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NO-donors, part X [1]: Investigations on the stability of pentaerythrityl tetranitrate (PETN) by HPLC-chemoluminescence-N-detection (CLND) versus UV-detection in HPLC

Andreas Seeling *, Jochen Lehmann

Institute of Pharmacy, Friedrich-Schiller-University Jena, Philosophenweg 14, D-07743 Jena, Germany

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Abstract

HPLC in combination with chemoluminescence-N-detection (CLND) is very useful for the analysis of pentaerythrityl tetranitrate (PETN) and its possible biological and chemical degradation products pentaerythrityl trinitrate (PETriN), pentaerythrityl dinitrate (PEDiN) and pentaerythrityl mononitrate (PEMonoN). Quantification is more convenient and sensitivity of this method is about four times higher compared to UV-detection. The present study demonstrates that PETN is a chemically more stable compound in vitro than expected. No degradation was observed in aqueous buffers (37 °C, pH 5.6, 7.4), human plasma, and simulated intestinal or gastric fluid. On the other hand, the addition of increasing amounts of thioles (cysteine, thioglycolic acid) induced an increasing degradation of PETN.

Keywords: HPLC; N-Selective detection; CLND; Organonitrate; PETN; Stability; Thioles

1. Introduction

Before starting a pharmacokinetic study it is obligatory to know all non-enzymatic degradation reactions of the substance to be investigated, because time-dependent chemical instabilities would compromise the determination of plasma levels, biotransformation-reactions, elimination half-lives, etc.

The organic nitrate pentaerythrityl tetranitrate (PETN), very commonly used in the treatment of cardiovascular diseases, is assumed to be a "double" prodrug, since it has to be converted to nitric oxide (NO) by bioactivation processes and, second, the nitric acid esters may be hydrolyzed enzymatically or non-enzymatically yielding the so called "lower" nitrates, i.e. pentaerythrityl trinitrate (PETriN), pentaerythrityl dinitrate (PEDiN) and pentaerythrityl mononitrate (PEMonoN) (Scheme 1). Thus, PETN can be considered to act in vivo as a set of four nitrates, which may contribute to its clinical advantages, such as lower occurrence of headaches and a lower nitrate tolerance [2,3]. Non-enzymatic hydrolysis of the nitrate ester PETN is assumed to go along with enzymatic biotransformation [4,5]. The main objective of our study was to establish an improved analytical method for quantifying PETN and the lower nitrates and to investigate whether non-enzymatic hydrolysis could play a role in the biotransformation of PETN.

Analysis of organic nitrates has not been performed very successfully in the past due to general obstacles. The primarily used analytical method, gas chromatography–mass spectroscopy (GC–MS) [6–8], shows a low limit of quantitation (LOQ) (PEMonoN 150 ng/ml, PEDiN and PETriN 30 ng/ml, but the mean recovery rate is not as high as described for other methods. Moreover, inaccuracy is about 10%, which may be caused by thermal instability of the analytes and/or by incomplete matrix extraction and derivatisation of PEMonoN, PEDiN and PETriN. The commonly used derivatisation reaction which is also used for GC-ECD and GC-FID is trifluoroacetylation [9] of free OH-groups.

A similar inaccuracy of about 6% has been described for a HPLC method combined with a thermal energy analyzer (TEA-detector) [10,11], which was mainly used for the determination of explosives in waste water. The disadvantages of the GC methods as well as the medium UV-absorption in HPLC prompted us to explore the possibilities of HPLC combined with

* Corresponding author. Tel.: +49 3641 949814; fax: +49 3641 949802.
E-mail address: b8sean@rz.uni-jena.de (A. Seeling).

