

E-ISSN: 2476-9606

Abstract Proceedings International Scholars Conference

Volume 7 Issue 1, October 2019, pp. 1901-1907

<https://doi.org/10.35974/isc.v7i1.1993>

Kinetic Analysis on Decomposition of Pentaerythrityl Tetranitrate under Influence of Thiols

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ABSTRACT

Pentaerythrityl tetranitrate (PETN) is an organic nitrate compound used in the therapy as general vasodilator in the treatment of angina pectoris. PETN acts as prodrug for nitric oxide as active component. However, in clinical association, it is known a decreased ability of organic nitrate to do chemical reduction, which is metabolic tolerance, by upregulation in endogenous thiol system. It has been proved that supplementation of thiol is able to overcome tolerance to nitrate, nevertheless there is no comprehensive reports on interaction between organic nitrate and thiol compounds. The goal of this research is to study decomposition reaction kinetic of PETN under influence N-acetyl cysteine (NAC) and thiosalicylic acid (TSA) as thiol compounds, in the physiological condition. PETN as tracing compound were monitored by chromatographic system, with ratio of thiols used was equal, doubled, five times, and ten times folds. Data were analyzed kinetically by using math equation for reaction order 1, 2, and composite order 1 (equivalence). The result showed the linearity values on kinetic derivative, where correlation coefficient, r , is higher than 0.9 ($r \geq 0.9$) for composite order 1. The result will give scientific contribution about tolerance phenomena in organic nitrate, particularly in the role of thiol compound to overcome nitrate tolerance.

Keywords: Pentaerythrityl Tetranitrate, N-Acetyl Cystein, Thiosalicylic Acid, Kinetic Study

INTRODUCTION

Pentaerythrityl tetranitrate (PETN) is an long acting organic nitrate used in the therapy as anti-anginal medication caused by endothelial dysfunction in patients with coronary artery disease (Rutherford and Struthers, 2013). In animal models of diabetes and hypertension, PETN showed to improved vascular function. PETN has fewer side effects on the vascular, particularly in endothelial cell, than other nitrates like NTG (nitroglycerin), isosorbide-5-mononitrate (ISMN), or isosorbide dinitrate (ISDN) (Steven et al., 2017).

Reducing compounds especially thiols, e.g. N-acetyl cysteine (NAC), play a role in the metabolism in activating organic nitrates to nitrite then liberate NO. Thiols acts as cofactors in the enzymatic bioactivation, and as reducing agents in the non-enzymatic via formation of nitro- and nitroso-thioles as precursors (Kartasasmita, 2002; Seeling and Lehmann, 2006).

In the formation of nitric oxide (NO) as the endothelium-derived relaxing factor (EDRF), high potency of organic nitrates e.g. nitroglycerin (NTG), pentaerythrityl tetranitrate (PETN) and pentaerythrityl trinitrate (PETriN), were suggested to be bioactivated by mitochondrial aldehyde dehydrogenase (ALDH-2), where cytochrome P450s is responsible for low potency of organic nitrate e.g. isosorbide dinitrate (ISDN), isosorbide mononitrate (ISMN), pentaerythrityl dinitrate (PEDN), and pentaerythrityl mononitrate (PEMN) (Münzel et al., 2011). Due to lack of technical aspect to detect direct NO, as well as for quantification of NO as primary product in the reaction of nitrate and thiol, thus, analysis is being focuses on its donor as indirect measurement of NO levels (Suwitono et al., 2011).

This study aims to study decomposition reaction kinetic of PETN under influence of thiol, e.g. N-acetyl cysteine (NAC) and thiosalicylic acid (TSA), in the physiological condition. High performance liquid chromatography (HPLC) method was used to quantify the amount of PETN change due to its reaction with thiols.

METHODS

Chemicals

Pentaerythrityl tetranitrate (PETN) (product of synthesis according to (Lange et al., 2009), thiosalicylic acid (TSA), N-acetyl-L-cysteine (NAC) (puriss, Fluka), NaCl, NaOH, H₃PO₄, NaH₂PO₄.12H₂O, Na₂HPO₄, sulfanilamide, N-(1-naphthyl)-ethylenediamine, NaNO₂, HCl, sulfanilic acid, acetic acid, alpha-naphthylamine. The chemicals used in this experiment were all analytical grade. All solvents were HPLC grade.

Solution

Solution of PETN 0.6 mM were prepared by dissolving PETN with methanol, and added with water to have 50% methanol solution of PETN. Then, the solution was filtered through 0.45 µm membrane filter. Solution of 6 mM of TSA or NAC were prepared by dissolving TSA or NAC with small amount of methanol, and PBS solution was added to have concentrations ratios, which is equimolar, two, five and ten times of nitrates concentration.

Phosphate buffered saline (PBS) solution (pH 7.4, 10 mmol) were prepared by mixing NaH₂PO₄ with Na₂HPO₄ and NaCl in aqua bidestillata, to have phosphate strength of 10 mmol in physiological solution, and adjusted with either 10% NaOH or 10% phosphoric acid solution to have pH 7.4.

