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MAKING ENERGETIC MATERIALS SAFER

BY

MICHELLE D`ASSUMPSOA GONSALVES

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

CHEMISTRY

UNIVERSITY OF RHODE ISLAND

2020

DOCTOR OF PHILOSOPHY IN CHEMISTRY DISSERTATION
OF
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2020

ABSTRACT

Canine (K9) units and scientists developing explosives trace detection devices (ETDs) work with and are exposed to energetic materials when imprinting the dog or building instrument libraries. However, access to extremely hazardous materials, such as explosives, is limited, with a great need for training aids that provide a safe-handling and long shelf-life material. In order to address these needs, encapsulation of energetic materials, such as triacetone triperoxide (TATP), erythritol tetranitrate (ETN) and trinitrotoluene (TNT) in a polymer matrix have been developed, and extensively characterized to ensure explosive desensitization, controlled release, clean background odor and delivery of the pure explosive as effective training aids. Although, peroxide explosives, such as TATP and hexamethylene triperoxide diamine (HMTD) are a prominent threat, and their detection a priority within K9 units and ETD manufacturers, their absorption, distribution, metabolism and excretion (ADME) in the body have not been investigated. TATP is volatile and HMTD is lipophilic allowing for their inhalation and dermal absorption. Their distribution to the body was evaluated with blood incubations, which determined that TATP is stable for at least a week, while HMTD is degraded within minutes. Hepatic metabolism was investigated with microsomal and recombinant enzyme incubations. The metabolism of TATP undergoes hydroxylation catalyzed by cytochrome P450 2B6 (CYP2B6) to form TATP-OH, which undergoes glucuronidation catalyzed by uridine diphosphoglucuronosyltransferase 2B7 (UGT2B7) to form TATP-*O*-glucuronide, which was excreted in the urine of laboratory personnel and bomb-sniffing dogs exposed to TATP. Detection of these peroxide explosives and/or their metabolites in biological matrices (e.g. blood and urine) can be used as forensic evidence to exposure; therefore,

paper spray ionization mass spectrometry was exploited as a robust analytical method for the analysis of peroxide explosives in *in vitro* and *in vivo* biological samples.

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DEDICATION

I would like to dedicate this dissertation to my family; my parents Marcos Antonio Gonçalves da Silva and Marcia d'Assumpção Gonçalves, my brother Marcos Antonio Gonçalves da Silva Filho, and my grandmother Maria da Conceição Fuina d'Assumpção, for encouraging me to pursue this degree and for bearing with me every step of the way.

PREFACE

This dissertation has been prepared in manuscript format in accordance with the guidelines of the Graduate School of the University of Rhode Island. The broad topic “Making energetic materials safer” was focused into four manuscripts. The first manuscript, “Characterization of Encapsulated Energetic Materials for Trace Explosives Aids for Scent (TEAS),” was submitted to the Journal of Energetic Materials. The second manuscript, “In vitro and in vivo studies of triacetone triperoxide (TATP) metabolism in humans,” has been published in the journal Forensic Toxicology. The third manuscript, “Paper spray ionization – high resolution mass spectrometry (PSI-HRMS) of peroxide explosives in biological matrices” was submitted to the journal Analytical and Bioanalytical Chemistry. The fourth manuscript, “In vitro blood stability and toxicity of peroxide explosives in canines and humans” will be submitted to the journal Xenobiotica.

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LIST OF ABBREVIATIONS

$^{13}\text{C}^3$ -TATP: carbon-13 labeled TATP

$^{15}\text{N}_2$ -HMTD: nitrogen-15 labeled HMTD

ABT: 1-aminobenzotriazole

ACN: acetonitrile

ADMET: absorption, distribution, metabolism, excretion and toxicity

AN: ammonium nitrate

APCI: atmospheric pressure chemical ionization

ATP: adenosine triphosphate

AU: arbitrary units

BSA: bovine serum albumin

BUP: bupropion

BUP-OH: hydroxybupropion

BZD: benzydamine

BZD-NO: benzydamine N-oxide

CID: collision induced dissociation

Cl : in vivo intrinsic clearance

Cl_{int} : in vitro intrinsic clearance

CYP: cytochrome P450

d_{12} -HMTD: deuterated HMTD

d_{18} -TATP: deuterated TATP

DART: direct analysis in real time

DCM: dichloromethane

DDI: drug-drug interaction

DESI: desorption electrospray ionization

DFT: density functional theory

DLM: dog liver microsomes

DMSO: dimethyl sulfoxide

DSC: differential scanning calorimetry

EC: ethoxyl ethyl cellulose

EGDN: ethylene glycol dinitrate

EESI: extractive electrospray ionization

EI: electron impact ionization

ESI: electrospray ionization

ETD: explosive trace detection device

ETN: erythritol tetranitrate

FA: formic acid

FMO: flavin monooxygenase

FTIR: Fourier-transform infrared spectroscopy

GC-MS: gas chromatography-mass spectrometry

GSH: glutathione

GST: glutathione S-transferase

HCD: high-energy collision dissociation

HLM: human liver microsomes

HLungM: human lung microsomes

HME: homemade explosive

HMTD: hexamethylene triperoxide diamine or 3,4,8,9,12,13-hexaoxa-1,6-diazabicyclo[4.4.4]tetradecane

HMX: 1,3,5,7-tetranitro-1,3,5,7-tetrazoctane

HPLC: high performance liquid chromatography

HRMS: high resolution mass spectrometry

IACUC: Institutional Animal Care and Use Committee

IMS: ion mobility spectrometry

IRB: Institutional Review Board

IS: internal standard

k_{cat} : turnover rate of an enzyme-substrate complex to product and enzyme

K_{m} : substrate concentration at half of V_{max}

LOD: limit of detection

LogP: partition coefficient

LOQ: limit of quantification

K9: police canine unit

LDH: lactate dehydrogenase

MeOH: methanol

MMI: methimazole

MRM: multiple reaction monitoring

MS/MS: tandem mass spectrometry

m/z: mass-to-charge ratio

NADPH: reduced nicotinamide adenine dinucleotide phosphate

NH₄OAc: ammonium acetate

OXC: oxcarbazepine

PC: polycarbonate

PEI: polyetherimide

PMMA: poly(methyl methacrylate)

PS: polystyrene

PVA: poly(vinyl alcohol)

PETN: pentaerythritol tetranitrate

PSI: paper spray ionization

QC: quality control

rCYP: recombinant cytochrome P450

RDX: 1,3,5-trinitro-1,3,5-triazinane or cyclotrimethylenetrinitramine

RESS: rapid expansion of supercritical solution

rFMO: recombinant flavin monooxygenase

RLM: rat liver microsomes

RSD: relative standard deviation

RT: retention time

rUGT: recombinant uridine diphosphoglucuronosyltransferase

[S]: substrate concentration

SAS: supercritical anti-solvent

sc-CO₂: supercritical carbon dioxide

SPE: solid-phase extraction

SRM: single reaction monitoring

$t_{1/2}$: half-life

TATP: triacetone triperoxide or 3,6,6,9,9-hexamethyl-1,2,4,5,7,8-hexoxonane

TATP-d₁₈: deuterated TATP

TATP-OH: hydroxy-TATP

T_d: decomposition temperature

T_g: glass transition temperature

TGA: thermal-gravimetric analysis

TIC: ticlopidine

TMDDD: tetramethylene diperoxide diamine dialdehyde or 1,2,6,7,4,9-tetraoxadiazecane-4,9-dicarbaldehyde

TMDDAA: tetramethylene diperoxide diamine alcohol aldehyde or 9-(hydroxymethyl)-1,2,6,7,4,9-tetraoxadiazecane-4-carbaldehyde

TMPDDD: tetramethylene peroxide diamine dialcohol dialdehyde or N,N'-(peroxybis(methylene))bis(N-(hydroxymethyl)formamide)

TNT: 2,4,6-trinitrotoluene

TPSA: topological polar surface area

UDPGA: uridine diphosphoglucuronic acid

UGT: uridine diphosphoglucuronosyltransferase

V_{max}: maximum formation rate

MANUSCRIPT 1

**Characterization of Encapsulated Energetic Materials for Trace
Explosives Aids for Scent (TEAS)**

by

Michelle D. Gonsalves, James L. Smith and Jimmie C. Oxley

This manuscript was submitted to Journal of Energetic Materials

Abstract

Encapsulation is proposed as a safer way of handling energetic materials. Different encapsulation methods for explosives, such as solvent evaporation, spray coating and supercritical carbon dioxide assisted encapsulation, were explored. Explosive training aids, where energetic materials, such as triacetone triperoxide (TATP), erythritol tetranitrate (ETN) and trinitrotoluene (TNT), are encapsulated in a polymer matrix were developed, followed by comprehensive quality control testing, including differential scanning calorimetry (DSC), thermogravimetric analysis-infrared spectroscopy (TGA-IR), gas chromatography-mass spectrometry (GC-MS), high resolution mass spectrometry (HRMS) and sensitivity testing, and finally field approved by canine units trained on the pure explosive.

Keywords

Training aids, encapsulation, insensitive explosives, dry ammonium nitrate, explosives characterization

Introduction

For detection instrumentation which functions by recognition of a compound by its unique vapor signature, a library of such signatures must be developed by training against authentic samples. In the case of detection of explosives, training aids may be necessary due to the uniquely hazardous nature of the material (J. C. Oxley, Smith, and Canino 2015); canine units (K9) or manufacturers of explosive trace detection (ETD) instruments may be deterred from working with them due to their limited availability and sensitivity. Encapsulation of a material is a well-recognized technique widely used in many industries (e.g. food, cosmetics, pharmaceuticals) for preservation and target-release (Madene et al. 2006). Herein, we describe encapsulation as a method of creating a barrier between the explosive and the environment in order to make the explosives less detonable or improve the materials physical property.

