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Determination of α-naphthylisothiocyanate and metabolites α-naphthylamine and α-naphthylisocyanate in rat plasma and urine by high-performance liquid chromatography

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Abstract

A rapid and sensitive high-performance liquid chromatographic (HPLC) assay for the determination of α-naphthylisothiocyanate (1-NITC) and two metabolites α-naphthylamine (1-NA) and α-naphthylisocyanate (1-NIC) in rat plasma and urine has been developed. The chromatographic analysis was carried out using reversed-phase isocratic elution with a Partisphere C_{18} 5-μm column, a mobile phase of acetonitrile–water (ACN–H_2O 70:30, v/v), and detection by ultraviolet (UV) absorption at 305 nm. The lower limits of quantitation (LLQ) in rat plasma, urine, and ACN were 10, 30, and 10 ng/ml for 1-NITC; 30, 100, and 30 ng/ml for 1-NA; and 30 ng/ml in ACN for 1-NIC. At low (10 ng/ml), medium (500 ng/ml), and high (5000 ng/ml) concentrations of quality control samples (QCs), the range of within-day and between-day accuracies were 95–106 and 97–103% for 1-NITC in plasma, respectively. Stability studies showed that 1-NITC was stable at all tested temperatures in ACN, and at −20 and −80 °C in plasma, urine, and ACN precipitated plasma and urine, but degraded at room temperature and 4 °C. 1-NA was stable in all of the tested matrices at all temperatures. 1-NIC was unstable in plasma, urine, and ACN precipitated plasma and urine, but stable in ACN. The degradation product of 1-NITC and 1-NIC in universal buffer was confirmed to be 1-NA. 1-NITC and 1-NA were detected and quantified in rat plasma and urine, following the administration of a 25 mg/kg i.v. dose of 1-NITC to a female Sprague–Dawley rat.

Keywords: α-Naphthylisothiocyanate; α-Naphthylamine; α-Naphthylisocyanate

1. Introduction

Many synthetic and naturally occurring organic isothiocyanates (ITCs; RN=C=S) can block chemical carcinogenesis in experimental animals and are being considered as chemopreventive agents for human use (see reviews in Refs. [1,2]). α-Naphthylisothiocyanate (1-NITC) (Fig. 1) was reported as a carcinogenesis inhibitor in rats as early as the 1960s [3–7]. Recently, we have found that 1-NITC can reverse the multidrug resistance (MDR) to antineoplastic agents in human cancer cell lines through inhibition of the ATP-dependent efflux proteins, P-glycoprotein and multidrug resistance associated-protein 1 (MRP1) [8]. These findings indicated the potential use of 1-NITC not only in cancer preven-

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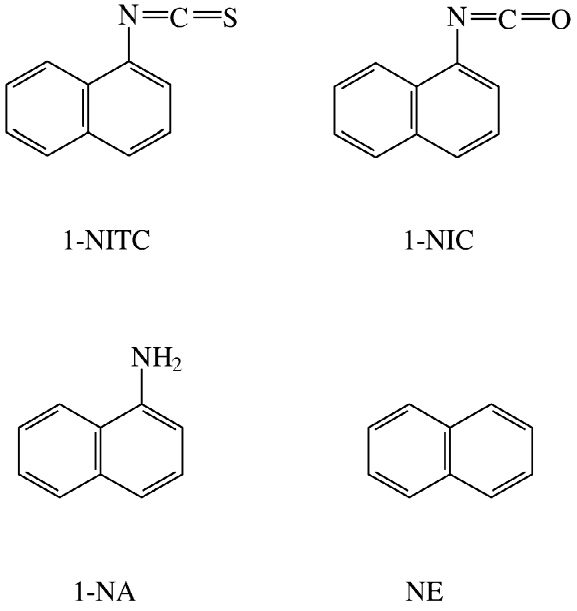

Fig. 1. Chemical structures of 1-NITC, 1-NA, 1-NIC and NE.

tion but as a chemosensitizing agent. High doses of 1-NITC (150–300 mg/kg) produce hepatic lesions resembling those occurring with biliary cirrhosis in humans [9–11]; this has led to the use of 1-NITC as a model chemical to study human cirrhosis and drug-induced cholestasis.

To our knowledge, the pharmacokinetics of 1-NITC and its metabolites in rat plasma and urine have not been studied, although it is reported that 1-NITC is eliminated in the bile and urine predominantly as metabolites [12]. The major metabolites of 1-NITC have been characterized by mass spectroscopy and found to be α-naphthylisocyanate (1-NIC) and α-naphthylamine (1-NA) [16]. Several analytical methods have been described for the determination of 1-NITC in biological samples, including assays based on radiolabelled drug [10–13], thin-layer chromatography [14], gas–liquid chromatography (GLC) [15], gas chromatography/mass spectrometry (GC–MS) [16], and high-performance liquid chromatography (HPLC) [17–22]. However, those methods were labor intensive, time consuming, inconvenient, or not sensitive enough. In this paper we report the development and validation of a rapid and sensitive HPLC assay able to detect the parent drug 1-NITC and its metabolites in rat plasma and urine, the stability of 1-NITC and its metabolites in rat plasma and urine samples, and the use of the assay to characterize the pharmacokinetics of 1-NITC in a preliminary rat study.

2. Experimental

2.1. Chemicals and reagents

1-NITC and 1-NA were purchased from Sigma (St. Louis, MO, USA) more than 99 and 98% purity, respectively. 1-NIC was purchased from Aldrich (Milkwaukee, MI, USA) at 98% purity. The internal standard naphthalene (NE) (Fig. 1) was purchased from Fisher Scientific (Fair Lawn, NJ, USA) at more than 99% purity. Acetonitrile (ACN) and methanol (MeOH) were HPLC grade from Fisher. Other chemicals are in analytical grade unless specified.