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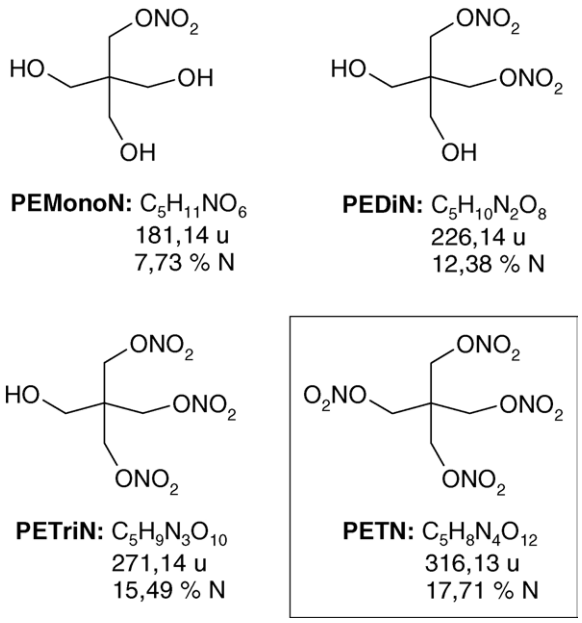


Scheme 1. PETN and its major degradation products.

an N-selective chemoluminescence-detection (CLND) for the analysis of PETN and its degradation products PETriN, PEDiN and PEMonoN. CLND was expected to detect these compounds more sensitively than UV-detection.

2. Materials and methods

2.1. Chemicals

LiChrosolv® methanol, gradient grade for liquid chromatography, sodium chloride, sodium hydroxide, sodium acetate-trihydrate, sodium dihydrogenphosphate-dodecahydrate, disodium hydrogenphosphate, phosphoric acid 85%, hydrochloric acid 37%, cysteine and thioglycolic acid, all of analytical grade, were obtained from VWR International (Darmstadt, Germany). Caffeine, pancreatine from hog pancreas (specification as described in USP 26 [12]) and pepsin from hog stomach (322 U/mg) were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). PETN, PETriN, PEDiN and PEMonoN (HPLC-purity >98%) were donated by ISIS-Alpharma (Monheim, Germany).

2.1.1. Solvents and solutions

All solutions were prepared in deionised water (Seralpur®). Acetate buffer, pH 5.5 and phosphate buffer, pH 7.4 were prepared in accordance to the general regulations of the European Pharmacopoeia [13], *Gastric Fluid, simulated, TS* (containing pepsine, pH 1.2) and *Intestinal Fluid, simulated, TS* (containing pancreatin, pH 7.5) are described in the USP 26 [12]. Human plasma was obtained from the clinic of the Friedrich-Schiller-University, Jena and stored at −25 °C.

The 10^{-3} M-stock solutions of caffeine (ext. standard), PETN, PETriN, PEDiN and PEMonoN were prepared in methanol and diluted with water and methanol to obtain lower concentrated HPLC-reference solutions. The standardized amount of methanol was 50% (v/v). For recording the nitrogen calibration curve caffeine dilutions in the concentration range of 2.79×10^{-8} to 2.79×10^{-5} g N/ml were used.

2.1.2. Investigated solutions

Investigations of the hydrolysis and redox stability were performed at 37 °C with 2×10^{-5} M PETN-solutions ($n = 3$) in aqueous solutions: demineralized water; acetate buffer, pH 5.5; phosphate buffer, pH 7.4; enzyme containing buffer solutions: gastric fluid, simulated, TS, according to USP 26 (pepsine containing, pH 1.2); intestinal fluid, simulated, TS, according to USP 26 (pancreatine containing, pH 7.5); human plasma pH 7.4; sodium hydroxide solution pH 10.0. To investigate the influence of thioles the following solutions were added: 10^{-5}, 5×10^{-5} and 10^{-4} M cysteine solutions in phosphate buffer pH 7.4; 10^{-5}, 5×10^{-5} and 10^{-4} M thioglycolic acid solutions in phosphate buffer, pH 7.4. Solutions without proteins were prepared by dispergation of PETN in the solvent at 37 °C, sonification for about 10 min and subsequent stirring of the suspensions for further 20 min. Ten times oversaturated 2×10^{-4} M solutions were obtained from which some PETN crystallized within 60 min. The resulting saturated solutions were stable and contained 2×10^{-5} mol/l PETN after filtration through a 0.2 μm membrane filter (Rotilabo®, Roth, Karlsruhe, Germany). Protein and thiol containing test solutions were freshly prepared by diluting the oversaturated PETN solution 1:10 with the respective media. No further crystallization was observed. The amounts of PETN dissolved were determined by HPLC using a PETN-calibration curve and taken as t_0-value. During the incubation period of 14 days (2 days, respectively in case of the protein containing solutions) the well closed sample flasks were held at 37 °C in a water bath.