Encapsulation can be accomplished by several techniques, which can be categorized into chemical, such as interfacial polymerization, physico-mechanical and physico-chemical processes (Jyothi et al. 2010). An example of physico-mechanical process includes fluidized bed coating (also called Wurster coating) which uses air jets to move the core material past a nozzle that sprays the material with a solubilized or molten polymer. A similar process is reported here to encapsulate ammonium nitrate (AN) (Jyothi et al. 2010; Suganya and Anuradha 2017).

Physico-chemical processes, such as solvent evaporation and supercritical CO₂ (sc-CO₂) assisted encapsulation, were used to develop the training aids. Solvent evaporation creates an emulsion using a volatile organic solvent for the dispersed phase containing the

polymer and core material and for the continuous phase, a solvent, usually aqueous, immiscible in first, to create droplets which harden into solid microspheres as the solvent evaporates (Li, Rouaud, and Poncelet 2008; O'Donnell and McGinity 1997). Supercritical CO₂ assisted encapsulation can be done by two methods: rapid expansion of supercritical solution (RESS) and supercritical anti-solvent (SAS). RESS consists of dissolving the polymer and core material into supercritical fluid. This pressurized solution is sprayed into a region at atmospheric pressure, thus forming particles. However, we found that polymer solubility in supercritical-CO₂ is challenging. More accommodating was the SAS method, where both the polymer and core material are dissolved in a soluble solvent, but become insoluble when the solution is introduced into supercritical fluid, forcing the materials to co-precipitate. This method was used to encapsulate explosives with poor solubility (Jyothi et al. 2010; Soh and Lee 2019).

The core materials that were investigated, encompassed most explosives classes: a nitroaromatic, e.g. trinitrotoluene (TNT); a nitrate-ester, e.g. erythritol tetranitrate (ETN); and a peroxide, e.g. triacetone triperoxide (TATP), were initially screened for training aids. TNT, the military explosive standard, is a secondary high explosive, insensitive to impact, friction and static but with a detonation velocity of 6.93 km/s (Dobratz 1972). On the other hand, an easily synthesized from household items material, TATP, is a primary high explosive, its sensitivity allows for easy initiation which may be used to trigger the secondary charge (J. C. Oxley et al. 2013). ETN has recently been emphasized because, similarly to TATP, it can be synthesized by amateurs and terrorists, raising its threat level

(J. C. Oxley et al. 2012), and similarly to TNT, it can be melt cast, expanding its potential usage (J. C. Oxley, Smith, and Brown 2017).

Explosives have been encapsulated for various purposes (Brewer, Staymates, and Fletcher 2016; Elzaki and Zhang 2016; Liu et al. 2017). Using encapsulated explosives as training aids for canine or detection instrumentation requires extensive quality control. This includes reproducible synthesis conditions to ensure clean background vapor which requires pre- and post-reaction processing to guarantee polymer compatibility and solvent removal. Furthermore, sufficient explosive vapor must be released only when triggered to provide a non-hazardous, safe to handle, material and deliver a pure explosive scent. We have previously demonstrated the encapsulation of TATP (J. C. Oxley, Smith, and Canino 2015). Herein, we report additional explosives which have been encapsulated, and more importantly, the details of their characterization.

Materials and Methods

Chemicals

Energetic materials, such as triacetone triperoxide (TATP) (J. C. Oxley et al. 2013), erythritol tetranitrate (ETN) (J. C. Oxley et al. 2012), trinitrotoluene (TNT), hexamethylene triperoxide diamine (HMTD) (J. C. Oxley et al. 2016), pentaerythritol tetranitrate (PETN) and cyclotrimethylenetrinitramine (RDX) were either purchased or synthesized. Optima LC-MS grade methanol, Optima LC-MS grade water, ACS grade acetone, ACS grade dichloromethane (DCM), ammonium nitrate (AN), ammonium acetate and ammonium chloride were purchased from Fisher Chemical (Fair Lawn, NJ, USA). Poly(vinyl alcohol) (PVA), polyetherimide (PEI) and ACS grade dichloromethane (DCM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polycarbonate (PC), polystyrene (PS) and poly(methyl methacrylate) (PMMA), ethoxyl ethyl cellulose (EC) and formic acid were purchased from Acros Organics (Morris Plain, NJ, USA).

Encapsulation processes

Solvent evaporation

The solvent evaporation method consisted of creating a dispersed organic phase in a continuous aqueous phase. The dispersed phase included the core material (1 g explosive) and the matrix material (2 g polymer) dissolved in a volatile solvent (20-30 mL DCM). The continuous phase was a surfactant aqueous solution (200 mL of 2% PVA aqueous solution). The dispersed phase was added to the continuous phase, which was vigorously

stirred (900 rpm) with the aid of a mixer. The two phases system were set to stir; the polymer, being insoluble in water, precipitates around the core material as the volatile solvent slowly evaporates from the solution, forming microspheres which are particles of the core material dispersed in a polymer matrix (Li, Rouaud, and Poncelet 2008; O'Donnell and McGinity 1997). The microspheres were washed with copious amounts of water prior to vacuum filtration and collection. Encapsulation of ETN and TNT was done using the solvent evaporation method previously described for TATP microspheres (J. C. Oxley, Smith, and Canino 2015). However, for optimization and potential scale-up some parameters were adjusted by varying volume and type of solvents, volume and concentration of surfactant, solvent-to-emulsifier ratio, polymer-to-explosive ratio, size of reaction vessel and reaction time. Different solvent removal techniques were tried to remove the large volume of solvent or co-solvent system required to dissolve HMTD, PETN or RDX, such as increasing the temperature of the continuous phase or continuous dilution of the continuous phase (Jyothi et al. 2010; Jeyanthi et al. 1996); however, encapsulation of these explosives was not successful.

Supercritical carbon dioxide (sc-CO₂) assisted encapsulation

A Waters Hybrid RESS/SAS (Rapid Expansion of Supercritical Solution/Supercritical Anti-Solvent) system (Waters Corporation, Milford, MA, USA) was used for the synthesis of training aids by the supercritical carbon dioxide (sc-CO₂) assisted encapsulation. The method, SAS (Prosapio, De Marco, and Reverchon 2018), consisted of creating a liquid solution of (0.5 g) polymer, (0.5 g) explosive and organic solvent (usually

100 mL DCM) and spraying it into a chamber filled with sc-CO₂, which was reached at the critical pressure, 74 bar, and critical temperature, 31.3 °C (Parhi and Suresh 2013). The sc-CO₂ extracted the solvent from the solution droplets, co-precipitating polymer and explosive into microspheres. Usual encapsulation procedure consisted of dissolving different ratios of polymer and the explosive in DCM and flowing the solution at a rate of 0.5mL/min into the sc-CO₂ filled chamber. The instrument settings were as follows: CO₂ flow rate of 20 g/min, the electric heat exchanger temperature was 80 °C, the reaction vessel heater temperature was 75 °C, the cyclone heater temperature was 10 °C, and the pressure was 150 bar. The main adjustment parameters included the concentration of the polymer explosive solution, the flow rate of the solution into the supercritical chamber, the pressure of the CO₂ chamber, the temperature of the CO₂ chamber, and addition of co-solvents to the CO₂ chamber, e.g. encapsulation of HMTD with PC was achieved by adding 10 mL water to the CO₂ chamber.

Spray coating

Ammonium nitrate (AN) encapsulation was done using a Resodyn LabRAM acoustic mixer coupled with an Sonozap Ultrasonic Atomizer spray coater (Resodyn, Butte, MT, USA) (Ivosevic, Coguill, and Galbraith 2009). Due to the hygroscopic nature of AN, a drying process before encapsulation was necessary. Pre-dried AN (core material, 200 g) was placed in the acoustic mixer under vacuum. A solution of a hydrophobic polymer (shell material, 2 g), either PMMA or EC in acetone (25 mL) was placed in the syringe pump and atomized into the vessel containing the AN. As the solution was sprayed

on the AN; the vacuum removed the solvent; and the polymer coated the AN surface. The LabRAM uses acoustic energy in the form of intense vibrations, that creates an almost fluidized state, allowing for an even surface coating (Bhaumik 2015). Encapsulation optimization included varying the acoustic mixer acceleration (10-20 G`s) and intensity (10-50 %), vacuum pressure (3-10 psi), syringe pump flow rate (0.1-0.5 mL/min), atomizer power (50-90%), and number of coatings (1-4).

Instrumental analyses

Imaging by polarized light microscope (PLM)

A Nikon Eclipse E400 POL Polarized Light Microscope (PLM; Nikon, Tokyo, Japan) was used to examine the particle size and shape of the training aids.