2.2. Preparation of rat plasma and urine samples for calibration of standards and quality control samples (QCs)

The stock solutions 10 mg/ml of 1-NITC, 1-NA, 1-NIC, and NE were freshly prepared for every validation run by dissolving a weighted amount of each compound in ACN. The 0.5 and 2.0 mg/ml working solutions of NE were prepared by diluting the stock solution with ACN as internal standard for validation of 1-NITC and 1-NA in rat plasma and urine samples, respectively.

Solutions of 1-NITC containing 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, and 250 μg/ml were prepared by serial dilution of the stock solutions with ACN. Each blank rat plasma sample (50 μl) was spiked with 5 μl of a NE solution (0.5 mg/ml), 5 μl of varying concentrations of 1-NITC, and 190 μl ACN, to prepare a series of standards (10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/ml as final concentration) for the calibration curve.

The working solutions of 1-NA containing 5, 10, 25, 50, 100, 250, 500, 1000, and 2500 μg/ml were prepared by serial dilution of the stock solutions with ACN. Each blank rat urine sample (50 μl) was spiked with 5 μl NE work solution (2.0 mg/ml), 5 μl appropriate 1-NA working solution, and added 190 μl ACN, to prepare a series of standards (100,

200, 500, 1000, 2000, 5000, 10 000, 20 000, and 50 000 ng/ml as final concentration) for the calibration curve.

Both spiked plasma and urine samples were vortexed for 10 s and centrifuged at 10 000 *g* for 5 min at 4 °C. The resulting supernatants were used for injection. QC samples at low (10 ng/ml for 1-NITC and 100 ng/ml for 1-NA), medium (500 ng/ml for 1-NITC and 5000 ng/ml for 1-NA), and high concentrations (5000 ng/ml for 1-NITC and 50 000 ng/ml for 1-NA), respectively, were prepared by the same procedures as previously described.

2.3. HPLC instrumentation and conditions

The Waters HPLC system (Milford, MA, USA) consisted of a model 1525 binary pump, a model 717plus autosampler (a 250-μl injector and a 200-μl loop) configured with a heater/cooler, a model 5HC column oven, and a model 2487 UV detector. The column and autosampler temperatures were kept at room temperature (21±1 °C) and 4 °C, respectively. The reversed-phase chromatography was performed with a Partisphere C_{18} 5-μm column 125×4.6 mm I.D. (Whatman, Clifton, NJ, USA) protected by a RP guard cartridge system C_{18} 5-μm (Whatman), and eluted isocratically with a mobile phase consisting of ACN–H_2O (70:30, v/v). The flow-rate was 1.0 ml/ml and the injection volume was 50 μl. The UV detector was set at a single wavelength of 305 nm. The Breeze System software version 3.2 (Waters) was used for instrument control and data analysis.

2.4. Assay validation

2.4.1. Lower limit of quantitation

The lower limit of quantification (LLQ) was determined during the evaluation of the linear range of calibration curve. LLQ was defined as the concentration of the lowest QC samples producing an assayed concentration within 10% of the theoretical value (i.e. accuracy between 90 and 110%) and yielding a precision of more than 90% for both within- and between-day evaluation.

2.4.2. Linearity of calibration curve

The linearity of calibration curve was evaluated by regression analysis of peak area ratios (1-NITC/NE and 1-NA/NE) to 1-NITC and 1-NA concentrations in blank plasma and urine samples, respectively.

2.4.3. Precision and accuracy

The assay was validated by within- and between-day accuracy and precision quantifying 1-NITC and 1-NA at QCs. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentrations. Within-day variability was assessed through the analysis of QCs in triplicate, and between-day variability was determined through the analysis of QCs on four consecutive days.

2.4.4. Recovery

The recovery of 1-NITC and 1-NA was established with QCs by comparing peak area ratios (1-NITC/NE and 1-NA/NE) to those of standards in ACN. The mean recoveries at low, medium, and high concentrations were determined for both within- and between-day analyses.

2.5. Stability

The stability of 1-NITC, 1-NA, and 1-NIC was studied in different matrices consisting of rat plasma, urine, ACN precipitated plasma and urine, ACN, and in a universal buffer (citrate–phosphate–borate–HCl, pH 2–12) at four designated temperatures over 96 h. 1-NITC, 1-NA, or 1-NIC (200 ng/ml as final concentration), along with internal standard NE (10 μg/ml), were added to plasma and ACN, respectively, for stability evaluations in plasma, ACN precipitated plasma, and ACN samples at room temperature (RT), 4, −20, and −80 °C. Samples were assayed at time points up to 96 h. The stability of 1-NITC, 1-NA, and 1-NIC in urine and ACN precipitated urine were tested at similar time intervals up to 96 h at a final concentration of 10 μg/ml for 1-NITC, 1-NA, and 1-NIC and 50 μg/ml for NE. The stabilities of 1-NITC, 1-NA, and 1-NIC in universal buffer were determined over a pH range from 2 to 12 at RT at times up to 96 h using the same concentrations as used for plasma samples. The compound was considered stable if the variation of

quantitation was less than 10% (i.e. 90–110% of initial time concentration).

2.6. 1-NITC pharmacokinetics in rat

The jugular vein cannula was inserted into a female Sprague–Dawley (Harlan, Indianapolis, IN, USA) rat following an i.m. injection of ketamine 90 mg/kg and xylazine 10 mg/kg (Henry Schein, Melville, NY, USA). Three days following surgery, a dose of 25 mg/kg 1-NITC (10 mg/ml) in a vehicle consisting of 10% ethanol (Pharmaco Products, Brookfield, CT, USA), 10% cremophor EL (Sigma), and 80% sterile saline (Braun Medical, Irvine, CA, USA) solution was administered as an intravenous (i.v.) bolus through the cannula.