2.2. Samples

After 30 min, 1, 2, 4, 8, 16, 24 h, 2, 7 and 14 days aliquots were sampled and diluted 1:1 with methanol. All the samples were centrifuged 15 min at 3000 rpm to separate denaturated proteins in case of the protein solutions. The clear supernatants were directly injected into the HPLC. Residues were tested for coprecipitations by extracting them three times with 1.0 ml methanol. The extracts were dried in a gentle stream of nitrogen, resolved in 1.0 ml methanol/water (1:1 v/v) and analyzed by HPLC.

2.3. Equipment

Separation of the analytes was performed with a LC-10 Series HPLC consisting of two LC-10AS-pumps in an isocratic mode, an autoinjektor SIL-10A, a UV-detector SPD-10A (Shimadzu Europe GmbH, Duisburg, Germany) and an Antek 8060 Nitrogen Specific HPLC Detector (Antek Instruments L.P., Houston, TX, USA). A Bus-Module SCL-10A VP with Class VP-Software, version 6.12 (Shimadzu Europe GmbH, Duisburg, Germany) was used for computer controlling and analysis. The simultaneous UV- and N-detection as well as the flow reduction

below the maximum eluent flow of 0.3 ml/min as required for the CLND was possible by using a Quick Split® post-column adjustable flow splitter 600-PO10-06 (Richard Scientific Inc., Novato, CA, USA).

Test solutions were stored in a thermostated water bath GFL 1042 (Gesellschaft für Labortechnik mbH, Burgwedel, Germany). For pH adjustment a pH 526 pH-meter (WTW Weilheim, Weilheim, Germany) was used. All samples were centrifuged with a Labofuge 200 (Haereus Instr., Osterode, Germany).

2.3.1. Chromatographic conditions

Analyses of the samples were performed isocratically on a Chromolith Performance RP-18e (100 × 4.6) column/ Chromolith RP-18e Guard Cartridge (5 × 4.6) (Merck, Darmstadt) with methanol/water (1:1) at a flow rate of 0.5 ml/min; injection volume 100 μl; split ratio UV:CLND = 3.2:1.8; detection A: UV 215 nm; detection B: CLND, calibrated with caffeine in the concentration range of 27.9 ng/ml N to 27.9 μg/ml N.

2.4. Reproducibility

For demonstrating the reproducibility concerning capacity-factors, response-factors and inter-assay variation the day to day-precision was determined and the experimental instructions were executed by several lab technicians ($n = 3$).

2.5. Linearity

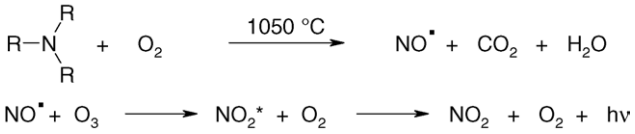
Standard curves of the organonitrates and caffeine, each diluted in the eluent were prepared in the concentration range from 0.1 to 100 μmol/l ($n = 6$). The UV- and CLND-responses were considered linear when adjusted to a correlation coefficient of at least 0.9990. The limit of detection (LOD) and limit of quantitation values (LOQ) were determined by calculating the signal/noise ratio (S/N (LOD) = 3; S/N (LOQ) = 10).

3. Results and discussion

3.1. Method development

Several HPLC methods have been described in the literature for the determination of PETN [10–15] which we screened to find a basic method for our analytical problem.