Thermal-gravimetric analyzer coupled to infrared spectrometer (TGA-IR)

A Q5000 thermal-gravimetric analyzer (TA Instruments, New Castle, DE, USA) was coupled to Nicolet 6700 Fourier transform infrared spectrometer (TGA-IR). The off-gas of core material from the TGA was routed through a heated transfer line to the infrared spectrometer for quantification and identification. The furnace and scale of the TGA were purged with nitrogen gas, at a flow rate of 25 and 10 mL/min, respectively. The TGA was set to ramp at 20 °C/min from 40 °C to 300 °C, for TATP and ETN analysis, and to 500 °C for TNT analysis. The transfer line and the 20 cm path length vapor cell were kept at 150 °C. IR spectra were collected at 32 scans with a spectral resolution of 4 cm⁻¹. Decomposition temperature (T_d) of polymers was determined by TGA, ramping at 20

°C/min from 40 °C to 1000 °C, with the off-gas uncoupled to the IR. Infrared (FT-IR) spectra with detection mode attenuated total reflection (ATR) were collected at 32 scans with a spectral resolution of 4 cm⁻¹. All samples (about 10 mg) were ran in duplicate, with two samples from each of the various training aids (n = 4).

Differential scanning calorimeter (DSC)

A TA Instruments differential scanning calorimeter (DSC) Q100 (TA Instruments, New Castle, DE, USA) was used to assess encapsulation efficiency. The DSC was set to ramp at 20 °C/min from 40 °C to 450 °C with nitrogen gas purge at 50 mL/min for TATP, ETN and TNT analysis. Decomposition temperature (T_d) of polymers was determined by DSC using the same heating method. AN analysis was done at a ramp of 20 °C/min from -30 °C to 300 °C. All samples (1 ± 0.05 mg) were sealed in hermetic aluminum pans and ran in duplicates, with two samples from each of the various training aids (n = 4). Glass transition temperature (T_g) of polymers was determined by ramping 5 °C/min from 40 °C to 250 °C, then ramping back down at a rate of 10 °C/min to -50 °C, and cycling back up at 5 °C/min to 250 °C.

Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography mass spectrometry (GC-MS), an Agilent 6890N GC system coupled to an Agilent 5973N Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA), was used to characterize the background vapor released from the training aids. Vapor from encapsulated materials was released thermally by baking training aids in a

headspace vial containing approximately 50 mg of the microspheres at 150 °C for 1 min, and injecting the headspace vapor (1mL) into a GC-MS. The method for TATP (J. C. Oxley, Smith, and Canino 2015) and ETN (J. C. Oxley et al. 2012) analysis were previously described. The method for TNT analysis was as follows: The vapor released from the training aids was injected into the splitless GC inlet set to temperature 260 °C. The GC column was an Agilent J&W VF-200ms (15m X 0.25mm i.d., particle size 0.25µm). The mode of operation was constant flow at 1.5 mL/min. The initial oven temperature, 110 °C, was held 5 min, followed by a ramp of 10 °C/min to 280 °C with a 5 min hold. The MS transfer line was kept at 285 °C. MS analysis was carried out using electron impact (EI) ionization (70 eV) with a scanning range of m/z 20-500 at a rate of 2 scan/s, and 1.5 min solvent delay.

Direct infusion high-resolution mass spectrometry (HRMS)

Direct infusion into the Thermo Scientific Exactive high resolution mass spectrometer (HRMS; Thermo Scientific, Waltham, MA, USA) was used to verify that intact explosive was present in the vapor phase. Since the encapsulated material is released by heat, the training aids were tested by baking a headspace vial containing approximately 50 mg of the microspheres at 150 °C for 1 min. The headspace vapor (5 mL) was dissolved in 500 µL of methanol/aqueous solution (1:1, v/v) and directly infused it into a HRMS at a flow rate of 10 µL/min. The aqueous solution consisted of 10mM ammonium acetate for TATP, or 200µM ammonium acetate, 200µM ammonium chloride, 200µM ammonium

nitrate and 0.1% formic acid for TNT and ETN. The MS tune conditions for TNT, TATP and ETN analyses are shown in **Table 1-1**.

Table 1-1. High resolution mass spectrometer tune conditions

Parameters	TNT (ESI ⁻)	TATP (APCI ⁺)	ETN (ESI ⁻)
Spray voltage (kV)	-4.2	N/A	-3
Discharge current (μ A)	N/A	5	N/A
Vaporizer temperature ($^{\circ}$ C)	N/A	220	N/A
N ₂ sheath gas flow rate (AU)	15	15	11
N ₂ auxiliary gas flow rate (AU)	2	4	0
Capillary temperature ($^{\circ}$ C)	250	220	150
Capillary voltage (V)	-25	30	-28
Tube lens voltage (V)	-80	45	-130
Skimmer voltage (V)	-18	14	-20

N/A not applicable; *AU* arbitrary units; *ESI⁻* electrospray ionization (negative mode); *APCI⁺* atmospheric chemical ionization (positive mode)

Explosive sensitivity tests

Explosive sensitivity tests were done to determine the sensitivity to detonation of the training aids when exposed to friction, electrostatic and impact.

Impact sensitivity (AOP-7 U.S. 201.01.001) was done using a custom type 12 system. Varying impact energy values, established by applying varying heights from which to drop a specific weight ($m = 3.9 \text{ kg}$), were applied to the sample (about $35 \pm 5 \text{ mg}$) until 50 % probability of impact initiation was reached, determined by the Bruceton method.

Friction sensitivity (AOP-7 U.S. 201.02.006) was measured using a BAM Friction Apparatus FSA-12 (OZM Research, Hrochuv Tyneč, Czech Republic). Varying friction force values, established by applying varying mass and distance, were applied to the sample ($2\text{-}5 \text{ mg}$) until 50 % probability of friction initiation was reached.

Electrostatic sensitivity was measured using an ESD 6 (UTEC Corporation, Riverton, KS, USA) in accordance to MIL-STD-1751 method 4. Varying electrostatic energy values, established by applying varying capacitance and voltages, were applied to the sample ($25 \pm 5 \text{ mg}$) until 50 % probability of electrostatic initiation was reached.

Results and Discussion

Trace Explosives Aids for Scent (TEAS)

Characterization

Thermoplastics were selected for the training aids because they are stable at high temperatures (Kawaguchi et al. 2005); nevertheless, the decomposition of the polymers selected for encapsulation was screened by TGA and DSC. Thermal degradation of the polymers by TGA showed one mass loss for PC and PEI with 77 % decomposition around 539 °C and 49% decomposition around 554 °C, respectively. PS completely decomposed around 429 °C, and PMMA had two mass losses, with complete decomposition around 397 °C (**Table 1-2, Figure A-1**). TGA traces indicated that PC, PEI and PS were stable in the desired temperature range with less than 0.1% mass loss by 300 °C. On the other hand, PMMA lost 0.2% mass by 100 °C, and the PMMA IR spectrum indicated decomposition as temperature increased, with methacrylate derivatives peaks matched to the IR library. DSC showed no decomposition from PC nor PEI until 450 °C (temperature limit of the instrument), but PS and PMMA showed initial decomposition around 378 °C and 344 °C, respectively (**Table 1-2, Figure A-2**). The similar thermal degradation patterns observed from both the TGA and DSC, suggest that PC, PEI and PS are suitable polymers for encapsulation release by heat.

Table 1-2. Summary of DSC results for thermoplastics

Polymers	TGA ramp 20 °C/min to 1000 °C								DSC cycle		DSC ramp			
	1 st Mass Loss - T (°C)	SD	1 st Mass Loss - Weight (%)	SD	2 nd Mass Loss - T (°C)	SD	2 nd Mass Loss - Weight (%)	SD	Polymer T _g (°C)	SD	1 st ↓ T (°C)	SD	1 st ↓ Heat Flow (J/g)	SD
PC	539	9	77	0					148	0				
PS	429	2	100	0					104	0	378	3	incomplete	
PMMA	302	6	5	0	397	9	95	0	118	0	344	2	859	166
PEI	554	11	49	2					216	0				

↓ endotherm; ↑ exotherm; *SD* standard deviation; *T* onset temperature

Microscopy of the encapsulated explosive showed them as spherical white particles with particle size, averaging around 50-150 μm (**Figure 1-1**). The solvent evaporation does not produce absolute capsules since the core material is distributed within the polymer matrix instead of encircled by it. Since the encapsulated material is the micrometer range and has a spherical shape, we referred to the encapsulated product as microspheres.

Figure 1-1. Image of TATP, ETN and TNT encapsulated with PEI

Release of the encapsulated explosive (the core material) is accomplished by heat; therefore, the first method to assess encapsulation was TGA-IR. The explosive was released from the polymer shell as the sample was heated at a set rate by TGA; the vapor released was channeled to the IR to confirm the release of the explosive. The method not only analyzed the purity of the vapor, but the weight loss, at the temperature releasing vapor, was used to evaluate the explosive loading of the microspheres, reported as percent mass loss (**Table 1-3**). The explosive release temperature was assigned from the TGA as the peak from the first derivative of the mass loss, which corresponded to the explosive vapor.