Blood samples (250 μl each) were collected at 5, 10, 20, 30 min, 1, 2, 4, 6, 9, 12, and 24 h following 1-NITC administration, and placed in heparinized 0.6-ml microcentrifuge tubes. The plasma was immediately separated from blood via centrifugation at 1000 *g* for 10 min at 4 °C and stored at −80 °C to prevent potential degradation of 1-NITC and metabolites. The internal standard (5 μl) was added to 50 μl of each plasma sample and treated as previously described. The data was fitted to obtain pharmacokinetic (PK) parameters using WinNonLin version 2.1 (Pharsight, Mountain View, CA, USA).

Urine samples were collected at 2, 4, 6, 9, 12, 24, and 25 h time points, and the volume was measured. After adding 0.1% sodium azide (Fisher), the urine samples were centrifuged at 1000 *g* for 10 min at 4 °C and stored at −80 °C to prevent potential degradation of 1-NITC and 1-NA. Five μl NE (2.0 mg/ml) was added to 50 μl of each urine sample before assay.

3. Results

3.1. Specificity and selectivity

Figs. 2 and 3 display typical chromatograms resulting from HPLC analysis of the ACN precipitated rat plasma and urine. Blank rat plasma and urine do not demonstrate any interference peaks (Figs. 2a and 3a). The mixture of 1-NITC, 1-NA and 1-NIC (200 ng/ml each) and internal standard in ACN solution are well separated from one another with retention times (t_R) of 1-NA (2.2 min), NE (3.2 min), 1-NIC (3.7 min), and 1-NITC (5.6 min) (Fig. 2b). The rat plasma and urine samples spiked with 1-NITC, 1-NA, 1-NIC and NE standards show similar results (Figs. 2c and 3b), except that 1-NIC is absent due to possible rapid degradation in plasma and urine samples (Figs. 2d and 3c). 1-NITC, 1-NA, and NE are separated well from potentially interfering endogenous plasma and urine compounds under the current optimal chromatographic conditions (Figs. 2a,c,d, and 3a–c). In biological samples obtained after the i.v. administration of 1-NITC to a rat, 1-NITC and 1-NA were the only compounds that could be detected in plasma (Fig. 2e) and urine (Fig. 3d), respectively.

3.2. Lower limit of quantitation (LLQ)

The LLQ of 1-NITC, 1-NA, and 1-NIC was determined in blank rat plasma and urine samples, as well as in ACN solution. As shown in Table 1, the lower limit of quantitation (LLQ) of 1-NITC, 1-NA, and 1-NIC are dependent on the matrix. The LLQ of 1-NITC is 10 ng/ml for plasma and ACN samples, and 30 ng/ml for urine samples. The LLQ of 1-NA is about three-fold more than 1-NITC, i.e. 30 ng/ml for blank rat plasma and ACN, and 100 ng/ml for blank rat urine. 1-NIC can be detected only in ACN with a LLQ of 30 ng/ml.

3.3. Linearity

The linear regression correlation coefficient *r* was more than 0.999 in every standard curve (data not shown). The linearity for 1-NITC and 1-NA was tested over a concentration range of 10–5000 ng/ml and 30–5000 ng/ml, respectively, in rat plasma. For rat urine samples, the calibration curves of 1-NITC and 1-NA were linear over the concentration range of 30–5000 and 100–50 000 ng/ml, respectively.

3.4. Accuracy, precision and recovery

As shown in Table 2, at low (10 ng/ml), medium (500 ng/ml), and high (5000 ng/ml) concentrations of 1-NITC, the within- and between-day accuracy were 95–106 and 97–103%, respectively. The with-

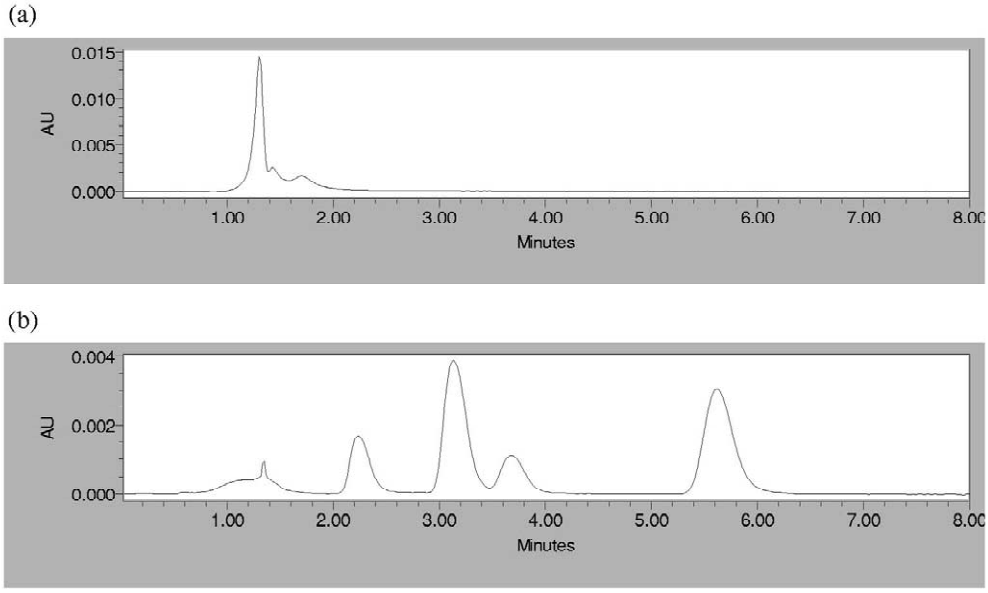

Fig. 2. Typical chromatograms for rat plasma samples obtained from the analysis of (a) blank plasma. (b) ACN containing 1-NITC (200 ng/ml), 1-NA (200 ng/ml), 1-NIC (200 ng/ml), and NE (10 μg/ml). (c) Blank plasma with added 1-NITC (200 ng/ml), 1-NA (200 ng/ml), 1-NIC (200 ng/ml), and NE (10 μg/ml), following protein precipitation with ACN; (d) blank plasma with added 1-NITC (200 ng/ml), 1-NA (200 ng/ml), and NE (10 μg/ml), following protein precipitation with ACN with the supernatant spiked with 1-NIC (200 ng/ml); (e) a 2-h rat plasma sample obtained after an i.v. bolus of 25 mg/kg 1-NITC. Chromatographic peaks were identified with the aid of pure reference standards based on retention time t_R, including 1-NA (2.2 min), NE (3.2 min), 1-NIC (3.7 min), and 1-NITC (5.4–5.9 min in different matrices).