In view of this some restrictions had to be taken in consideration. Because using an N-selective detector we had to avoid N-containing eluents such as acetonitrile as well as N-containing buffer substances. The differences in polarity and the absence of ionisable structures in the organonitrate molecules led us to a method described in the US Pharmacopoeia [12] which is used for the assay of PETN in PETN triturations. We reduced the MeOH amount in the MeOH/water mixture from 60 to 50%. So we got a buffer free eluent with high polarity that could differentiate among the lower nitrates PEMonoN, PEDiN and PETriN very well. Furthermore the retention times even of the most lipophilic analyte PETN were short enough to guarantee a high sample throughput. The standard calibration method for the CLND using caffeine as external standard was adapted to the expected quantification ranges and evaluated in comparison to the UV-calibration curves recorded separately for every organonitrate. The examination of possible interactions between the eluent and sample matrix residues, such as insolubilities, precipitations or coprecipitations has shown none such side reactions. This was of main interest, because the eluent should also be used as sample solvent that means 50% MeOH plus 50% aqueous media. So a sensitive and selective separation of PETN and its lower nitrate derivatives could be achieved (Fig. 1). Quantification was more convenient because the CLND is calibrated on ng/ml nitrogen by using a nitrogen standard (Fig. 1a). Individual calibration curves for every compound are not required. Knowing the nitrogen-amount by the formula mass of every analyte, the obtained AUC-values could be converted into any other unit e.g. concentration, mass or [mole]. In contrast to UV-detection, the size of the detector signal is independent to the oxidation number, ion charge and hybridisation of the nitrogen (Scheme 2). Fig. 1 demonstrates the ability to detect PETN and its degradation products by HPLC/CLND. Fig. 1b shows the baseline of a protein containing sample. Validation parameters such as linearity, LOD and LOQ are given in Table 1. The LOD- and LOQ-values were in the range as expected for the determined response-factors. Depending on the detection mode the signals of equimolar organonitrate-samples correlated with the percentage of nitrogen calculated from formula mass.



Scheme 2. The nitrogen containing analytes are pyrolyzed in an O_2/Ar stream at 1050 °C forming selectively NO, which is further oxidized to excited nitrogen dioxide (NO_2^*) by in situ generated ozone. During transition to the ground state (NO_2) a quantum of red light, proportional to the injected amount of nitrogen, is emitted and recorded by the CLND.

3.2. In vitro-experiments

In the first incubation experiments it was demonstrated, that PETN is obviously stable against hydrolysis not only under physiological pH-conditions but also in a pH-range from 1.2 to 7.4. Consequently, a non-enzymatic degradation of PETN even in case of long-time-incubations and extraction from acidified biological matrices can be excluded. The slopes observed at the beginning of the concentration/time curves of PETN in aqueous and buffered solutions (pH 5.5 and 7.4) (Fig. 2) are not due to an initial hydrolysis, but to the precipitation of PETN from the oversaturated solutions on the glass surface. After removing the supernatant liquid these deposits of PETN were resolved with methanol and examined by HPLC. Only PETN but none of the lower nitrates were detected. After 90 min the concentration of PETN remained stable at 2×10^{-5} mol/l and this concentration was used in all of the following degradation experiments. Degradation was found in alkaline solutions (Fig. 2). In 10^{-4} M sodium hydroxide solution at 37 °C, all nitric acid ester moieties