Table 1-3. Summary of TGA results for all training aids

Sample	1 st Mass Loss - T (°C)	SD	1 st Mass Loss - Weight (%)	SD	2 nd Mass Loss - T (°C)	SD	2 nd Mass Loss - Weight (%)	SD	3 rd Mass Loss - T (°C)	SD	3 rd Mass Loss - Weight (%)	SD
TATP	94	6	98	1								
PC-TATP	148	1	15	0								
PS-TATP	98	1	1	0	175	1	14	1				
PMMA-TATP	136	1	6	0	215	1	39	1				
PEI-TATP	194	0	32	0								
ETN	177	4	100	0								
PC-ETN	192	1	27	0								
PS-ETN	185	1	26	0								
PMMA-ETN	194	0	33	0	267	1	24	0				
PEI-ETN	198	1	30	0								
TNT	242	10	100	0								
PC-TNT	241	10	32	0								
PS-TNT	240	14	27	0								
PMMA-TNT	259	11	35	0								
PEI-TNT	115	3	1	0	202	1	11	2	286	5	21	2

SD standard deviation; *T* temperature

The TGA thermograms of the TATP microspheres with different polymers are shown in **Figure 1-2**. PC-TATP had a mass loss of about 15 % at 148 °C (**Table 1-3**), indicating that the microspheres can be triggered to release the explosive by heating them at this temperature, and that the explosive loading is about 15 % of the total microspheres. The IR spectrum from the vapor released from PC-TATP around 148 °C matches the IR spectrum of TATP vapor (**Figure 1-3**), with corresponding characteristic peaks at 942 cm^{-1} (ring O-O asymmetric stretch), 1194 cm^{-1} (O-C-O and $\text{H}_3\text{C-C-CH}_3$ asymmetric stretch), 2953 cm^{-1} (methyl substituent C- CH_3 symmetric stretch) and 3007 cm^{-1} (aliphatic C-C symmetric stretch) (Mamo and Gonzalez-Rodriguez 2014; J. Oxley et al. 2008), indicating that the explosive, can be controllably released from its encapsulated shell.

Figure 1-2. TGA thermograms of TATP encapsulated with various polymers

Figure 1-3. Overlaid IR spectra of the vapor released from pure TATP compared with the vapor released from TATP encapsulated with various polymers (corresponding to the largest mass loss from the thermograms illustrated on **Figure 1-2**)

Curiously, the thermogram of PS-TATP indicated two consecutive mass losses, first 1 % at 98 °C and second 14 % at 175 °C (**Table 1-3**), but the IR spectrum from both mass losses corresponded to that of TATP. The TGA of PEI-TATP showed an abnormally high mass loss of about 32 % at 194 °C (**Table 1-3**); however, the IR spectrum of the vapor released at that mass loss indicated a contaminant, since it exhibited an extra peak at 1776 cm^{-1} (**Figure 1-3**), which is characteristic of an imide carbonyl stretch consistent with the chemical structure of PEI (Chen et al. 2006). Considering PEI did not decompose until higher temperatures (554 °C), this PEI leaching around 194 °C suggests chemical interaction with TATP is promoting early degradation (**Figure 1-3**).

The IR spectra of the vapor released from PMMA-TATP (**Figure 1-3**) and PMMA-ETN (**Figure A-3**) contained peaks characteristic of methyl methacrylate at 1167 cm^{-1} (C-O-C), 1636 cm^{-1} (C=C vinyl) and 1749 cm^{-1} (C=O ester) (Sugumaran, Juhanni, and Karim 2017). Kashiwagi have described the thermal degradation of PMMA into its monomer at temperatures above 200 °C (Kashiwagi et al. 1986), suggesting that the heating process necessary to release the core material from the microspheres is also degrading the polymer and contaminating the background odor of the spheres. Evaluations such as this, led to final selection of the encapsulating polymers.

The TGA thermograms of PC-ETN, PS-ETN and PEI-ETN indicated the explosive loading of 27, 26, and 30 %, with vapor release at 192, 185 and 198 °C, respectively (**Figure A-4**). The IR spectra from the vapor released from these microspheres matches the IR spectrum of pure ETN (**Figure A-3**), with corresponding characteristic peaks at 837 cm^{-1}

¹ (ester O-N stretch), 1276 cm⁻¹ (symmetric NO₂ stretch) and 1630 cm⁻¹ (asymmetric NO₂ stretch) (Oleske et al. 2015; Manner et al. 2014; McNesby and Pesce-Rodriguez 2002; Urbanski and Witanowski 1963), indicating that the mass losses observed in the TGA corresponds to the explosive being released.

The core material is released from the microspheres by heat; however, little is known about its mechanism. Glass transition temperature has been suggested to be involved in the release mechanism (Omelczuk and McGinity 1992). The TATP release temperature from the PC-TATP and PS-TATP (**Table 1-3**) coincided with the glass transition temperature of those polymers, 148 and 104 °C, respectively (**Table 1-2, Figure A-5**), suggesting the mechanism of release occurs as the polymer changes from a glassy to a soft material. On the other hand, the ETN release temperature from all polymers was around 192 °C (**Table 1-3**), which correlates to the exothermic decomposition of ETN, 185 °C (**Table 1-4**). This data suggests the mechanism of release is dependent on the properties of the explosive and not on that of the encapsulation material. Therefore, neither the glass transition temperature of the polymer nor the vaporization temperature of the explosive can explain the mechanism of release for all explosive microspheres examined.

Table 1-4. Summary of DSC results for all training aids

Sample	1 st ↓ T (°C)	SD	1 st ↓ Heat Flow (W/g)	SD	1 st ↑ T (°C)	SD	1 st ↑ Heat Flow (W/g)	SD	2 nd ↓ T (°C)	SD	2 nd ↓ Heat Flow (W/g)	SD	3 rd ↓ T (°C)	SD	3 rd ↓ Heat Flow (W/g)	SD
TATP	98	0	121	4	193	9	1245	80								
PC-TATP					198	2	174	46								
PS-TATP					204	0	205	14	379	3	210	5				
PMMA-TATP	180	3	10	1					217	3	29	2	367	3	222	36
PEI-TATP	91	0	9	0	177	1	398	60								
ETN	61	0	121	8	185	5	796	22								
PC-ETN	61	0	20	3	183	1	297	23								
PS-ETN	61	1	38	4	182	2	479	203	360	7	205	4				
PMMA-ETN					184	2	744	106								
PEI-ETN					188	2	668	75								
TNT	81	0	95	3	311	5	1164	62								
PC-TNT	58	1	5	0	325	2	669	116								
PS-TNT	80	0	23	1	290	8	390	191	383	10	129	8				
PMMA-TNT					296	2	570	112								
PEI-TNT					297	1	684	33								

↓ endotherm; ↑ exotherm; *SD* standard deviation; *T* onset temperature

The thermograms of PC-TNT, PS-TNT and PMMA-TNT had a mass loss of 32 % at 241 °C, 27 % at 240 °C and 35 % at 259 °C, respectively (**Figure A-6**); furthermore, the TGA of PEI-TNT showed three mass losses of 1, 11 and 21 % at 115, 202 and 286 °C, respectively. Unfortunately, IR vapor analysis at these specific mass losses could not be obtained because the TNT vapor condensed in the transfer line connecting the TGA to the IR vapor cell.

Trace Explosive Aids for Scent (TEAS) are intended for construction of ETD library databases and dog scent imprinting; therefore, a clean background odor is essential. For that reason, GC-MS verification of the vapor released from the microspheres was performed. The chromatograms of the vapor released from the TATP (**Figure A-7**) and TNT (**Figure A-8**) microspheres with PC, PS and PEI showed a clean background with peaks corresponding to the retention time of only the respective explosive. The chromatograms of the vapor released from PMMA-TATP, PMMA-TNT and PMMA-ETN included an additional peak that did not match the explosives; and although no mass spectral library match was obtained, polymer degradation was likely, as suggested from the IR data.

In addition to the issues pertaining to polymer thermal degradation, there were additional problems with contaminants present in the polymers' starting material. Unfortunately, these impurities did not necessarily appear under all chromatographic conditions. Using the gentle chromatographic method employed for the easily degradable ETN (**Figure 1-4**), i.e. a short chromatographic column and low starting temperature,

styrene, the monomer of PS, and dichlorobenzene, the solvent used in polymerization of PEI (Chiong et al. 2017; Bookbinder, Peters, and Cella 1988), were observed in the blank microspheres of their corresponding polymers (**Figure A-9**). This emphasizes the need for pre-cleaning the polymers before encapsulation which was accomplished by heating the polymer under vacuum, or heating the polymer under high pressure using the Rapid Expansion of Supercritical Solutions (RESS), or dissolving and re-precipitating the polymer (J. C. Oxley, Smith, and Canino 2015).

Figure 1-4. Overlaid GC chromatogram, with magnification around the baseline, of the vapor released from pure ETN compared with the vapor released from ETN encapsulated with various polymers at 150 °C for 1 min

The mass spectra of the vapor released from the TATP, TNT and ETN microspheres observed from GC-MS experiments matched the retention time and literature mass spectra by EI ionization of the pure explosive. The EI fragmentation pattern (**Figure A-10**) of TATP (characterized by m/z 43, 58, 59, 75) (ATF 2018), ETN (characterized by m/z 30, 46, 60, 76, 89, 151) (J. C. Oxley et al. 2012), TNT (characterized by m/z 63, 89, 134, 149, 164, 180, 193, 210) (ATF 2018), all matched the mass spectra of the vapor released from the corresponding microspheres. However, molecular ions for these explosives were not observed under standard EI conditions.

To confirm that the explosives did not degrade while the microspheres were heated, their released vapor were dissolved in a solvent and directly infused into an HRMS. All TATP microspheres had m/z 240.1442, corresponding to $[\text{TATP}+\text{NH}_4]^+$; all ETN microspheres had m/z 363.9866, corresponding to $[\text{ETN}+\text{NO}_3]^-$; and all TNT microspheres had m/z 226.0106, corresponding to $[\text{TNT}-\text{H}]^-$, indicating that the explosives did not degrade during heating and was present as an intact molecule in the vapor phase (**Figure 1-5**).