in- and between-day precision values were 97–100 and 93–97%, respectively. Moreover, the protein precipitation with ACN for plasma samples resulted in the recovery of 1-NITC between 93 and 97% for both within- and between-day analysis.

At low (100 ng/ml), medium (5000 ng/ml), and high (50 000 ng/ml) concentrations of 1-NA, the within- and between-day accuracy was 96–106%, precision 97–99%, and recovery 95–110% (Table 3).

3.5. Stability

1-NITC was stable at temperatures of −20 °C and −80 °C in plasma, urine, ACN precipitated plasma and urine (Fig. 4a–d), and at all tested temperatures in ACN over 96 h (data not shown). However, 1-NITC degraded at RT and 4 °C in plasma, urine, and ACN precipitated plasma and urine (Fig. 4a–d). The faster degradation at RT than at 4 °C indicated a temperature-dependent pattern in each matrix (Fig. 4a–d). Moreover, the degradation of 1-NITC in plasma (Fig. 4a) and urine (Fig. 4c) was greater than that in ACN precipitated plasma (Fig. 4b) and urine (Fig. 4d) at same temperatures (RT and 4 °C). The degradation of 1-NITC in ACN diluted urine (Fig. 4d) was much slower than ACN precipitated plasma (Fig. 4b); 1-NITC was stable when prepared in ACN at all temperatures (Fig. 4e). 1-NITC degraded with very similar patterns over the pH range of 2–10 over a 96-h period (Fig. 4f). A different pattern of degradation was observed at pH 11 (Fig. 4f); at pH 12 there was instantaneous degradation (data not shown). The degradation product of 1-NITC in universal buffer was confirmed to be 1-NA (data not shown). The degradation product of 1-NITC in plasma, urine, and ACN extracts of plasma and urine was not identified.

1-NA was stable in all matrices at RT, 4, −20, and −80 °C with quantitation variation less than 10% during individual test periods (plasma data only is shown in Fig. 4g); it was also stable over the pH

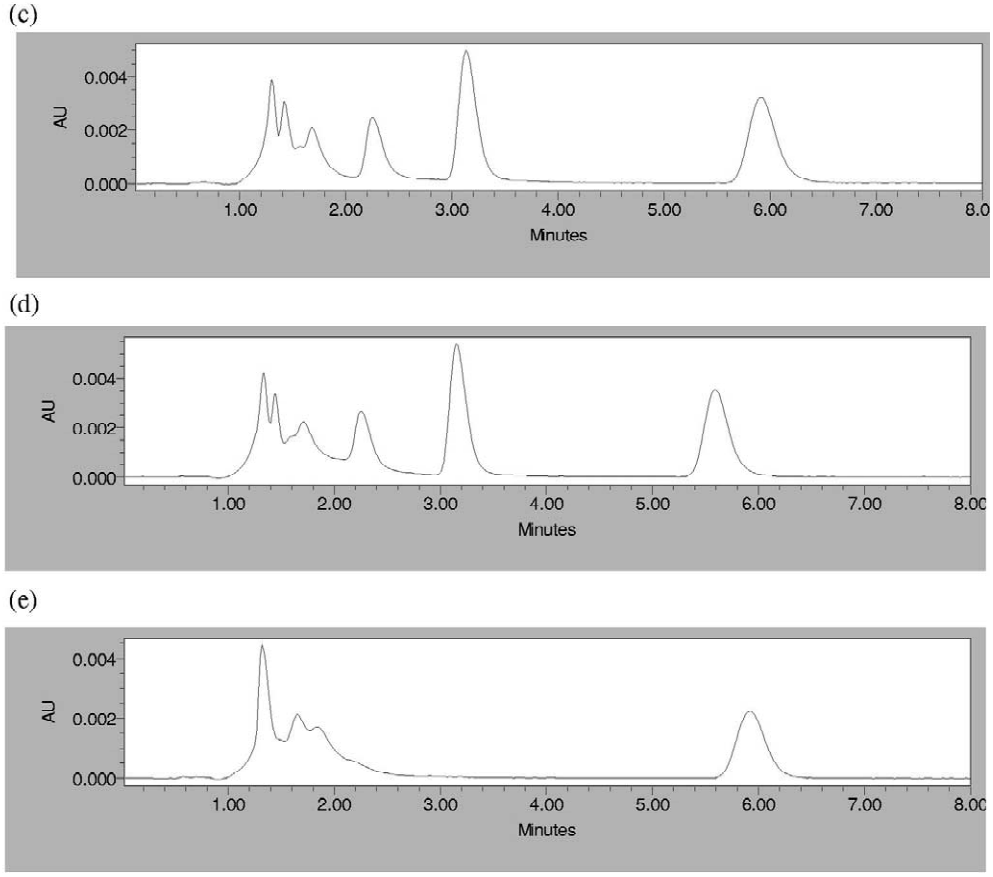

Fig. 2. (*continued*)

range of 2–12 (data not shown). In comparison, 1-NIC was stable when prepared in ACN (data not shown) but rapidly degraded in plasma (Fig. 2d), urine (Fig. 3c) and in ACN precipitated plasma and urine (data not shown). In universal buffer, 1-NIC was rapidly degraded to form 1-NA (data not shown).

