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Simultaneous determination of nitroglycerin and dinitrate metabolites in metabolism studies using liquid chromatography–mass spectrometry with electrospray ionization

Takashi Miyayama [1], Pei-Suen Tsou [1], Sun-Mi Fung, Ho-Leung Fung *

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, Buffalo, NY 14260-1200, USA

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Abstract

We have developed a liquid chromatographic–mass spectrometric method for the simultaneous determination of nitroglycerin (NTG) and its active metabolites, glyceryl 1,2-dinitrate (1,2-GDN) and glyceryl 1,3-dinitrate (1,3-GDN), for metabolism studies in cell cultures. 1,2,4-Butanetriol-1,4-dinitrate was chosen as an internal standard. Using a linear gradient of water/methanol containing 0.025 mM NH_4Cl, the compounds were eluted within 12.5 min on an Allure Aqueous C_{18} column (100 mm × 2.1 mm). Detection and quantification was achieved with multiple reaction monitoring in the negative ion mode. Intra- and inter-day variabilities for simultaneous determination of the three nitrates were below 10 and 18%, respectively, over a range of NTG and GDN concentrations of 0.5–15 ng/ml. The lower limit of quantification was found to be about 0.01 ng on column. Application of this method was illustrated through in vitro metabolism studies of NTG in culture media bathing LLC-PK1 cells and human vascular smooth muscle cells (HA-VSMC) at 37 °C. The degradation half-life of NTG was found to be 4.5 ± 0.4 h and 39.2 ± 0.02 h, respectively, for LLC-PK1 cells versus HA-VSMC. At 5 h, the 1,2-GDN versus 1,3-GDN metabolite distribution ratio in the bathing medium was found to be 1.5 ± 0.1 and 0.2 ± 0.02 for LLC-PK1 and HA-VSMC cells, respectively. With this method, the degradation half-life of NTG in rat plasma at 37 °C was shown to be 26.8 ± 1.8 min, consistent with previous values obtained using gas chromatography.

Keywords: LC–MS; Nitroglycerin; Dinitrate; Metabolism; LLC-PK1 cells; Rat plasma; Human vascular smooth muscle cells

1. Introduction

Nitroglycerin (NTG) has been used extensively in treating cardiovascular diseases for over a century. In various organs and tissues, including blood vessels, NTG is extensively metabolized via sequential denitration, yielding dinitrates and mononitrates, NO, and inorganic NO_2^- ions. The production of glyceryl 1,2-dinitrate (1,2-GDN) has been used as an index of "mechanism-based" metabolism because its production has been shown to parallel that of vasorelaxation [1,2], while the production of glyceryl 1,3-dinitrate (1,3-GDN) appears to be unrelated to relaxation and has been associated with nonenzymatic degradation of NTG [3]. In metabolism studies of NTG, therefore, it is useful to monitor 1,2-GDN and 1,3-GDN concurrently with NTG in order to provide possible mechanistic insights. Presently, most investigators employ either thin layer chromatography-liquid scintillation spectrometry (TLC-LSS) [1,4,5] or gas chromatography (GC) with either electron capture detector or mass spectrometry [6-9] for these studies. The TLC-LSS method produces good resolution but suffers from low sensitivity [10] and lack of an internal standard, while the GC system requires solvent extraction and may be affected by problems such as thermal decomposition and adsorption [11]. Although the detection of organic nitrates using liquid chromatography–mass spectrometry (LC–MS) has been recently reported [11], an LC–MS analytical method that separates and quantifies NTG

* Corresponding author. Tel.: +1 716 645 2842x222; fax: +1 716 645 3693.
E-mail address: hlfung@buffalo.edu (H.-L. Fung).
[1] Contributed equally to the project.

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and its dinitrate metabolites in metabolism studies has not been developed.