Figure 1-5. Full scan mass spectrum of TATP, ETN and TNT encapsulated with PS using APCI+ for TATP and ESI- for ETN and TNT on a Thermo Exactive HRMS

DSC was another quality control technique used to evaluate encapsulation (**Table 1-4**). Encapsulation consists of non-covalent interactions between the core and shell material, in which both retain their intrinsic properties (e.g. melting point). However, the disappearance of the melting endotherm of the core material suggests an inclusion complex, where the shell material completely surrounds the core (Grandelli et al. 2013). The DSC thermogram of TNT encapsulated with PS (**Figure 1-6**) indicates their intrinsic properties are preserved; only the TNT endothermic and exothermic peaks were observed (no polymer peaks should be observed in this range). In the thermograms of PMMA-TNT and PEI-TNT, the TNT melting peak disappeared, suggesting the formation of an inclusion complex. In the thermogram of TNT encapsulated with PC, the melting point of TNT shifted to lower temperature, which is usually an indication of impurities contaminating the thermogram. Encapsulated ETN either exhibited an endotherm which was an exact match to that of pure ETN, observed with PC and PS; or the endotherm of melt completely disappeared, observed with PMMA and PEI (**Figure A-11**). In the DSC thermograms of PC-TATP and PS-TATP the TATP melting endotherm was absent. However, the DSC of PMMA-TATP did not resemble the thermograms of either the core or shell material; and the DSC of PEI-TATP showed a decrease in the endothermic peak of TATP from 98 °C to 91 °C (**Figure A-12**), suggesting some decomposition and/or impurities, which was noted in the IR data (**Figure 1-3**).

Figure 1-6. DSC thermogram of pure TNT compared with TNT encapsulated with different polymers

Explosives are classified based on their sensitivity to detonation when exposed to impact, friction and/or electrostatic. DFT calculations of energetic materials encapsulated in carbon nanotubes have predicted that explosives are stabilized upon encapsulation, proposing that, as a consequence, their sensitivity would be reduced (Smeu et al. 2011). Therefore, sensitivity testing (**Table 1-5**) was done to ensure that encapsulation decreased the explosives sensitivity, converting them to a less hazardous material that can safely be handled. TATP is a primary explosive (Gerber, Walsh, and Hopmeier 2014), considered extremely sensitive, but encapsulation desensitized TATP. All polymers significantly decreased the sensitivity of TATP to impact, friction and electrostatic, although PEI appears to be least effective polymer in decreasing the impact and friction sensitivity of TATP. Encapsulation of ETN greatly decreased its impact (Matyas and Künzel 2013), friction (Matyas and Künzel 2013) and electrostatic sensitivity, especially with PMMA and PEI which prevented ETN from reacting even at the maximum friction force (360 N) or drop height (2.838 m). TNT is a secondary explosive (Trzciński et al. 2014), deemed insensitive, and although encapsulation did not affect its friction sensitivity, encapsulation reduced its impact and electrostatic sensitivity.

Table 1-5. Summary of impact, friction and electrostatic sensitivity results for all training aids

Sample	Impact Energy (J)	Friction Force (N)	Electrostatic Energy (mJ)
TATP	12	<0.5 ^b	3-4
PC-TATP	>108 ^a	>360 ^a	563-606
PS-TATP	>108 ^a	>360 ^a	792-3001
PMMA-TATP	>108 ^a	>360 ^a	309
PEI-TATP	34	28-30	563
ETN	24	14-56	29-46
PC-ETN	48	120-144	309
PS-ETN	41	144-360	276-309
PMMA-ETN	>108 ^a	>360 ^a	203
PEI-ETN	>108 ^a	>360 ^a	124-150
TNT	63	>360 ^a	141-360
PC-TNT	>108 ^a	>360 ^a	446-456
PS-TNT	>108 ^a	>360 ^a	456
PMMA-TNT	>108 ^a	>360 ^a	309
PEI-TNT	>108 ^a	>360 ^a	446

^a greater than instrument maximum

^b less than instrument minimum

Field Testing

IMS and canines are placed in most ports of entry for screening of people and cargo to detect explosives and other contraband (Brown et al. 2016). Therefore, IMS testing of these encapsulated explosives was imperative to ensure that they would perform as effective training aids in field. Encapsulated explosives were deposited on a swab, and the swab was analyzed using a Morpho Detection Itemizer IMS (Morpho Detection, Newark, CA, USA). TATP and TNT encapsulated with any of the polymers alarmed as the corresponding explosive in tests run in triplicate. However, ETN training aids indicate some false negatives, once with PS and PEI, and twice with PC and PMMA.

Bomb-sniffing dogs are imprinted on explosive odor by “operant conditioning” (K. G. Furton and Myers 2001; K. Furton, Greb, and Holness 2010). Briefly, small containers with explosives are hidden in multiple locations, as the handler slowly walks the dog, allowing it to sniff and sit when it recognizes the explosive odor, expecting a positive reward. The K9 unit evaluation of our training aids consisted of: 1) placing about 50 mg of microspheres in a small vial (7×50 mm) plugged with a pre-cleaned cotton swab, 2) heating the microspheres with a purpose-built heater (DetectaChem®), and 3) once the explosive had condensed on the swab, proceeding with the normal training routine, using the swab instead of bulk explosives (**Figure 1-7**). The bomb-sniffing dogs, imprinted on bulk explosives were able to unmistakably recognize the odor released from the training aids.

Encapsulation allows the training aids a long shelf-life, controlled release, and safe handling of otherwise sensitive materials. Containing only trace amounts of pure explosives, they are a valuable alternative to handling hazardous materials in the field.

Figure 1-7. Steps for field testing of training aids

Supercritical CO₂ assisted encapsulation

Since post-reaction treatment to eliminate solvent remnants from the training aids decreased throughput, an encapsulation method that did not require solvent was needed. In addition, a method that would allow encapsulation of HMTD, PETN and RDX was wanted, since they have poor solubility in DCM and other suitable solvents, required for the solvent evaporation method. In order to improve the production of training aids, supercritical CO₂ assisted encapsulation was attempted. The advantages of using supercritical CO₂ for the encapsulation of explosives include use of low temperatures which minimizes the risk of decomposition, solvent absence which reduces the processing time and increases throughput, and polar and nonpolar compounds suitability which accommodates most explosives and co-solvents.

Although the thermogram of TNT (Teipel, Gerber, and Krause 1998) encapsulated in PEI demonstrated a small mass loss of 4-5 %, it was consistent within trials, demonstrating proof-of-concept (**Figure 1-8**). Reproducibility issues of HMTD encapsulation were observed using both solvent evaporation (**Figure A-13**) and sc-CO₂ assisted encapsulation, as illustrated in the **Figure 1-9** thermogram where the first trial indicated a mass loss of 6.5 %, demonstrating encapsulation and release of the core material around 150 °C, but the second trial shows a minimum mass loss of 1.4 %, pointing to a failed encapsulation.

Figure 1-8. TGA thermogram of sc-CO₂ PEI-TNT made under the same SAS method conditions

Figure 1-9. TGA thermogram of sc-CO₂ PC-HMTD made under the same SAS method conditions

Spray coating

The energetic material community can benefit from using encapsulation in areas other than manufacturing training aids. For example, encapsulation of AN can significantly reduce its hygroscopicity, and, as a consequence, improve its fuel efficiency to be used as a green solid rocket propellant (Chaturvedi and Dave 2013). **Figure 1-9** illustrates the standard five phase transitions of AN. At 32 °C, AN undergoes the phase transition IV → III, which is associated with a large volume change. This volumetric change may cause material cracking, which can initiate irregular burns, rendering the material impractical as a propellant (Lang and Vyazovkin 2008; Chaturvedi and Dave 2013). However, in the absence of water, phase transition III is absent; and the transition IV → II occurs around 50 °C (Lang and Vyazovkin 2008; Kiiski 2009). Therefore, a polymer shell of PMMA (**Figure 1-10**) or EC (**Figure A-14**) was spray coated onto AN to prevent the uptake of water (**Figure 1-10**), avoid the phase transition III, decrease the AN volume variation, and solve the issues with abnormal burning. Spray coating produced a capsule which surrounded the AN with a hydrophobic barrier that prevented AN from absorbing water. The IR spectrum of PMMA coated AN demonstrated the presence of the polymer on the surface of the encapsulated AN. PMMA-AN has two peaks at 1727 and 1754 cm⁻¹. The latter corresponds to the AN peak at 1754 cm⁻¹ (**Figure 1-11**); and the former, to the PMMA peak at 1722 cm⁻¹ (C=O stretching vibration) which became more prominent as the number of coatings was also increased.

Figure 1-10. DSC thermogram of pure AN compared with AN coated with PMMA stored at room temperature for 6 months

Figure 1-11. Overlaid IR spectrum of AN coated PMMA compared with either pure AN or pure PMMA

Conclusions

TATP, TNT and ETN have been encapsulated using the solvent evaporation method. The use of these encapsulated materials as training aids for ETD manufacturers and K9 units training has several advantages: the polymer protective shell allows the material to be stored at room temperature and provides explosivity protection, since the microspheres are insensitive to impact, friction and electrostatic, rendering them safe to handle; and thorough quality control tests ensure a clean background odor, no decomposition upon heating release, and a pure explosive vapor.