3.6. Application of assay in rat pharmacokinetic studies

The described analytical method was used to analyze plasma and urine samples following the administration of 1-NITC (25 mg/kg i.v.) to a rat. The parent drug 1-NITC and metabolite 1-NA were the only compounds that could be detected in plasma and urine samples, respectively (Figs. 2e and 3d). The concentration of 1-NITC in plasma over 24 h and 1-NA in urine over 25 h are given in Tables 4 and 5 and plasma data are plotted in Fig. 5. Using this HPLC assay, 1-NITC and 1-NA were quantified in rat plasma and urine, respectively (Tables 4 and 5). Analysis of plasma samples allowed the determination of the pharmacokinetic parameters for 1-NITC (clearance of 2.07 l/kg/h, apparent volume of distribution of 14.3 l/kg, and elimination half life of 4.76 h). The metabolite 1-NA was present in urine samples but the total recovery was about 0.4%.

4. Discussion

A rapid and sensitive high-performance liquid chromatographic (HPLC) assay for the determination

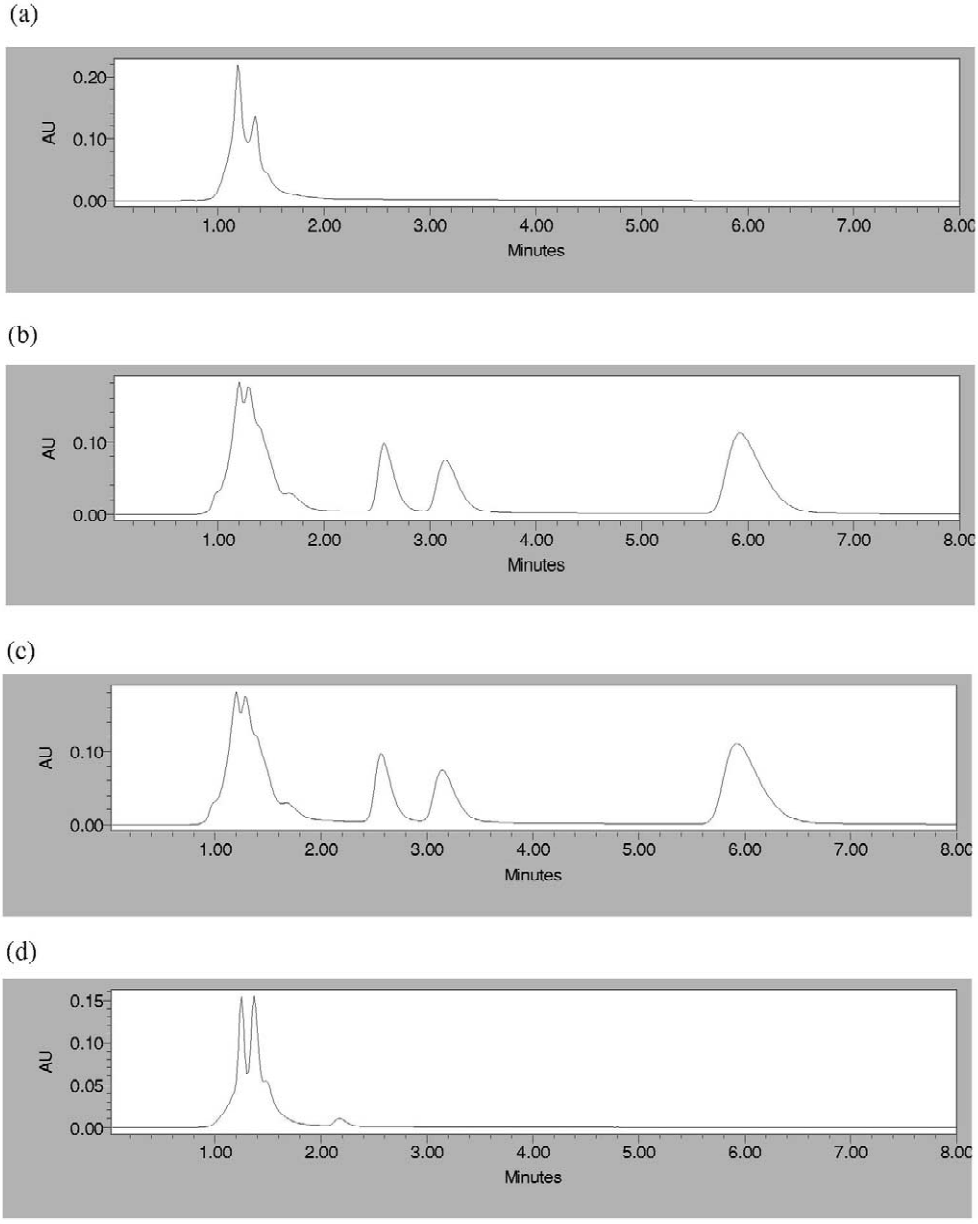

Fig. 3. Typical chromatograms for rat urine samples obtained from the analysis of (a) blank urine. (b) Blank urine with added 1-NITC (10 μg/ml), 1-NA (10 μg/ml), 1-NIC (10 μg/ml), and NE (40 μg/ml), followed by dilution with ACN; (c) blank urine with added 1-NITC (10 μg/ml), 1-NA (10 μg/ml), and NE (40 μg/ml), following dilution with ACN with the supernatant spiked with 1-NIC (10 μg/ml). (d) A urine sample obtained 2–4 h after an i.v. bolus of 25 mg/kg 1-NITC. Chromatographic peaks were identified with the aid of pure reference standards based on retention time t_R, including 1-NA (2.2–2.6 min), NE (3.2 min), and 1-NITC (6.0 min).

of α-naphthylisothiocyanate (1-NITC) and two metabolites α-naphthylisothiocyanate (1-NA) and α-naphthylisocyanate (1-NIC) in rat plasma and urine has been developed. The features of the assay include the use of a reversed-phase column, UV detection, protein precipitation using ACN, and the

Table 1
The lower limit of quantitation of 1-NITC, 1-NA, and 1-NIC in rat plasma, urine and ACN

Compounds	LLQ in plasma (ng/ml)	LLQ in urine (ng/ml)	LLQ in ACN (ng/ml)
1-NITC	10	30	10
1-NA	30	100	30
1-NIC	ND	ND	30

ND: not detected in blank plasma and urine samples.

use of an internal standard. Through an extensive evaluation of the stabilities of 1-NITC and its metabolites in different biological matrices, we have optimized the conditions for the collection and storage of biological samples.