2. Experimental

2.1. Chemicals and reagents

NTG stock solution was obtained as a 5 mg/ml injection in 50% (v/v) each of dehydrated alcohol and propylene glycol (Abbott Laboratories, North Chicago, IL) and diluted before use. In the plasma degradation study, NTG was obtained through dissolving a sublingual tablet (NitroQuick®, 0.6 mg, Ethex Corp., St. Louis, MO) in water, and the supernatant was diluted after centrifugation. 1,2-GDN, 1,3-GDN, 1,2-propanediol dinitrate (PDDN), pentaerythritol tetranitrate (PETN) and 1,2,4-butanetriol trinitrate (BTTN) were purchased from Cerilliant (Austin, TX). 1,2,4-Butanetriol-1,4-dinitrate (BTDN) was obtained from Absolute Standards, Inc. (Hamden, CT). Isosorbide-2-mononitrate (IS-2-MN), isosorbide-5-mononitrate (IS-5-MN) and isosorbide dinitrate (ISDN) were previously obtained from Schwarz Pharma (Monheim, Germany). HPLC grade water and methanol were obtained from Burdick & Jackson (Muskegon, MI). Ammonium chloride was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). All cell culture chemicals/media were obtained from Gibco (Grand Island, NY), except otherwise stated. The F-12 medium was composed of 48 substances, including inorganic salts, amino acids, vitamins, etc., at varying concentrations [12].

2.2. Calibration standards

Solutions containing NTG, 1,2-GDN, 1,3-GDN and the eventual internal standard (BTDN, IS) were prepared, with final concentrations of the three nitrate analytes ranging from 0.5 to 15 ng/ml, and the IS concentration was set at 2.5 ng/ml. The solvent mixture contained 5% F-12 medium, 45% water and 50% methanol.

2.3. Incubation of NTG with cell cultures

LLC-PK1 cells (porcine kidney epithelial cells, American Type Culture Collection, Manassas, VA) were grown in F-12 culture medium supplemented with 15% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin at 37 °C in a humidified atmosphere with 5% CO_2/95% air. The cells were grown to confluence in 35 mm tissue culture dishes, and were washed twice with phosphate-buffered saline before the experiment. Human vascular smooth muscle cells (HA-VSMC, CRL-1999) were purchased from American Type Culture Collection (ATCC, Manassas, VA), and maintained in F-12K medium (ATCC, Manassas, VA), supplemented with 50% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 5.5 mg/L sodium selenite, 10 mg/L insulin, 6.7 μg/L transferrin (Gibco. Grand Island, NY), and 20 mg/L endothelial cell growth supplement (Sigma, St. Louis, MO). These cells were grown to confluence in T25 flasks, and were washed twice with phosphate-buffered saline before the experiment.

Nitroglycerin (final concentration 1 μM, $n = 3$ for each cell type) was added to the respective fresh culture medium. In these incubations, the medium contained 0.002% each of ethanol and propylene glycol (from the NTG stock solution). Samples were collected at 0, 1, 2, 5 and 10 h (for LLC-PK1 cells), and 0, 1, 2, 5, 12, 24, and 48 h (for HA-VSMC). The samples were stored in the freezer at −20 °C until analysis. They were then diluted 20 times with 50% methanol and 50% water containing IS, and subjected to LC–MS analysis. For the determination of intracellular nitrate concentrations, the cells were collected after incubation, and 5 ng/ml BTDN was added. The mixture was sonicated for 10 min on ice, and centrifuged at ∼10,000 × g at 4 °C for 20 min. The supernatant was then diluted with an equal volume of water, and subjected to LC–MS analysis.

2.4. Rat plasma study

Fresh plasma from Sprague–Dawley rats was obtained after centrifuging rat blood at 2000 × g for 11 min. After incubating the plasma ($n = 3$ replicates) at 37 °C for 10 min, nitroglycerin (1 μM, prepared by dissolving sublingual tablets in water) was added. Samples were collected at 0, 0.25, 0.5, 1, 2 and 3 h, and stored at −20 °C until analysis. A 50 μL aliquot was mixed with 50 μL of water and 300 μL methanol containing IS, to create an identical co-solvent ratio as used in calibration standards. After shaking on a rocking platform for 20 min, samples were centrifuged at ∼10,000 × g at 4 °C for 30 min. A volume of 100 μL of the supernatant was collected, diluted with 100 μL of water and 50 μL of methanol, mixed and subjected to LC–MS analysis.

