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# Identification, solubility enhancement and *in vivo* testing of a cyanide antidote candidate

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ABSTRACT

Present studies focused on the *in vitro* testing, the solubility enhancement and the *in vivo* testing of methyl propyl trisulfide (MPTS), a newly identified sulfur donor to treat cyanide (CN) intoxication. To enhance the solubility of the lipophilic MPTS, various FDA approved co-solvents, surfactants and their combinations were applied. The order of MPTS solubility in the given co-solvents was found to be the following: ethanol >> PEG 200 ≈ PEG400 ≈ PEG300 > PG. The maximum solubility of MPTS was found at 90% ethanol of 177.11 ± 12.17 mg/ml. The order of MPTS solubility in different surfactants is Cremophor EL > Cremophor RH40 > polysorbate 80 > sodium deoxycholate > sodium cholate. The maximum solubility of 40.99 mg/ml was achieved with 20% Cremophor EL. A synergistic solubilizing effect encountered with the combination of 20% Cremophor EL + 75% ethanol lead to a 2900-fold increase (compared to water solubility) in solubility. The *in vivo* efficacy using intramuscular administration was determined on a therapeutic mice model and expressed as a ratio of CN LD50 with and without the test antidote(s) (APR). Intramuscular administration was shown to be effective and the therapeutic antidotal protection by MPTS alone and MPTS + thiosulfate (TS) was significantly higher than the present therapy of TS.



## 1. Introduction

The major mechanism which removes cyanide (CN) from the body is its biotransformation to the less toxic thiocyanate (SCN) in the presence of a sulfur donor (SD) and a sulfurtransferase enzyme such as rhodanese (Rh) (Way, 1983). The SD component of the present therapy of Nithiodote™, the inorganic sodium thiosulfate (TS), has limitations due to its high Rh dependency, relative low SCN formation efficacy, and low cell penetration ability to reach the endogenous Rh localization. The antidotal approach of co-administering TS with purified Rh encapsulated within various enzyme carriers such as erythrocytes (Way et al., 1985), and polymeric nano-delivery systems (Petrikovics et al., 2010) made the SD and Rh available in the blood stream to react immediately with the absorbed CN before it reaches its target points in the body. This way, the two components of the CN antidotal systems: (a) an appropriate SD and (b) Rh enzyme, protected from adverse immunologic reactions by macrophages, are readily available in the circulation. This approach proved to be significantly efficient: a prophylactic antidotal protection of over 9× LD50 was achieved when sodium nitrite (SN) was co-administered with TS and encapsulated external Rh (Way et al., 1991). However, still there were some limitations with the encapsulated Rh and TS due to the product inhibition by the formed sulfite. This approach was further improved by the application of organic thiosulfonates with superior SCN formation efficacy and superior cell penetration capability to that of the inorganic TS (Petrikovics et al., 1994). When butane thiosulfate was administered with encapsulated Rh in combination with SN, a prophylactic antidotal protection of 14× LD50 was achieved (Petrikovics et al., 1995). Sulfur donors with higher lipophilicity can penetrate cell membranes and reach the mitochondrial Rh, and are expected to be efficient even without external Rh administration. Various synthetic and naturally occurring organo-sulfur molecules were tested *in vitro* and *in vivo* and compared to the inorganic TS (Baskin et al., 1999; Frankenberg, 1980; Iciek, 2001). Several garlic originated organo-sulfur molecules were evaluated as SDs and CN acceptors (Ashani et al., 2006; Block, 1985; Iciek et al., 2005).

Although great progress was achieved in the field, especially in the prophylactic treatment of cyanide intoxication, there are still numerous factors that could be improved, including the need to identify further, possibly more effective organo-sulfur molecules

*Abbreviations:* MPTS, methyl propyl trisulfide; CN, cyanide; SCN, thiocyanate; SD, sulfur donor; Rh, rhodanese; TS, thiosulfate; PEG, polyethylene glycol; PG, propylene glycol; APR, antidote potency ratio; RAPR, relative antidotal potency ratio.

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and the need of an intramuscular preparation for therapeutic treatment. Latter is important since the presently used antidotes are all intravenous preparations, which in the case of a mass casualty scenario are difficult to administer in time due to the large number of people involved. An intramuscular preparation would be easier and quicker to administer or even self-administer which in turn would be more favorable in such a situation.

One of the main drawbacks of the organo-sulfur donors is their very low water solubility, which hinders their application in liquid dosage forms. To overcome this issue, an appropriate solubility enhancing method or solvent system has to be developed that is capable of dissolving the compounds at therapeutically relevant concentrations. In the case of parenterals this poses extra difficulties as the available excipients for solubilizing lipophilic molecules is limited and their applicable concentration range is also restricted (Liu, 2008; Strickley, 2004).