Based on the features of chemical structures (Fig. 1), naphthylene (NE) was selected as an ideal internal standard candidate. Additionally we found that other chemically unrelated compounds, such as acetophenone and propiophenone, could also be used as the internal standard in this assay. A single UV wavelength of 305 nm was used for the detection of 1-NITC, 1-NA, and 1-NIC in rat plasma and urine samples since we obtained the greatest sensitivity and minimal interference by endogenous compounds present in plasma and urine at this wavelength. Under the current HPLC conditions, the LLQ values were 0.5 ng (10 ng/ml) and 1.5 ng (30 ng/ml) for 1-NITC in plasma and urine, 1.5 ng (30 ng/ml) and 5 ng (100 ng/ml) for 1-NA in plasma and urine, respectively (Table 1).

The extraction of plasma samples was optimized by the use of a protein precipitation step with ACN at 4 °C. Using protein precipitation of plasma samples was more convenient and time-saving than liquid–liquid extraction and solid-phase extraction, and resulted in the least amount of interference with endogenous compounds, while retaining high extraction efficiency. Other organic solvents, such as methanol and acetone, were also investigated in our preliminary studies but produced endogenous interferences and/or variability in recovery. An extraction step for urine samples using ACN, methanol, acetone, and acetyl acetate (EtOAc) was also investigated, since the direct injection of urine supernatant resulted in tailing peaks of 1-NITC, 1-NA, and NE (data not shown). Extraction of urine samples with ACN at 4 °C resulted in the best accuracy, precision, and recovery.

The isothiocyanate group (N=C=S) in 1-NITC and the isocyanate group (N=C=O) in 1-NIC are highly reactive, undergoing hydrolysis. Therefore, the stabilities of 1-NITC, 1-NA, and 1-NIC were systematically investigated with regards to matrix and temperature effects over time. 1-NA was stable in all tested matrices at all tested temperature. However,

Table 2
The within- and between-day accuracy, precision, and recovery for 1-NITC in rat plasma

	QC (ng/ml)	Accuracy (%)	Precision (%)	Recovery (%)
Within-day	10	106	97.6	93.2
	500	97.5	99.8	97.4
	5000	95.4	98.9	94.6
Between-day	10	102	92.9	95.9
	500	97.3	96.5	96.7
	5000	99.3	96.7	96.1

Table 3
The within- and between-day accuracy, precision, and recovery for 1-NA in rat urine

	QC (ng/ml)	Accuracy (%)	Precision (%)	Recovery (%)
Within-day	100	106	98.7	102
	5000	98.2	99.4	107
	50 000	100	98.4	110
Between-day	100	105	97.4	95.4
	5000	96.3	97.7	104
	50 000	100	99.3	107