2.5. LC–MS conditions

LC–MS analysis was conducted on a PE/SCIEX API 3000 triple quadrupole mass spectrometer (Forster City, CA), equipped with a turbo electrospray ionization source and Perkin-Elmer Series 200 HPLC system (Norwalk, CT). HPLC separation was achieved with an Allure Aqueous C_{18} column (100 mm × 2.1 mm I.D., 5 μm particle size, Restek Corporation, Bellefonte, PA) and a SecurityGuard cartridge (C_{18}, 4.0 mm × 3.0 mm I.D., Catalog #AJ0-4287, Phenomenex, Torrance, CA) at room temperature using solvent A (0.025 mM NH_4Cl in water) and solvent B (0.025 mM NH_4Cl in methanol). The analytes were eluted using the following linear gradient: 20–90% B over 10 min; then held at 90% B over 12.5 min, followed by 90–20% B over 12.6 min and held at 20% B over 15.5 min for re-equilibration prior to the next sample injection. The flow rate was maintained at 150 μl/min, and the sample injection volume was 20 μl. The turbo electrospray ionization source was set at a voltage of −4100 V and a temperature of 300 °C with both of the curtain gas and the nebulizing gas set at 8. The mass spectrometer was used in the negative ion mode and multiple reaction monitoring (MRM). Mass/charge ratios of precursor ions for NTG, 1,2-GDN, 1,3-GDN and IS were chosen at 262, 217, 217 and 231 ($[M + Cl]^-$, with M denoting

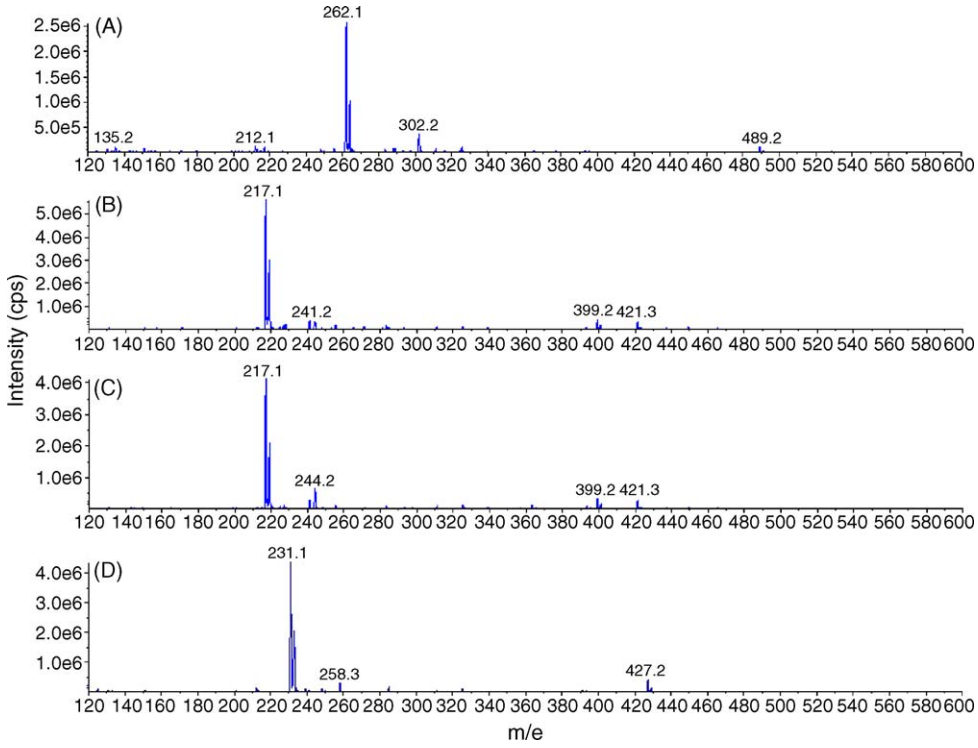

Fig. 1. Precursor ion mass spectra of the nitrates ionized with 0.025 mM NH_4Cl. (A) NTG, (B) 1,2-GDN, (C) 1,3-GDN and (D) BTDN (IS).

each compound, respectively (Fig. 1). Product ion was chosen at $m/e = 62$ (NO_3^-) for all analyzed compounds (Fig. 2). All data were acquired and analyzed using the Analyst® 1.4 software (Applied Biosystems, Forster City, CA).