Results from each of the quality control techniques used to characterize these training aids determined that PC and PS are suitable polymers for TATP encapsulation with about 15 % TATP released around 162 °C, PS and PEI are suitable solvents for TNT encapsulation with about 30 % TNT released around 242 °C, and PC, PS and PEI are suitable polymers for ETN encapsulation with about 28 % ETN released around 192 °C (**Table 1-3**); however, considering the impurities found in the pure polymers, PS and PEI, these are not adequate for encapsulation unless a thorough pre-cleaning process is implemented.

Supercritical CO₂ assisted encapsulation was performed with TNT, showing proof-of-concept. However, to date, this technique has not increased throughput, nor allowed for scale-up of the training aids made using the solvent evaporation method, nor did it expand the training aids selection to permit more explosives to be encapsulated. Spray coating successfully encapsulated AN with PMMA and EC, preventing the uptake of water,

avoiding the phase transition III, and decreasing the AN volume variation. As a consequence, the coating increased the shelf-life of the dry AN and, perhaps, solving the issues with abnormal burning, thus, making it an effective green propellant. Encapsulation is a versatile tool that can be further exploited, in addition to the practical application demonstrated, the use of training aids, from encapsulating explosives.

Acknowledgments

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MANUSCRIPT 2**In vitro and in vivo studies of triacetone triperoxide (TATP) metabolism
in humans**

by

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Abstract

Purpose Triacetone triperoxide (TATP) is a volatile but powerful explosive that appeals to terrorists due to its ease of synthesis from household items. For this reason, bomb squad, canine (K9) units, and scientists must work with this material to mitigate this threat. However, no information on the metabolism of TATP is available.

Methods In vitro experiments using human liver microsomes and recombinant enzymes were performed on TATP and TATP-OH for metabolite identification and enzyme phenotyping. Enzyme kinetics for TATP hydroxylation were also investigated. Urine from laboratory personnel collected before and after working with TATP was analyzed for TATP and its metabolites.

Results While experiments with flavin monooxygenases were inconclusive, those with recombinant cytochrome P450s (CYPs) strongly suggested that CYP2B6 was the principle enzyme responsible for TATP hydroxylation. TATP-*O*-glucuronide was also identified and incubations with recombinant uridine diphosphoglucuronosyltransferases (UGTs) indicated that UGT2B7 catalyzes this reaction. Michaelis–Menten kinetics were determined for TATP hydroxylation, with $K_m = 1.4 \mu\text{M}$ and $V_{\max} = 8.7 \text{ nmol/min/nmol CYP2B6}$. TATP-*O*-glucuronide was present in the urine of all three volunteers after being exposed to TATP vapors showing good in vivo correlation to in vitro data. TATP and TATP-OH were not observed.

Conclusions Since scientists working to characterize and detect TATP to prevent terrorist attacks are constantly exposed to this volatile compound, attention should be paid to its metabolism. This paper is the first to elucidate some exposure, metabolism and excretion

of TATP in humans and to identify a marker of TATP exposure, TATP-*O*-glucuronide in urine.

Keywords

Triacetone triperoxide (TATP), Terrorists, Human in vitro and in vivo metabolism for TATP exposure, TATP-*O*-glucuronide, CYP2B6 hydroxylation, UGT2B7 glucuronidation

Introduction

Triacetone triperoxide (3,3,6,6,9,9-hexamethyl-1,2,4,5,7,8-hexoxonane, TATP) is a homemade explosive, easily synthesized from household items [1]. For this reason, TATP has often been used by terrorists [2, 3], necessitating its research by bomb squad, canine (K9) units, and scientists [4]. In addition to being extremely hazardous, this peroxide explosive is highly volatile, with partial pressure of 4-7 Pa at 20 °C [5, 6]. Personnel exposed to TATP will most likely absorb it through inhalation and/or dermal absorption. However, no information on the human absorption, distribution, metabolism, excretion and toxicity (ADMET) of TATP is available. Therefore, this paper will investigate the in vitro metabolism of TATP and the in vivo excretion through urine analysis.

The toxicity of most military explosives has been well characterized [7]. The biotransformation of trinitrotoluene, for example, has been thoroughly investigated. It is metabolized by cytochrome P450 (CYP) reductase, forming nitroso intermediates, and yielding 4-hydroxylamino-2,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene. These primary metabolites are further reduced by CYP to 2,4-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene [8, 9]. In vivo studies of Chinese ammunition factory workers found metabolites such as 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene, in urine and bound to the hemoglobin in blood [9, 10]. TATP has been studied for almost two decades, but its metabolism and toxicity are still unknown. TATP characterization is problematic since it is an extremely sensitive explosive, difficult to handle and, due to its high volatility, difficult to concentrate in biological samples [5].

Most xenobiotics are metabolized by CYP which is a family of heme-containing enzymes found in all tissues, particularly the liver endoplasmic reticulum (microsomes). CYPs catalyze phase I oxidative reactions (among others) in the presence of oxygen and a reducing agent (usually reduced nicotinamide adenine dinucleotide phosphate, NADPH). NADPH provides electrons to the CYP heme via CYP reductase. This oxidation generally produces more polar metabolites that are either excreted in the urine or undergo phase II biotransformation, further increasing their hydrophilicity [11]. One of the most common phase II reactions is glucuronidation, which is catalyzed by uridine diphosphoglucuronosyltransferase (UGT) in the presence of the cofactor uridine diphosphoglucuronic acid (UDPGA). In this reaction, glucuronic acid is conjugated onto an electron-rich nucleophilic heteroatom, frequently added to the substrate by phase I metabolism. Glucuronide metabolites increase the topological polar surface area (TPSA) and reduce the partition coefficient (LogP) of xenobiotics to be ionized at physiological pH, thus, increasing the aqueous solubility of the compound for excretion [11].

TATP is a cyclic peroxide, a motif shared with the antimalarial drug, artemisinin. The endoperoxide functionality of artemisinin is thought to be crucial for its antimalarial activity [12]. In the presence of ferrous ions, artemisinin undergoes homolytic peroxide cleavage to yield an oxygen radical that may be lethal to malaria parasite, *Plasmodium falciparum*. Biotransformation studies indicate that artemisinin is primarily metabolized by CYP2B6 to deoxyartemisinin, deoxydihydroartemisinin, dihydroartemisinin and ‘crystal-7’ [13, 14]. Similarly, we have previously shown that TATP is metabolized in vitro by canine CYP2B11, another CYP2B subfamily enzyme [15]. Artemisinin is further

metabolized by glucuronidation, particularly by UGT1A9 and UGT2B7, to dihydroartemisinin-glucuronide, the principal metabolite found in urine, suggesting endoperoxides, like TATP, may be glucuronidated and excreted in urine [16].

Laboratory personnel who work on synthesizing, characterizing and detecting TATP are inevitably exposed to this volatile compound. Even the small-sized samples that they work with can result in buildup of TATP in a confined space. Furthermore, bomb-sniffing dogs and their handlers are purposely exposed to these vapors for the sake of training. Our previous study revealed TATP metabolism in dog liver microsomes (DLM) [15]. Now we evaluate its in vitro biotransformation in human liver microsomes (HLM) and recombinant enzymes, identifying phase I and phase II metabolites, estimating enzyme kinetics and also detecting urinary in vivo metabolites excreted from scientists exposed to TATP in their work environment.

Materials and Methods

Chemicals

Optima HPLC grade methanol, Optima HPLC grade water, Optima HPLC grade acetonitrile, American Chemical Society (ACS) grade acetone, ACS grade methanol, ACS grade pentane, hydrochloric acid, ammonium acetate, dipotassium phosphate, monopotassium phosphate, magnesium chloride (MgCl_2) and reduced glutathione (GSH) were purchased from Fisher Chemical (Fair Lawn, NJ, USA); NADPH, 1-aminobenzotriazole, methimazole, 1-naphthol and hydroxyacetone from Acros Organics (Morris Plain, NJ, USA); UDPGA, saccharolactone and 2,4-dichlorophenoxyacetic acid from Sigma-Aldrich (St. Louis, MO, USA); bupropion, benzydamine and alamethicin from Alfa Aesar (Ward Hill, MA, USA); oxcarbazepine from European Pharmacopoeia Reference Standard (Strasbourg, France); ticlopidine from Tokyo Chemical Industry (Tokyo, Japan); hydroxybupropion from Cerilliant Corporation (Round Rock, Texas, USA); deuterated acetone (acetone- d_6) from Cambridge Isotope Labs (Cambridge, MA, USA); hydrogen peroxide (50%) from Univar (Redmond, WA, USA); HLM, rat liver microsomes (RLM), DLM and human lung microsomes (HLungM) from Sekisui XenoTech (Kansas City, KS, USA); human recombinant CYP (rCYP) bacosomes expressed in *Escherichia coli* (*E. coli*) from Cypex (Dundee, Scotland); human recombinant flavin monooxygenase (rFMO) supersomes and human recombinant UGT (rUGT) supersomes expressed in insect cells from Corning (Woburn, MA, USA).

TATP, deuterated TATP (TATP- d_{18}) and hydroxy-TATP (TATP-OH) synthesis

TATP was synthesized following the literature methods using hydrochloric acid as the catalyst [1]. TATP was purified by recrystallization, first with methanol/water (80:20, w/w) and then with pentane. TATP- d_{18} was synthesized as above using acetone- d_6 . TATP-OH was synthesized as above using hydrogen peroxide (50 wt%)/acetone/hydroxyacetone (2:1:1, mol ratio) [15]. TATP-OH was purified using a CombiFlash RF+ system with an attached PurIon S MS system (Teledyne Isco, Lincoln, NE, USA), followed by two cycles of drying and reconstituting in solvent to sublime away the TATP. Separation was performed using a C-18 cartridge combined with a liquid chromatograph flow of 18 mL/min with 10% methanol (A) and 90% aqueous 10 mM ammonium acetate (B) for 1 min, before ramping to 35%A/65%B over 1 min, followed by another ramp to 95%A/5%B over the next 1 min, holding for 2 min, before a 30 s transition to initial conditions, with a hold of 2 min [15].