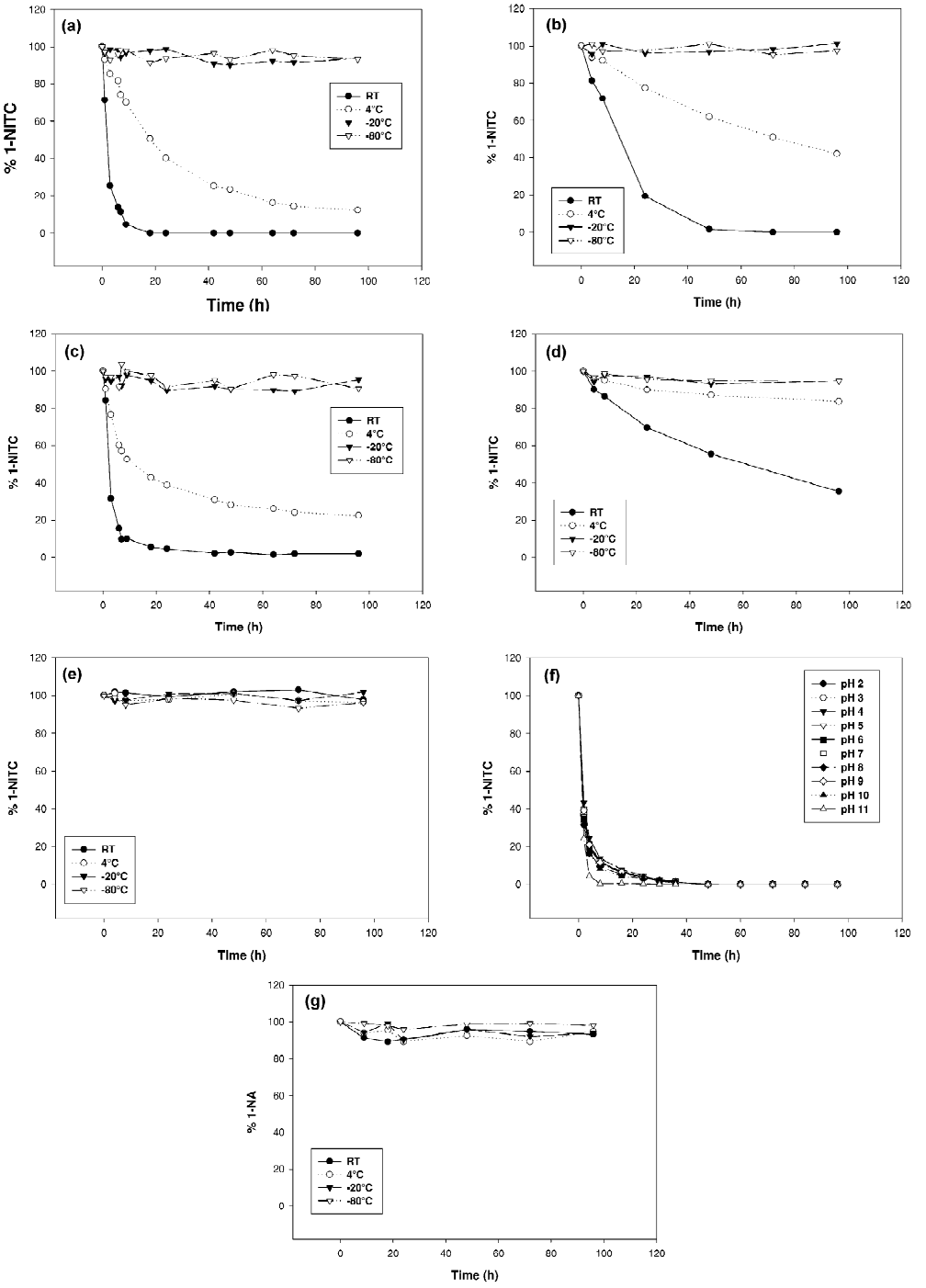

Fig. 4. The stability of 1-NITC, 1-NA, and 1-NIC in rat plasma, urine, ACN precipitated plasma and urine, ACN, and universal buffer at RT, 4, −20, and −80 °C over 96 h. (a) The stability of 1-NITC in rat plasma at RT, 4, −20, and −80 °C. (b) The stability of 1-NITC in ACN precipitated rat plasma at RT, 4, −20, and −80 °C. (c) The stability of 1-NITC in rat urine at RT, 4, −20, and −80 °C. (d) The stability of 1-NITC in ACN diluted rat urine at RT, 4, −20, and −80 °C. (e) The stability of 1-NITC in ACN at RT, 4, −20, and −80 °C. (f) The stability of 1-NITC in universal buffer pH 2–11 at RT. (g) The stability of 1-NA in rat plasma at RT, 4, −20, and −80 °C.

Table 4
Concentrations of 1-NITC in rat plasma samples following a 25 mg/kg i.v. dose

Time	Conc. (ng/ml)
5 min	10 490±400
10 min	7405±498
20 min	4172±140
30 min	3312±118
1 h	1692±77
2 h	1016±48
4 h	702±36
6 h	620±29
9 h	351±15
12 h	150±12
24 h	

Data is mean±SD; $n=3$.

Table 5
Urinary excretion of 1-NA following a 25 mg/kg i.v. dose of 1-NITC to a female rat

Time interval (h)	Vol. (ml)	Conc. (μg/ml)	Amount (μg)
0–2	8.2	0.42±0.04	3.44±0.33
2–4	3.8	2.02±0.23	7.68±0.87
4–6	1.5	2.64±0.35	3.96±0.52
6–9	1.5	2.17±0.25	3.26±0.37
9–24	30	–	–
24–25	3.2	–	–
0–25			18.34±2.09

Data is mean±SD, $n=3$. –: below detection limit.

the stabilities of 1-NITC and 1-NIC varied under different experimental conditions. The stability of 1-NITC was temperature-dependent in plasma, urine and ACN extracts of plasma and urine, i.e. stable at −20 and −80 °C but degraded at RT and 4 °C. Therefore, the plasma and urine samples obtained in our animal study were centrifuged at 4 °C and stored immediately at −80 °C. The standards of 1-NITC in plasma and urine for calibration curves and QCs were prepared individually on ice and assayed immediately at 4 °C using an autosampler. Under these conditions, the degradation of 1-NITC was less than 5% within 1 h for plasma samples and within 4 h for ACN extracts of plasma at 4 °C.

Our stability studies showed that 1-NITC and 1-NA were stable in plasma and urine at −80 °C when stored for more than 2 months (data not shown). The temperature-independent stability of 1-NITC in ACN indicated that ACN is an ideal extraction solvent for 1-NITC. In addition, the pH-independent degradation of 1-NITC in universal buffer further confirmed its high lability to hydrolysis. The degradation of 1-NITC at pH values of 2–10 was very similar to that of 1-NITC in plasma and urine samples at RT.

The isocyanate group was more reactive than the isothiocyanate group based on our study results. 1-NIC instantly degraded in aqueous matrix, i.e. plasma, urine, ACN precipitated plasma and urine,

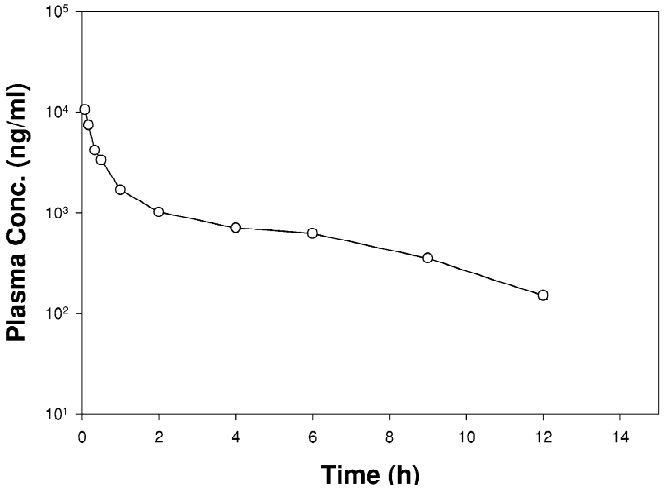

Fig. 5. Log plasma concentration vs. time relationship for 1-NITC after an i.v. bolus dose of 25 mg/kg.

and universal buffer. Although the degradation product of 1-NIC in plasma, urine, and ACN precipitated plasma and urine was not identified, the degradation product in universal buffer was confirmed to be 1-NA. In addition, the information on the stability in ACN indicated that 1-NIC (t_R 3.7 min) is stable in the mobile phase (ACN–H_2O 70:30, v/v) for at least 4 min, but probably shorter than 15 min (10 min for sample preparation and 5 min for mobile phase elution). Therefore the lack of detection of 1-NIC was probably due to its instability in the plasma and urine samples.