3. Results and discussion

In the absence of NH_4Cl, the analytes exhibited poor detectability. With NH_4Cl, the precursor ion of the organic

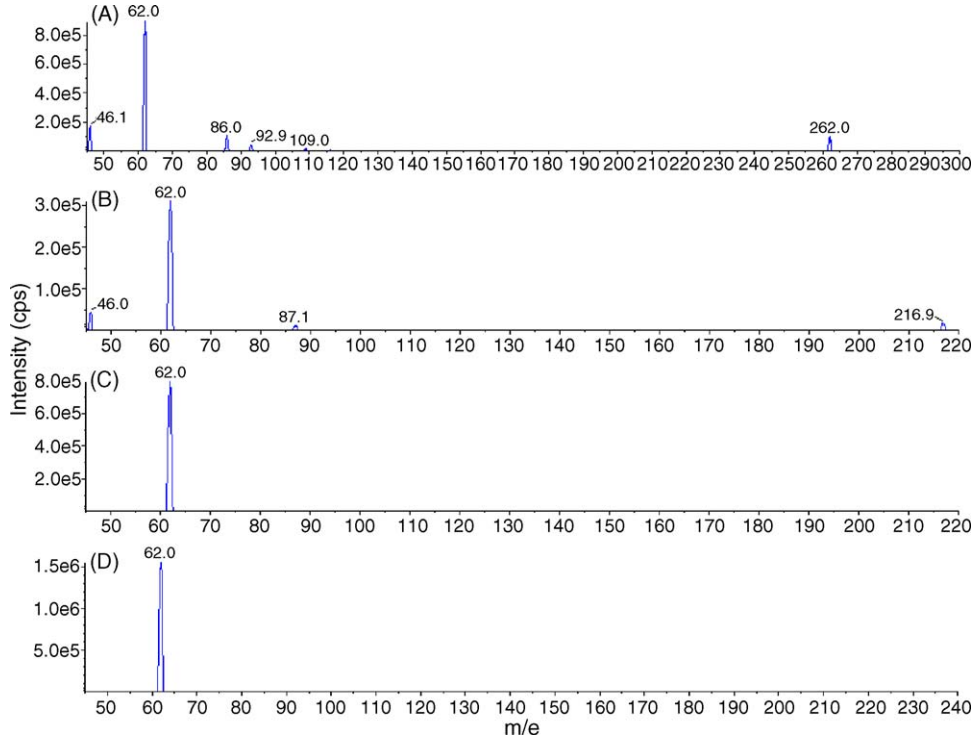

Fig. 2. Product ion mass spectra obtained by collision-induced dissociation of the nitrates at the precursor ion of $[M + Cl]^-$. (A) NTG, (B) 1,2-GDN, (C) 1,3-GDN and (D) BTDN (IS).

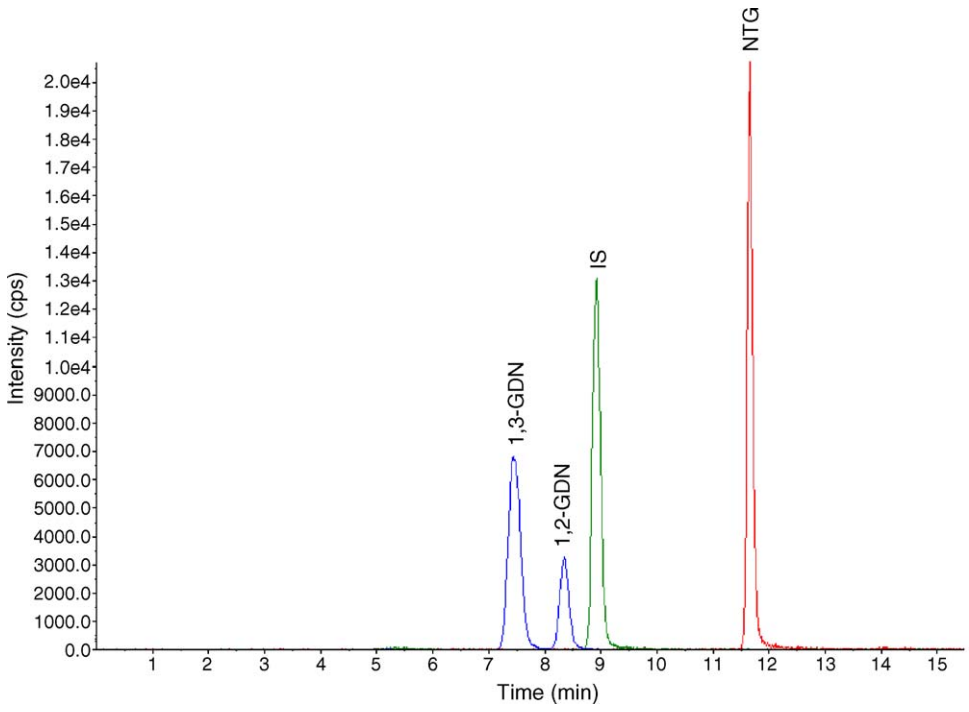

Fig. 3. The MRM chromatogram of a mixture containing NTG (5 ng/ml), 1,2-GDN (5 ng/ml), 1,3-GDN (5 ng/ml) and BTDN (2.5 ng/ml, IS).