Instrumental analyses

Metabolite identification was performed by high-performance liquid chromatography coupled to Thermo Scientific Exactive or Thermo Scientific LTQ Orbitrap XL high resolution mass spectrometers (HPLC–HRMS) (Thermo Fisher Scientific, Waltham, MA, USA). A CTC Analytics PAL autosampler (CTC Analytics, Zwingen, Switzerland) was used for LC injections, solvent delivery was performed using a Thermo Scientific Accela 1200 quaternary pump, and data collection/analysis was done using Xcalibur software (Thermo Scientific, version 2.1).

Metabolite quantification was performed by high-performance liquid chromatography coupled to AB Sciex Q-Trap 5500 triple quadrupole mass spectrometer (HPLC–MS/MS) (AB Sciex, Toronto, Canada). A CTC Analytics PAL autosampler was used for LC injections, solvent delivery was performed using a Thermo Scientific Accela 1200 quaternary pump and data collection/analysis was done with Analyst software (AB Sciex, version 1.6.2).

The HPLC method for all TATP derivatives was as follows: sample of 40 μ L (Exactive and LTQ Orbitrap XL) or 20 μ L (Q-Trap 5500) in acetonitrile/water (50:50, v/v) were injected into LC flow at 250 μ L/min of 10%A/90%B for introduction onto a Thermo Synchronis C18 column (50 $\times$ 2.1 mm i.d., particle size 5 μ m). Initial conditions were held for 1 min before ramping to 35%A/65%B over 1 min, followed by another ramp to 95%A/5%B over the next 1 min. This ratio was held for 2 min before reverting to initial conditions over 30 s, which was held for additional 2 min. The Exactive MS tune conditions for atmospheric pressure chemical ionization (APCI) in positive mode were as follows: N₂ sheath gas flow rate, 30 arbitrary units (AU); N₂ auxiliary gas flow rate, 30 AU; discharge current, 6 μ A; capillary temperature, 220 °C; capillary voltage, 25 V; tube lens voltage, 40 V; skimmer voltage, 14 V; and vaporizer temperature, 220 °C. The Exactive MS tune conditions for electrospray ionization (ESI) in negative mode were as follows: N₂ sheath gas flow rate, 30 AU; N₂ auxiliary gas flow rate, 15 AU; spray voltage, -3.4 kV; capillary temperature, 275 °C; capillary voltage, -35 V; tube lens voltage, -150 V; and skimmer voltage, -22 V. The LTQ Orbitrap XL MS tune conditions for ESI–, used for TATP-*O*-glucuronide verification, were as follows: N₂ sheath gas flow rate, 30 AU; N₂ auxiliary gas

flow rate, 15 AU; spray voltage, -4 kV; capillary temperature, 275 °C; capillary voltage, -15 V; and tube lens voltage, -84 V. The single-reaction monitoring settings were as follows: m/z 413.13 with isolation width m/z 1.7, activated by higher-energy collision dissociation at 35 eV. The Q-trap 5500 MS tune and multiple reaction monitoring (MRM) conditions are shown in **Table 2-1**. TATP and TATP-OH quantification was done as the area ratio to TATP- d_{18} (internal standard, IS) using a standard curve ranging 10-20,000 ng/mL and 10-500 (**Figure B-1**) or 500-8,000 ng/mL, respectively. TATP-*O*-glucuronide relative quantification was done as the area ratio to 2,4-dichlorophenoxyacetic acid (IS).

The HPLC method for bupropion, hydroxybupropion, benzydamine, benzydamine *N*-oxide, and oxcarbazepine (IS), was as follows: sample of 10 μ L in acetonitrile/water (50:50, v/v) were injected into LC flow at 250 μ L/min with 30%A/70%B for introduction onto a Thermo Scientific Acclaim Polar Advantage II C18 column (50 $\times$ 2.1 mm i.d., particle size 3 μ m). Initial conditions were held for 1 min before instant increase to 95%A/5%B, held for 2.5 min, and then reversed to initial conditions over 30 s, with a hold of 1 min for the bupropion, hydroxybupropion and oxcarbazepine method or with a hold of 3 min for the benzydamine, benzydamine *N*-oxide and oxcarbazepine method.

Table 2-1. Triple quadrupole mass spectrometer (Q-trap 5500) operating parameters

Parameters	Method 1		Method 2	Method 3			
Source type	APCI+		ESI–	ESI+			
Source temperature °(C)	300		300	260			
Ion spray voltage (V)	N/A		-4500	4500			
Nebulizer current (μA)	0.8		N/A	N/A			
Ion source gas 1 (psi)	50		50	20			
Ion source gas 2 (psi)	2		2	2			
Curtain gas (psi)	28		28	30			
Collision gas (psi)	6		6	5			
Declustering potential (V)	26		-26	30			
Entrance potential (V)	10		-10	10			
Internal standard MRM transitions (<i>m/z</i>)	258 → 80, 46		219 → 161, 125	253 → 208, 180			
Collision energy (V)	11, 27		-13, -27	27, 39			
Collision cell exit potential (V)	14, 20		-36, -20	22, 14			
Analyte	TATP	TATP-OH	TATP-O-gluc	BUP	BUP-OH	BZD	BZD-NO
Analyte MRM transition (<i>m/z</i>)	240 → 74, 43	256 → 75	413 → 113, 87	240 → 184, 166	256 → 238, 139	310 → 86	326 → 102
Collision energy (V)	11, 28	13	-22, -34	17, 23	15, 33	21	19
Collision cell exit potential (V)	10, 11	36	-13, -9	20, 10	12, 16	10	12

APCI+ positive mode atmospheric pressure chemical ionization, *BUP* bupropion, *BUP-OH* hydroxybupropion, *BZD* benzydamine, *BZD-NO* benzydamine *N*-oxide, *ESI+* positive mode electrospray ionization, *ESI–* negative mode electrospray ionization, *MRM* multiple reaction monitoring, *N/A* not applicable, *TATP* triacetone triperoxide, *TATP-O-gluc* triacetone triperoxide-*O*-glucuronide, *TATP-OH* hydroxyl-triacetone triperoxide

Metabolite identification

All incubations were performed in triplicates in a Thermo Scientific Digital Heating Shaking Drybath set to body temperature 37 °C and 800 rpm. An incubation mixture containing phosphate buffer (pH 7.4), MgCl₂ [17], and NADPH (CYP cofactor [11]) was prepared so that at a final volume of 1 mL, their concentrations were 10, 2 and 1 mM, respectively. When the incubation times were relatively long (greater than 15 min), it was thought necessary to use closed vessels to avoid loss of the volatile TATP or TATP-OH; as a result, prior to incubation, oxygen gas was bubbled through the buffer to ensure ample oxygen availability [15]. To this mixture, microsomes or recombinant enzymes were added and equilibrated for 3 min before the reaction was initiated by adding the substrate. Substrates included TATP in acetonitrile, TATP-OH in methanol, and bupropion and benzydamine in water. Organic solvents can disrupt metabolism, but the catalytic activity of most CYP enzymes is unaffected by less than 1% acetonitrile or methanol [18]. At the end point, an aliquot was transferred to a vial containing equal volume of ice-cold acetonitrile and immediately vortex-mixed to quench the reaction. The sample was centrifuged for 5 min at 14,000 rpm, and the supernatant was analyzed by LC–MS.

Metabolite identification studies used microsomes (HLM, RLM and DLM) at protein concentrations of 1 mg/mL in the incubation mixture. The substrate, TATP, TATP-OH, or TATP -*d*₁₈ (10 µg/mL) was allowed to incubate for several min before MS analysis. Negative controls consisted of the incubation mixture excluding either microsomes or NADPH. Positive control used 100 µM bupropion, a probe substrate for CYP2B6 [19–21].

Phase II TATP metabolism was examined by two studies. Metabolism by glutathione S-transferase (GST) was probed by equilibrating 5 mM GSH (GST cofactor [11]) for 5 min in the incubation mixture including HLM before the substrate (TATP) was added. Ticlopidine (10 μ M) was the substrate for the GST positive control (**Figure B-2**) [22]. To examine metabolism by UGT, HLM, buffer, and alamethicin (50 μ g/mL in methanol/water) were equilibrate cold for 15 min, before saccharolactone (1 mg/mL, β -glucuronidase inhibitor [23]), $MgCl_2$, and NADPH were added, and the mixture was warmed to 37 °C and shaken at 800 rpm. After 3 min equilibration, the substrate (TATP or TATP-OH) was added, and in 2 min, the reaction was started by the addition of 5.5 mM UDPGA (UGT cofactor [11]) [24, 25]. Positive control used 100 μ M 1-naphthol, an UGT1A6 substrate (**Figure B-3**) [26, 27]. Alamethicin was employed to replace membrane transporters, in allowing UGT (located in the endoplasmic reticulum lumen) easy access to the UDPGA cofactor [11].