Using this HPLC assay, the concentrations of 1-NITC and 1-NA in rat plasma and urine, respectively, were determined (Tables 4 and 5). Our results agree with previous investigations demonstrating no unchanged 1-NITC in urine samples [12]. Analysis of plasma samples allowed the determination of the pharmacokinetic parameters for 1-NITC (clearance of 2.07 l/kg/h, apparent volume of distribution of 14.3 l/kg, and elimination half life of 4.76 h). The metabolite 1-NA was present in urine samples but the total recovery was low (0.4% of the injected dose of 1-NITC) indicating that 1-NITC and its metabolites may be eliminated by other mechanisms such as biliary excretion and CO_2 expiration, as reported by Capizzo and Roberts [11]. As well, there may be other unidentified metabolite(s) in urine rather than 1-NA.

5. Conclusion

In this paper, we have described a reversed-phase HPLC method for the quantitative determination of 1-NITC and metabolites 1-NA and 1-NIC in rat plasma and urine. The sample pretreatment procedure is based on a rapid precipitation step with ACN for both plasma and urine, thereby eliminating the need of laborious liquid–liquid extraction and solid-phase extraction techniques. The assay provides high sensitivity with LLQ values of 10, 30 and 10 ng/ml for 1-NITC in plasma, urine and ACN. The analysis method is precise and accurate, with the within- and between-day precision and accuracy within the range of 90–110% for QCs at low, medium and high concentration levels. The stability studies showed that 1-NITC was stable at all tested temperatures in ACN, and at −20 and −80 °C in plasma, urine, and ACN extracts of plasma and urine, but degraded at RT and 4 °C. In universal buffer (pH 2–12) at RT, 1-NITC degraded with similar patterns at pH values ranging from 2 to 10; there was rapid degradation at pH 12. 1-NA was stable in all tested matrix at all temperatures (RT to −80 °C). 1-NIC was unstable with rapid degradation in plasma, urine, and ACN extracts of plasma and urine; however, 1-NIC was stable in ACN. The HPLC assay was successfully used in a preliminary rat pharmacokinetic study to analyze plasma and urine samples following the i.v. administration of 25 mg/kg 1-NITC.

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References

[1] P. Talalay, Y. Zhang, Biochem. Soc. Trans. 24 (1996) 806.
[2] S.S. Hecht, Drug Metab. Rev. 32 (2000) 395.
[3] S. Sasaki, J. Natl. Med. Assoc. 14 (1963) 101.
[4] H. Sidransky, N. Ito, E. Verney, J. Natl. Cancer Inst. 37 (1966) 677.
[5] N. Ito, Y. Hiasa, Y. Konishi, M. Marugami, Cancer Res. 29 (1969) 1137.
[6] A. Lacassagne, L. Hurst, M.D. Xuong, C.R. Seances Soc. Biol. Fil. 164 (1970) 230.
[7] S. Makiura, Y. Kamamoto, S. Sugihara, K. Hirao, Y. Hiasa, Gann 64 (1973) 101.
[8] E. Tseng, A. Kamath, M.E. Morris, Pharm. Res. 19 (2002) 1509.
[9] S. Goldfarb, E.J. Singer, H. Popper, Am. J. Pathol. 33 (1962) 685.
[10] F. Capizzo, R.J. Roberts, Toxicol. Appl. Pharmacol. 17 (1970) 262.
[11] F. Capizzo, R.J. Roberts, Toxicol. Appl. Pharmacol. 19 (1971) 176.
[12] S. Lock, H. Witschi, F.S. Skelton, G. Hanasono, G.L. Plaa, Exp. Mol. Pathol. 21 (1974) 237.
[13] F.S. Skelton, H. Witschi, G.L. Plaa, Exp. Mol. Pathol. 23 (1975) 171.

[14] M.C. Breschi, M. Ducci, M. Tacca, L. Mazzanti, M. Giusiani, G. Poggi, U. Palagi, Arzneimittelforschung 27 (1977) 122.
[15] G.J. Traiger, K.P. Vyas, R.P. Hanzlik, Chem.-Biol. Interact. 52 (1985) 335.
[16] Y. Li, I.M. Yousef, G.L. Plaa, Liver 15 (1995) 271.
[17] A.K. Connolly, S.C. Price, D. Stevenson, J.C. Connelly, R.H. Hinton, Liver Cells Drugs 164 (1988) 191.
[18] L. Carpenter-Deyo, D.H. Marchand, P.A. Jean, R.A. Roth, D.J. Reed, Biochem. Pharmacol. 42 (1991) 2171.
[19] P.A. Jean, M.B. Bailie, R.A. Roth, Biochem. Pharmacol. 49 (1995) 197.
[20] P.A. Jean, R.A. Roth, Biochem. Pharmacol. 50 (1995) 1469.
[21] D.A. Hill, R.A. Roth, Toxicol. Appl. Pharmacol. 148 (1998) 169.
[22] D.A. Hill, P.A. Jean, R.A. Roth, Toxicol. Sci. 47 (1999) 118.