nitrate [M] could be readily monitored as $[M+Cl]^-$, and the product ion as NO_3^- ($m/e = 62$). The stated HPLC conditions enabled the separation of the three analytes, 1,3-GDN, 1,2-GDN, and NTG, with corresponding retention times of 7.4, 8.3 and 11.6 min, respectively (Fig. 3). No interference peak was found in the blank samples during the chromatographic run. Several organic nitrates were examined as potential internal standards; these were IS-2-MN and IS-5-MN (both mononitrates), ISDN, PDDN and BTDN (dinitrates), BTTN (trinitrate), and PETN (tetranitrate). Among these compounds, both BTTN (retention time = 12.0 min, precursor ion $[M+Cl]^-$, $m/e = 276$, product ion $m/e = 62$) and BTDN (retention time = 9.1 min, precursor ion $[M+Cl]^-$ $m/e = 231$, product ion $m/e = 62$) exhibited suitable characteristics as internal standards. BTDN was eventually selected because its retention time was situated among the analytes.

We examined the effect of NH_4Cl concentration in the eluting solvent on the sensitivity of the assay. Higher concentrations of NH_4Cl in the eluent decreased the sensitivity of the analytes (Fig. 4). Of the concentrations tested, 0.025 mM NH_4Cl was found to be satisfactory, and was therefore chosen in the final analytical procedure. Under the conditions used, the lowest quantifiable concentration (signal/noise ratio >3) was estimated to be 0.5 ng/ml (and with a 20 μl injection volume, an on-column amount of 0.01 ng). Intra- and inter-day variabilities were less than 10 and 18%, respectively, over the concentration range of 0.5–15 ng/ml for all three compounds ($n = 6$, Table 1). The calibration curves for all three analytes were linear within the range of 0.5–15 ng/ml, with $r^2 > 0.995$. The slope and intercept (mean ± S.D., $n = 3$) for the calibration curves of NTG (peak area ratio versus ng/ml) were 0.178 ± 0.036 and 0.0420 ± 0.0234, respectively. These parameters for 1,2-GDN were 0.0568 ± 0.0013 and 0.0003 ± 0.0069, and those for 1,3-GDN were 0.145 ± 0.010 and 0.0138 ± 0.0059, respectively. In a separate study using quality control samples in F-12 culture medium (Table 2) recovery of the three organic nitrates (at different concentration ratios when compared to the previous study) was found to be essentially complete, and the inter- and inter-day variabilities were all under 20% (using only triplicates). Thus, this method appears to exhibit satisfactory sensitivity, precision and accuracy.

Using this method with a one-point standard ($n = 4$ determinations), we found the NTG stock solution to contain 0.220 ± 0.204% of 1,2-GDN, and 0.945 ± 0.160% of 1,3-GDN

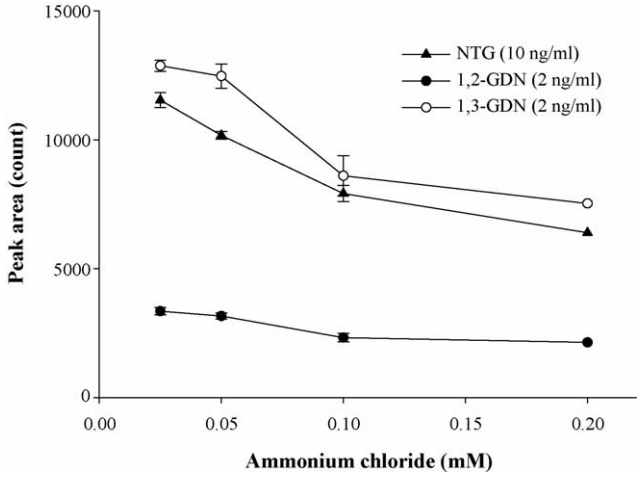

Fig. 4. Detector response of NTG, 1,2-GDN and 1,3-GDN as a function of NH_4Cl concentration in the mobile phase. Each point presents mean ± S.D. of three samples, except for 0.20 mM NH_4Cl (only duplicate samples).