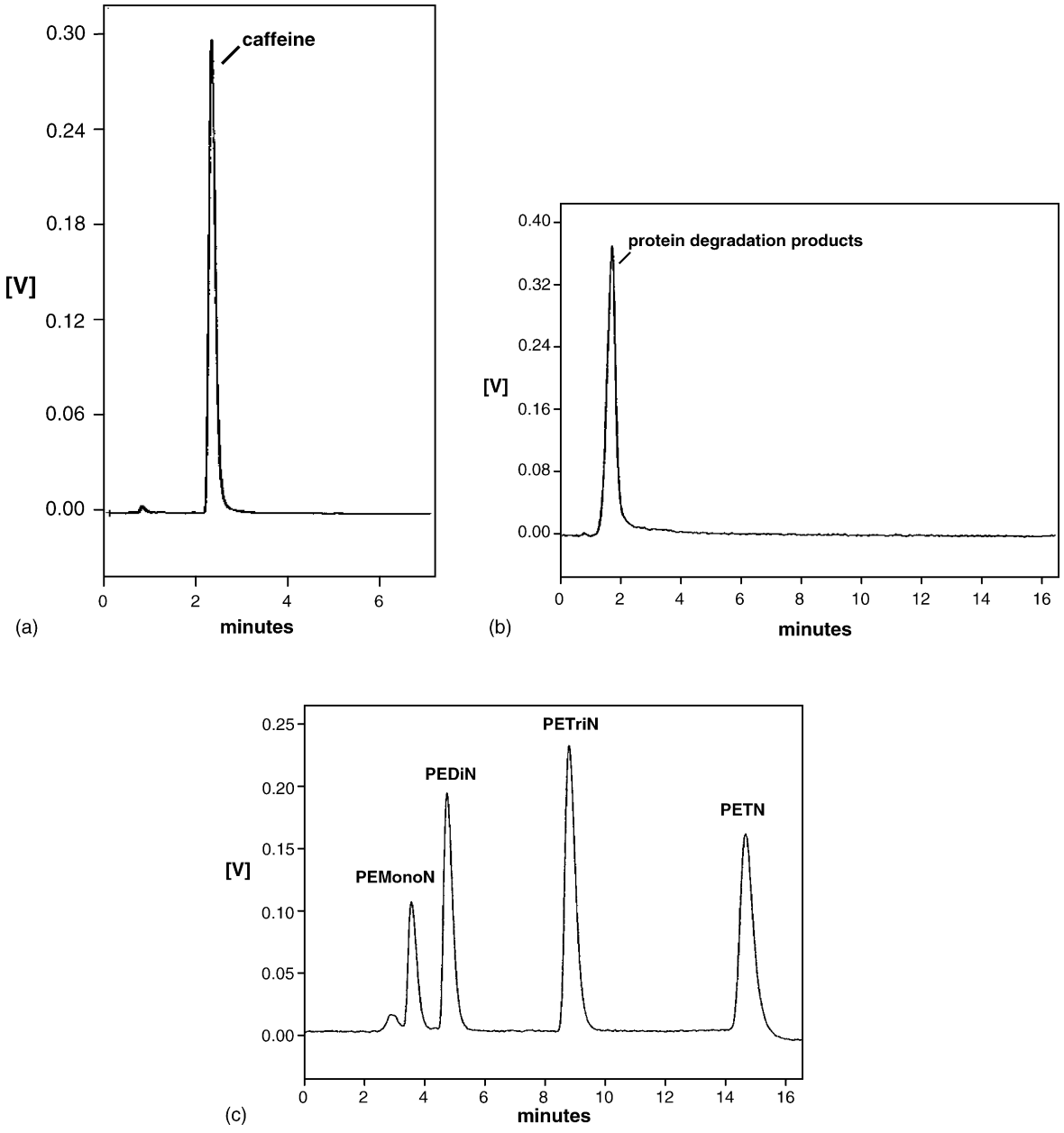


Fig. 1. (a) HPLC-chromatogram of caffeine (internal standard, $c = 2 \times 10^{-5}$ M) recorded by CLND, (b) HPLC-chromatogram of an organonitrate-free human plasm blanc; recorded by CLND after sample preparation (see Section 2.2) and (c) HPLC-chromatogram of PETN ($c = 10^{-5}$ M) and its possible hydrolysis products ($c = 2 \times 10^{-5}$ M) recorded by CLND.

were hydrolyzed within ~75 h. The intermediate formation and subsequent degradation of lower nitrates was observed but not analyzed quantitatively. In order to simulating the gastric and intestinal situation, PETN was incubated in artificial, enzyme containing digestive fluids. In the strong acidic gastric fluid as well as in the weak basic intestinal fluid, PETN remains unchanged for at least 50 h which was definitely longer than the physiological time of residence in the digestive tract (Fig. 3).