Present study focused on the *in vitro* efficacy characterization of methyl propyl trisulfide (MPTS), an SD molecule that to our present knowledge has never been used in combating cyanide intoxication, and on its *in vivo* antidotal efficacy determined on a therapeutic mice model. Furthermore, since the identified SD is a highly lipophilic molecule it was the aim of the study to design a solvent system that is capable of dissolving the drug candidate in therapeutically effective doses. In developing the system another aim of the study was realized, namely to identify a vehicle/vehicle mixture that could serve the animal studies in which the intramuscular administration was tested and later be the base of an appropriate kit for a mass casualty scenario. This was achieved by enhancing the solubility of the lipophilic MPTS with the application of FDA approved co-solvents, surfactants and their combinations. The aim of the animal studies was therefore dual as the test not only gave answer to the *in vivo* efficacy of the drug candidate but would also answer the question of whether the drug shows a fast enough absorption from an intramuscular injection for combating cyanide intoxication.

## 2. Materials and methods

### 2.1. Materials

Materials for the conversion test were potassium cyanide (KCN), formaldehyde, ferric nitrate reagent, monobasic sodium phosphate monohydrate and dibasic sodium phosphate anhydrous (VWR International, Suwanee, GA, USA). Methyl propyl trisulfide (50% purity; water solubility = 0.15 ± 0.003 mg/ml) was purchased from Sigma–Aldrich (St. Louis, Missouri, USA), TS were purchased from VWR International (Suwanee, GA, USA). Ethanol, PEG 200, PEG 300, PEG 400, PG (VWR International, Suwanee, GA, USA), Cremophor EL, Cremophor RH40, sodium cholate, sodium deoxycholate, polysorbate 80 (Sigma Aldrich, St. Louis, MO, USA) were used as solubilizers. Cyclohexanone (Sigma–Aldrich, St. Louis, MO, USA) was used as solvent for the GC–MS measurements. KCN solutions (1.0 mg/ml and 3.5 mg/ml) were used throughout the animal studies. 250, 100 and 50 μl Hamilton Luer-lock syringes (VWR International, Suwanee, GA, USA) were used in the animal studies with 27G 1/2 needles for intramuscular and 25G 1½ needles (VWR International, Suwanee, GA, USA) for subcutaneous injection.

### 2.2. In vitro efficacy test

*In vitro* efficacy of MPTS was determined based on its ability to convert CN to SCN. The method applied was a spectrophotometric measurement of the formed SCN based on the method of Westley (1981) with minor modifications (Petrikovics et al., 1995). Briefly, 200 μl of various concentrations of SDs, 200 μl of 10 mM phosphate buffered saline, 200 μl of 250 mM KCN and 400 μl of deionized water were mixed. The reaction was incubated for 5 min and was quenched with 500 μl of 15% (v/v) formaldehyde. 1.5 ml of ferric nitrate reagent was added to form a reddish brown complex ($Fe(SCN)_3$) that was quantitatively determined at 464 nm using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Tests were performed with MPTS and TS at concentrations ranging from 25 mM to 0.156 mM with two fold serial dilutions in between.

### 2.3. Solubility studies

The solubility of MPTS was determined in co-solvents, surfactants and their combinations. Aqueous solutions of co-solvents and surfactants were prepared at 10%, 25%, 50%, 75%, 90% and 1%, 5%, 10%, 15%, 20% respectively. Based on the solubility enhancing efficacy of the co-solvent/water and surfactant/water systems the most effective excipients were combined into one system forming a co-solvent/surfactant/water system. Thus combinations of Cremophor EL, ethanol and PEG200 were prepared, where the concentration of the surfactant was 5%, 10%, 15%, 20% and the concentration of the co-solvents was 50%, 62.5% and 75%.

Triplicates of the solvent systems were prepared in glass vials, excess MPTS was added to the solutions and the vials were sealed to eliminate the possibility of evaporation. The samples were then vortexed (Heidolph Multi Reax, Heidolph Instruments, and Cinnaminson, NJ, USA) for 20 min and left to equilibrate at room temperature. After equilibration (determined as 1 week) an aliquot of the samples was centrifuged (Galaxy 20R, VWR International, Suwanee, GA, USA) at 5000 rpm for 5 min to ensure sedimentation of the excess MPTS and the drug content of the saturated solution was measured using a GC–MS method detailed in Section 2.4. Prior to GC–MS measurements the internal standard (1 mg/ml of dibuthyl disulfide; DBDS) was added to the samples and dilution with ethanol and cylcohexanone was performed.

### 2.4. GC–MS measurement

A GC–MS method was chosen for the quantitative determination of MPTS. The system consisting of an Agilent Technologies 7890A GC with a 7683 autosampler and a 5975C VL MSD,

**Table 1**
Gas chromatograph parameters.

| | |
|---|---|
| Injection source | GC auto-loading sampler (ALS) |
| Injection volume | 1.0 μl |
| Injection port temperature | 250 °C |
| Injection mode | Split |
| Split ratio | 60:1 |
| Carrier gas | Helium |
| Carrier gas velocity | 1.0 ml/min |
| Carrier gas pressure | 7.6522 psi |
| Initial temperature of column | 50 °C |
| Initial temperature duration | 2 min |
| Temperature ramp | 5 °C/min |
| Final temperature of column | 250 °C |
| Final temperature duration | 5 min |

**Table 2**
Mass spectrometer parameters.

| | |
|---|---|
| EMV mode | Relative (+200) |
| EM voltage | 1118 |
| Solvent delay | 2.00 min |
| Source temperature | 230 °C |
| Quadrupole temperature | 150 °C |
| Electron energy | 70 eV |