Table 1
Intra- and inter-day variabilities of NTG and its dinitrate metabolites using the LC–MS assay ($n = 6$)

Analyte	Concentration (ng/ml)	Variability (in CV%)	
		Intra-day	Inter-day
NTG	0.5	9.1	17.4
	2.5	4.1	11.3
	15.0	3.2	12.2
1,2-GDN	0.5	3.4	7.6
	2.5	3.7	7.1
	15.0	2.5	6.2
1,3-GDN	0.5	3.6	15.8
	2.5	2.1	10.7
	15.0	2.1	11.9

The solutions were prepared in F-12 cell cultured medium as described.

as impurities. Similarly, the 1,2-GDN stock, as obtained, contained $0.435 \pm 0.193\%$ NTG, and $0.046 \pm 0.032\%$ 1,3-GDN as impurities. The 1,3-GDN contained $0.676 \pm 0.195\%$ NTG, and $3.16 \pm 0.61\%$ 1,2-GDN as impurities. These modest contaminations did not affect the establishment of the standard curves significantly.

Application of this analytical method was illustrated by in vitro metabolism studies using LLC-PK1 cells. The degradation of NTG (initial concentration = 1 μM) and the formation of 1,2-GDN and 1,3-GDN were simultaneously monitored in culture medium bathing LLC-PK1 cells at 37 °C as a function of time (Fig. 5). The half-life of NTG was found to be 4.5 ± 0.4 h ($n = 3$). The ratio of 1,2-GDN versus 1,3-GDN from NTG at 5 h was 1.5 ± 0.1. In the culture medium alone without LLC-PK1 cells, the degradation of NTG over the same time period was negligible. Analysis of intracellular amounts of NTG and its dinitrate metabolites revealed little accumulation of all three compounds, since their concentrations were all below the quantification limits.

LLC-PK1 cells have been used as a model to study vascular nitrate tolerance [13], and the typical protocol involved incubation of NTG at 1 μM for 5 h. This protocol produces a reduced biochemical response, as measured by cellular production of

Table 2
Intra- and inter-day variabilities observed in a recovery study for the determination of the three organic nitrates in quality control samples

Analyte	Concentration (ng/ml)	Recovery ($n = 3$ each)	
		Intra-day study (CV%)	Inter-day study (CV%)
NTG	1.5[a]	112 ± 9 (8.4)	92.4 ± 4.4 (4.8)
	4[b]	112 ± 14 (12.1)	105 ± 9 (8.2)
	12[c]	111 ± 7 (6.1)	114 ± 4 (3.4)
1,2-GDN	1.5[c]	116 ± 4 (3.7)	117 ± 1 (0.9)
	4[a]	96.0 ± 3.3 (3.4)	98.6 ± 2.4 (2.4)
	8[b]	97.1 ± 2.6 (2.7)	96.0 ± 2.4 (2.5)
1,3-GDN	1.5[a]	107 ± 7 (6.5)	98.7 ± 4.7 (4.7)
	4[b]	102 ± 3 (3.1)	107 ± 5 (4.9)
	8[c]	109 ± 2 (2.1)	95.5 ± 3.9 (4.1)

Letters (a–c) denote separate quality control solutions containing the specified amounts of the three analytes.

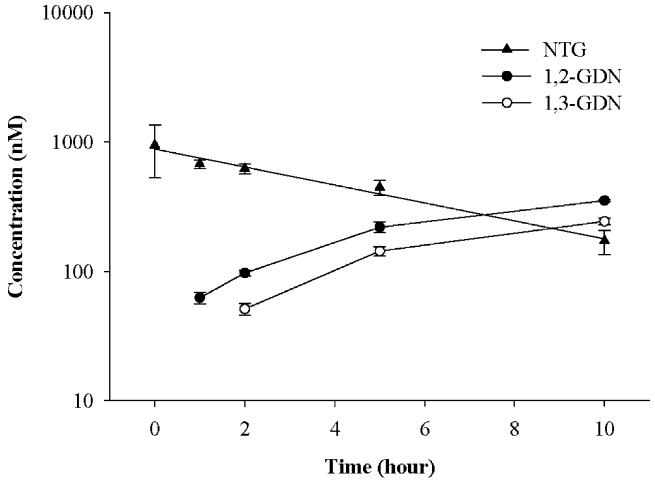

Fig. 5. Concentration–time profile of NTG, 1,2-GDN and 1,3-GDN in culture medium bathing LLC-PK1 cells. Each point represents mean ± S.D. of three samples except for 1,3-GDN at 2 h (one sample only).

cyclic guanosine-5′-monophosphate, cGMP. The present study shows that about more than half of the NTG added was lost during the 5 h incubation protocol, indicating that replenishment of NTG during the protocol may enhance the biochemical effect observed. The preference of formation of the 1,2-dinitrate metabolite versus its 1,3-isomer was consistent with the presence of mechanism-based clearance [1,4] of NTG by cellular proteins.