Table 1
Analytical data of PETN and its degradation products; caffeine is used as external standard

Substance	M_{rel}	N [%] (m/m)	k'	Linearity (μM)	Correlation coefficient	LOD (nmol)	LOQ (nmol)
PEMonoN	181.14	7.73	1.45	0.57–100	0.9995	16.2	53
PEDiN	226.14	12.38	2.42	0.5–100	0.9997	10.7	36
PETriN	271.14	15.49	5.58	0.35–100	0.9999	8.9	30
PETN	316.13	17.71	11.35	0.2–100	0.9999	5.4	22
Caffeine	194.19	28.8	5.20	0.18–400	>0.9999	6.2	20

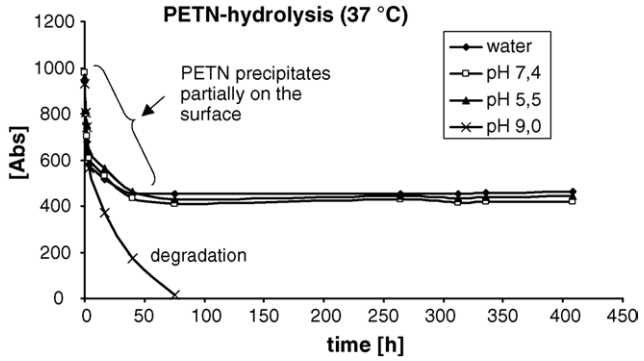


Fig. 2. PETN-concentration/time-diagram depending on solvent pH.

Quantification of xenobiotics in biological, that means protein containing matrices, can be impaired by enzymatic reactions following the defined incubation period. For this reason a denaturation of the proteins, e.g. by phosphoric acid/methanol is essential, which on the other hand may induce coprecipitations with compounds such as PETN. So PETN was examined before and after denaturation. In human plasma a small but significant decrease of PETN was observed (Fig. 3), obviously caused by degradation reactions with the proteins, since the complete extraction of the denaturated precipitate has not shown any incidence of absorbed or coprecipitated PETN.

A limited number of free thiol groups in the human plasma may be responsible for the limited degradation of PETN. The interaction of nitrates with thioles could indeed be confirmed by our experiments. Addition of cysteine or thioglycolic acid caused a rapid decrease of PETN (Fig. 4).

To understand the meaning of these results it is necessary to explain the role of thioles during bioactivation of organonitrates in vivo. It was found that an organonitrate treatment increases the formation of superoxide ($O_2^{-\bullet}$) in blood vessels and that this seems to inactivate NO; further the vascular NADH-oxidase activity is elevated. The latter significantly enhances the basal generation of $O_2^{-\bullet}$ radicals and promotes the inactivation of NO and the formation of peroxynitrite. Superoxide radicals ($O_2^{-\bullet}$) react effectively with NO ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$) to form peroxynitrite, which is a strong oxidant [16] that mediates oxidation of both non-protein and protein sulfhydryls to disulfides and in case of protein bound SH-groups probably to sulfenic acid [17].

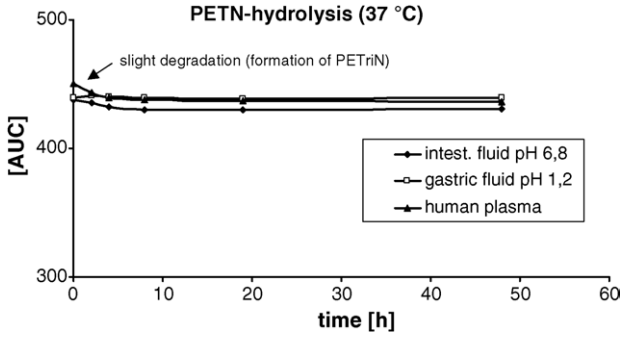


Fig. 3. Level of PETN after addition of enzymes in acidic or neutral solutions and proteins.

