 μg/mL. Obviously, in NHBE cells, both (+)-NNAL and (−)-NNAL could be metabolically formed from NNK. Furthermore, NNK was preferentially reduced to (+)-NNAL. Fig. 4A and B displayed the metabolic tendencies of NNK at 50 μg/mL and its metabolite (+)-NNAL, respectively. As the exposure time increased, the concentration of NNK was decreased and that of (+)-NNAL was increased as expected. However, the ratio of [(+)-NNAL]/[(−)-NNAL] was constant and independent of the incubation time of NNK (shown in Fig. 4C). On the other hand, it was slightly changed with the original incubation concentration of NNK. When the original concentration of NNK altered from 50 to 100 μg/mL, the ratio of [(+)-NNAL] and [(−)-NNAL] varied from 6.87 ± 0.08 to 8.52 ± 0.06. Therefore, the ratio of (+)/(−)-NNAL was NNK concentration-dependent to certain extent.

4. Conclusion

In this paper, chiral CE modified with S-β-CD was developed to investigate the metabolism of NNK to enantiomers of NNAL in vitro in NHBE cells for the first time, without the use of complicated sample derivatization with chiral reagents and the high expense of chiral columns in chiral LC and GC methods. The SPE method was used to extract the determinants from the cell matrices with good recoveries. NNK and four NNAL isomers, namely (*E*)-(+)-NNAL, (*E*)-(−)-NNAL, (*Z*)-(+)-NNAL and (*Z*)-(−)-NNAL, were baseline separated by CE. With satisfactory quantitation capability and reproducibility, this chiral CE method has been utilized to investigate metabolic tendencies of NNK and its metabolite (+)-NNAL. Taken together, our results demonstrated a metabolic profile of NNK to enantiomers of NNAL. (+)-NNKL has been verified to be the major metabolite of NNK through carbonyl reduction. The ratio of (+)-NNAL/(−)-NNAL was independent of HBE cells' incubation time and related to the concentration of the original incubation concentration of NNK to some extent. In conclusion, the proposed chiral CE method could be used for the study on toxicology and metabolic transformation of related TSNAs in biosystems.

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