Incubation

Decomposition of PETN was analyzed by adding PETN solution with equal volume to each concentration ratios of TSA and NAC in a well-stoppered glass apparatus. The mixture was then incubated in thermostated water bath at 37 °C. Solution of PETN was added equivolume with PBS solution, and treated as control. The aliquot was sampled (n=3) at certain intervals of up to 240 minutes of incubation time, samples then freeze below -20 °C until ready to be analyzed by HPLC, but not more than 1 week.

HPLC Condition

HPLC condition is optimized and validated to analyzed PETN. The HPLC system was Hitachi D-7000 with L-7400 UV/Vis detector, using RP C-18 column (phenomenex bond clone, 300 x 3.9 mm, 10 µm). Volume of HPLC sample loop was 20 µl. PETN was detected at 215 nm wavelength. Mobile phase was isocratic acetonitrile:water with ratio 1:1, 1 ml/min

RESULTS

Validation of HPLC Condition

Validation of HPLC method was performed, and some parameter of HPLC condition to analyze PETN were obtained (PETN showed significant linear regression along its concentration from 12.5 µM up to 300 µM, (Figure 1) thus, this calibration equation could be used in conversion of concentration while detecting PETN.

Table 1). PETN was eluted at 12.7 min, where TSA and NAC were around 2.6 min. Examination of either TSA and NAC could not implement since newly product peak emerged at around TSA and NAC retention time, and piled up each other, therefore PETN used as tracing compound. PETN showed significant linear regression along its concentration from 12.5 µM up to 300 µM, (Figure 1) thus, this calibration equation could be used in conversion of concentration while detecting PETN.

Table 1. **Validation Parameter of Chromatographic System for Detection of PETN**

Parameter	Result
Model	Hitachi D-7000, Chromatography Data Station Manager V 4.0,
Wavelength	215 nm
Column	reverse phase C-18 (Phenomenex), 300 mm

	x 3,9 mm i.d., particle size: 10 µm
Retention time	PETN = 12.7 min., TSA & NAC = 2.6 min.,
Selectivity	21.2
Linearity	0,999
Detection limit	11,88 µM
Quantification limit	33.57 µM
Accuracy	0.70% (of <5%)
Variation coef.	2.82 (of <5%)

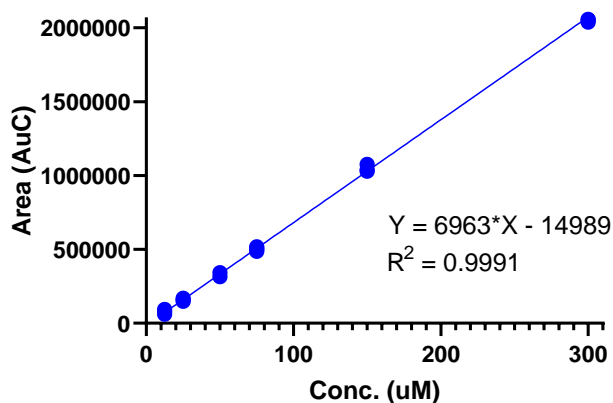


Figure 1. Calibration curve of PETN

Kinetic Decomposition

Decomposition of PETN were analyzed using validated HPLC method, under influence of TSA and NAC, in phosphate buffered-saline, mimic vascular buffer condition. Concentration of PETN showed extensive decreased in concentration while incubated with TSA and NAC, compared with its starting concentration, although at the end of incubation period there are still PETN remains (data is presented in Figure 2).

This condition applied for each variation ratios of TSA and NAC. Contrary, without neither TSA nor NAC, the profile showed stability by giving just slightly decreases of PETN. This result shows that TSA and NAC plays an important role in the degradation of PETN.