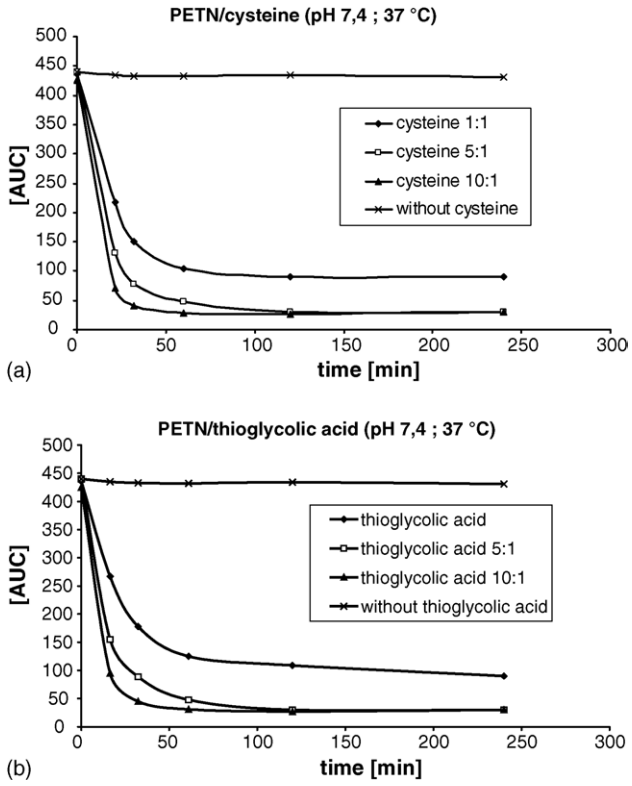


Fig. 4. The PETN concentration decreases more (a) or less (b) rapidly with increasing thiol/PETN-ratio.

So this mechanism is supposed to be one factor in development of nitrate tolerance.

Up to now nitrate tolerance has not been observed during PETN therapy [18] because PETN provokes only a minimal stimulation of superoxide radical formation. Since the development of nitrate tolerance was reported to be associated with increased vascular oxidative stress, induced by organic nitrates and antioxidant co-treatment (e.g. ascorbic acid) had beneficial effects [19], the lack of tolerance upon PETN treatment was explained by antioxidative side-effects of PETN itself.

On the other hand thioles have repeatedly been discussed to act as cofactors in the enzymatic [5] bioactivation as well as reducing agents in the non-enzymatic [20] bioactivation of organic nitrates via formation of nitro- and nitroso-thioles as precursors of nitric oxide [21]. Because this has been observed only for other organonitrates like GTN or ISDN but not for PETN itself the rapid degradation of PETN after addition of cysteine and thioglycolic acid in our studies is very surprising. In former in vitro-studies it could only be proved, that a cystine-pool reacts as a selective moderator inside the reduction of the PETN metabolite PEDiN without being interfered by superoxide, yielding pentaerythityl mononitrate (PEMonoN) meanwhile in the absence of cystine only pentaerythrite (PE) is formed [22]. Clarifying the reaction mechanism and the structures of the intermediates and the final products resulting from the reaction of PETN with thioles has to be the subject of further investigations.

4. Conclusions

HPLC combined with CLND is a convenient method for the identification and quantification of PETN and its metabolites. Sensitivity showed to be about four times higher than the parallel performed UV-detection. The detection limits found were 1.6×10^{-7} M = 50 ng PETN/ml for CLND and 6.4×10^{-7} M = 0.2 μg PETN/ml for UVD. Furthermore, oligopeptides, but not the usual organic solvents needed for extraction, or other compounds without nitrogen interfere with the chromatograms. With regard to quantification, CLND proved to be highly favourable, since only one calibration curve using caffeine in the range of 3 ng–30 μg N/injection had to be established. A peaks size only depends on the amount of nitrogen in the compound.

In our study, PETN showed to be surprisingly stable at 37 °C in aqueous systems of pH 1.2, 5.5, 6.8 and 7.4 and in simulated intestinal and gastric fluids. According to this, a non-enzymatic hydrolysis in vivo can be ruled out. Total hydrolysis of PETN takes place at 37 °C at pH 10 within 75 h. A rapid, dose-dependent degradation of PETN was observed after addition of thioglycolic acid and cysteine. This confirms the discussion, that endogenous thioles are involved in the bioactivation or at least in the biotransformation of organic nitrates.

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