In a second application of this analytical method, we examined the rate of NTG metabolism in HA-VSMC culture. Interestingly, although this cell line was established from a normal human aorta, and blood vessels are highly sensitive to the pharmacological effects of NTG [14], NTG metabolism was comparatively slower ($t_{1/2} = 39.2 \pm 3.5$ h) in this cell line when compared to that in LLC-PK1 cells (data not shown). Additionally, the 1,2-GDN/1,3 GDN ratio at 5 h of incubation was found to be 0.2 ± 0.02, indicating that NTG degradation was more mediated by chemical degradation [3] than by metabolic activation [1,4]. Thus, although this cell line has been used as a model to study vascular function, e.g., response to angiotensin stimulation [15], its utility for studying NTG metabolism may be somewhat limited. It should be noted however that, in these studies, the culture medium contained 0.002% each of ethanol and propylene glycol, which might inhibit the activities of cellular degradation enzymes. Nevertheless, since the concentrations of these two solvents were identical for both cell lines, the relative ability of these cells to degrade NTG was likely reflected by our results.

To avoid the possible effects of these solvents, we employed sublingual NTG tablets to conduct a metabolism study in rat plasma. In this application, plasma calibration standards containing 0.5, 1, 2.5, 5, 10 and 15 ng/ml of each of the analytes were used. The r^2 of all standard curves for each analyte was ≥ 0.993 ($n = 3$–6 calibration standards on different days). The limit of quantitation in this study was set at the lowest concentration of the standards, i.e., at 0.5 ng/ml for all three analytes. The inter-day variability (CV%) of plasma standards at 0.5, 5

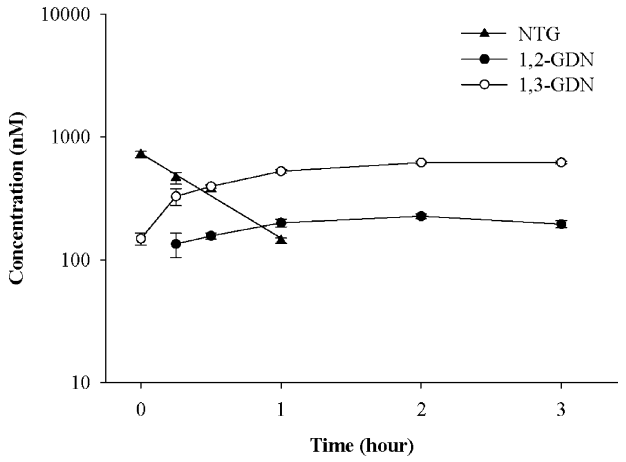

Fig. 6. Concentration-time profile of NTG, 1,2-GDN and 1,3-GDN in rat plasma. Each point represents mean ± S.D. of three replicates.

and 15 ng/ml of NTG, 1,2-GDN, 1, 3-GDN was <21% for each analyte (range = 3.2–20.2%, $n = 3$ replicates for each concentration). The intra-day variability (CV%) of the same standards was <12% for each analyte (range = 1.45–11.7%, $n = 6$ replicates for each concentration). Fig. 6 shows that NTG degrades with a half-life of 26.8 ± 1.8 min in rat plasma, consistent with the results ($t_{1/2}$ of 22 min) that we previously reported using gas chromatography [16]. We found also that the ratio of 1,3-GDN to 1,2-GDN to be about 3.

4. Conclusion

We have developed a new LC–MS method capable of simultaneous quantitative determination of NTG and its dinitrate metabolites. The method exhibits satisfactory sensitivity, precision and accuracy. We believe that this method provides an alternative to the current TLC method using radiolabeled NTG; it can be applied to various metabolism studies of NTG in vitro, as well as for the simultaneous determination of NTG and its dinitrates in rat plasma.

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