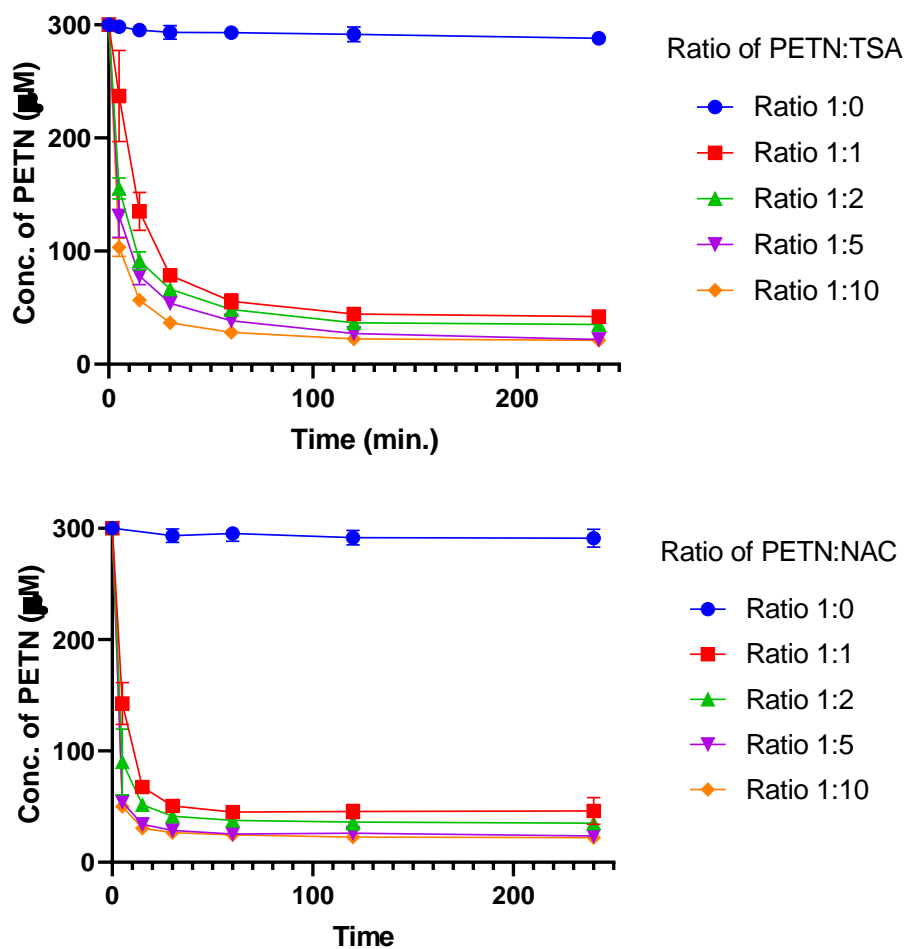


Figure 2. Decomposition profile of PETN under influence of a) TSA, and b) NAC

Kinetic Order

Reaction order kinetic were calculated based on equilibrium condition, which is showing a nearly stable condition (no more significant reduction of PETN). Calculation of reaction rate is attempted with several kinetic models with curve fitting method, using several reaction order equations, which is narrowed to 1st, 2nd and composite 1st order, showed in Table 2. Result of kinetic calculation from ordinate equation were shown in

Table 3, showing linearity significant value r, which is indicating acceptance of reaction order, with graphical plot of its mathematical derivation were shown in

Figure 3.

Table 2. Reaction Order Equations

Reaction order	1 st order	2 nd order	Composite 1 st order
Ordinate Equation	$\ln [A]$	$\frac{x}{[A]_0([A]_0 - x)}$	$\ln \frac{\{[A] - [A]_{eq}\}}{\{[A]_0 - [A]_{eq}\}}$

Table 3. Recapitulation of Significance Value (r) of the Trend Line

Reaction	Molar ratio	Significant value		
		Order 1	Order 2	Composite
PETN:NAC	1:1	0,80	0,87	0,93
	1:2	0,75	0,87	0,95
	1:5	0,69	0,83	0,90
	1:10	0,69	0,83	0,93
PETN:TSA	1:1	0,95	0,99	0,99
	1:2	0,89	0,97	0,96
	1:5	0,88	0,98	0,95
	1:10	0,85	0,96	0,96

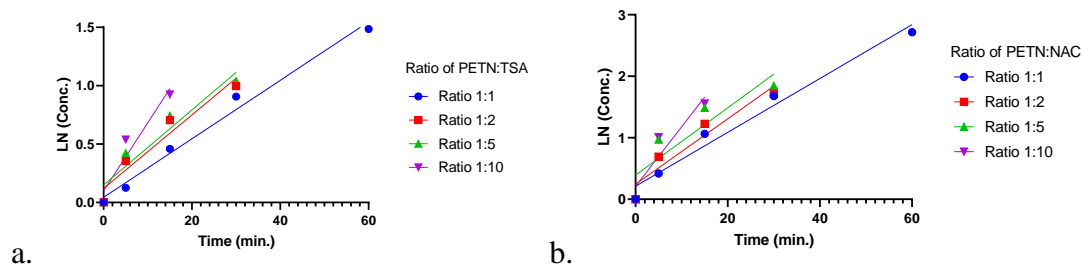


Figure 3. Plot of mathematical derivatization of PETN concentration, with a) TSA, and b) NAC

Composite first order was chosen as best fit kinetic order for PETN decomposition scheme. This result could be explained as reaction of PETN with TSA and NAC were showed equilibrium at certain concentration of PETN (about 20%). This might need further study to understand this phenomenon, including strength of phosphate buffer used, molar concentration of PETN and thiols used (TSA and NAC), different solvent, etc.

DISCUSSION

Conclusion

Analysis of PETN in this reaction scheme were performed using validated HPLC method. Chromatogram peak of PETN were completely separated from TSA and NAC, although simultaneous determination would not be possible due to the presence of products of reaction which are deleterious to NAC peak.

Result of this research confirmed that nitrate tolerance is occur when organic nitrate reacted with thiols, i.e. thiosalicylic acid (TSA) and n-acetyl-cystein (NAC). Nevertheless, the result showed that thiols plays important role in bioactivation of nitrates, as key on NO pathway.

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