Enzyme identification

TATP was incubated as described in the previous section with various enzyme inhibitors in HLM (1 mg/mL). TATP-OH formation was first monitored and benchmarked against incubations without inhibitors. Chemical inhibitors, such as 1-aminobenzotriazole (1 mM) [28, 29], methimazole (500 μ M) [30, 31], or ticlopidine (100 μ M) [32, 33], were pre-equilibrated in the incubation mixture for 30 min prior to the addition of the substrate (100 μ M, TATP or controls). TATP was also tested as a possible CYP2B6 inhibitor; TATP or bupropion was pre-equilibrated in the incubation mixture before starting the reaction

with a known CYP2B6 substrate (bupropion) or TATP, respectively. FMO inhibition by heat was also tested [11, 34]. In that experiment, HLM was mixed with buffer and pre-heated at 37 or 45 °C for 5 min. After an hour, cooling on ice, the incubation procedure was resumed. Bupropion, a CYP2B6 substrate [19–21], and benzydamine, an FMO substrate [34], were used as positive and negative control substrates to assess CYP, FMO and CYP2B6 inhibition. Samples not pre-incubated with chemical inhibitors nor heated to 45 °C were used as 100% TATP-OH formation. Inhibition studies were quenched after 15 min incubation.

Recombinant CYP and FMO enzymes were employed to identify the isoform responsible for the NADPH-dependent metabolism. Human bactosomes expressed in *E. coli* were used for CYP isoform identification; CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (100 pmol CYP/mL) were tested. Human supersomes expressed in insect cells were used for FMO isoform identification; FMO1, FMO3 and FMO5 (100 µg protein/mL) were examined. Both TATP and TATP-OH were tested as the substrate (10 µg/mL) in the incubation mixture with the recombinant enzymes. Negative control incubations were done in *E. coli* control or insect cell control. Positive control incubations were done in HLM (200 pmol CYP/mL), which contains all CYP and FMO enzymes. Recombinant enzymes studies were quenched after 10 min incubation.

Recombinant UGT enzymes were used to identify the isoform responsible for phase II metabolism. Human supersomes expressed in insect cells were used for UGT isoform identification; UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 and UGT2B7 were examined. The incubation mixture was similar to the glucuronidation incubation

previously described, except saccharolactone was not added [35]; 500 μg protein/mL was used; and the substrate was 10 $\mu\text{g}/\text{mL}$ TATP-OH. Negative control incubations were done in insect cell control. Positive control incubations were done in HLM (1 mg protein/mL), which contains all UGT enzymes. Glucuronidation with recombinant enzymes was quenched after 2 h incubation.

Enzyme kinetics

Kinetics experiments were done to determine the affinity of the enzyme CYP2B6 to the substrate TATP. The human CYP2B6 bacosomes used contained human CYP2B6 and human CYP reductase co-expressed in *E. coli*, supplemented with purified human cytochrome b_5 . CYP reductase is responsible for the transfer of electrons from NADPH to CYP, a task sometimes extended to cytochrome b_5 [11]. The incubation mixture (1 mL) contained 10 mM phosphate buffer (pH 7.4), 2 mM MgCl_2 , 50 pmol/mL rCYP2B6, 1 mM NADPH, and various concentrations of 0.1 to 20 μM TATP. The reaction was initiated by adding TATP after a 3 min pre-equilibration and stopped at different end points (up to 5 min) to determine rate of TATP hydroxylation. The rate of TATP hydroxylation in lungs was also investigated by incubating TATP (100 μM) in the incubation mixture containing 1 mg/mL HLM or HLungM, instead of rCYP2B6, for up to 10 min.

TATP-OH metabolism by CYP2B6 was evaluated by incubating 10 $\mu\text{g}/\text{mL}$ TATP-OH in CYP2B6 bacosomes according to the above procedure, with or without NADPH, except that the buffer was pre-oxygenated so that the incubation could be performed in a closed vessel. Aliquots were removed and quenched at different time points, up to 30 min.

TATP-OH depletion by HLM was determined using the same procedure, except that 1 μ M TATP-OH and up to 60 min reaction times were used.

Urine analysis

Laboratory personnel testing TATP are constantly exposed to this volatile compound. Explosive sensitivity experiments, such as drop weight impact tests, are done in a small brick-walled room for explosivity precautions, unlike synthesis reactions which are performed inside a fume hood for coverage protection. Also, portable explosive trace detection devices that are meant to be used in the field are tested as such, which also contribute to exposure. Urine from laboratory workers was tested for TATP and its metabolites after TATP exposure in the laboratory environment. Urine was collected at the beginning of the work week and 2 h after performing activities that could lead to high TATP exposure. To determine the longevity of TATP in the body, urine from the day following TATP exposure was also tested. The fresh urine was cleaned and concentrated for analysis using solid-phase extraction. Restek RDX column (Restek, Bellefonte, PA, USA) was conditioned with 6 mL of methanol, followed by 6 mL of water, and sample introduction (20-250 mL urine). The sample was washed with two cycles of 3 mL methanol/water (50:50, v/v). Extraction was achieved with two cycles of 1 mL acetonitrile. Both eluents were tested by LC-MS, because the lipophilic TATP and TATP-OH were extracted with acetonitrile, but the hydrophilic TATP-*O*-glucuronide was present in the methanol/water wash.

Results

Metabolite identification

When TATP was incubated in HLM, TATP was depleted, and one observable product, TATP-OH, was formed over time (**Figure 2-1**). Metabolism of TATP in HLM consists of hydroxylation at one methyl group with the peroxide bonds and nine-membered ring structure preserved (**Figure 2-2**) [15]. Opsenica and Solaja [12] reported various monohydroxylated and dihydroxylated products during microsomal incubations with cyclohexylidene and steroidal mixed tetraoxanes where the peroxide bond was also preserved. A TATP-OH standard (**Figure B-4**) was chemically synthesized to confirm the metabolite by retention time and mass-to-charge ratio (m/z). TATP-OH, identified as $[\text{TATP-OH} + \text{NH}_4]^+$ (m/z 256.1391) by accurate mass spectrometry, increased in incubation samples as time progressed. Attempts to confirm the hydroxylated metabolite with the deuterated substrate were unsuccessful due to the persistence of a contaminant with the same mass that the metabolite would have had, even in samples where TATP- d_{18} was not used as the substrate. As previously observed, TATP incubations in different species (dogs and rats) yielded the same metabolite, TATP-OH (**Figure B-5**) [15]. Other suspected metabolites, including the dihydroxy-species and additional oxidation of the TATP-OH to the aldehyde and carboxylic acid were not observed. Small polar molecules, such as acetone and hydrogen peroxide, the synthetic reagents of TATP, could not be chromatographically separated or are below the lower mass filter limit.

Figure 2-1. Triacetone triperoxide (TATP) biotransformation into hydroxy-TATP (TATP-OH) monitored over time in human liver microsomes (HLM), performed in triplicate

Figure 2-2. TATP metabolic pathways in HLM. *CYP* cytochrome P450, *UGT* uridine diphosphoglucuronosyltransferase

TATP was investigated for phase II metabolism routes of glutathione and glucuronide conjugation. Incubation in HLM with GSH produced no detectable glutathione metabolite conjugates, indicating that TATP is most likely not a substrate for microsomal GSTs (**Figure B-6**). When TATP was incubated with UDPGA, the TATP-OH glucuronic acid metabolite (TATP-*O*-glucuronide) was observed (**Figure 2-2**). The m/z 432.1712 for $[\text{TATP-}O\text{-glucuronide} + \text{NH}_4]^+$ was observed at very low levels after 2 and 3 h of incubation. When the sample was dried and reconstituted in low volume, the intensity of $[\text{TATP-}O\text{-glucuronide} + \text{NH}_4]^+$ increased, but TATP and TATP-OH were evaporated along with the solvent. TATP and TATP-OH are volatile, limiting their use in quantification experiments since sample concentration is not feasible [5]. However, TATP-*O*-glucuronide is a non-volatile TATP derivative that is amenable to sample preparation. Formation of TATP-*O*-glucuronide over time was monitored, using concentrated samples, as an intensity increase of m/z 432.1712 (**Figure B-7**). Even though TATP and TATP-OH generally form ammonia adducts under positive ion APCI, the glucuronide favors negative ion mode ESI. The m/z 413.1301 for $[\text{TATP-}O\text{-glucuronide} - \text{H}]^-$ was easily seen at 1-3 h without sample concentration. The common fragments of glucuronic acid, m/z 175, 113, and 85, were observed in the fragmentation pattern of m/z 413.1301, confirming the presence of a glucuronic acid conjugate (**Figure 2-3**) [36]. TATP-*O*-glucuronide was not observed in negative controls without UDPGA or NADPH, indicating that TATP-OH must be formed and then be further metabolized into TATP-*O*-glucuronide. Glucuronide conjugates are highly polar compounds that are easily eliminated by the kidney, suggesting that TATP-*O*-glucuronide would likely progress via urinary excretion [11].

Figure 2-3. Product ion spectrum of [TATP-*O*-glucuronide - H]⁻ (m/z 413.1301), fragmented with 35 eV using electrospray ionization in negative mode (ESI⁻). Proposed structures are shown

Enzyme identification

TATP was only metabolized into TATP-OH in HLM in the presence of NADPH, indicating that the metabolism is NADPH-dependent. The predominant microsomal enzymes that require NADPH for activity are CYP and FMO [11].

The usual roles of CYP are hydroxylation of an aliphatic or aromatic carbons, epoxidation of double bonds, heteroatom oxygenation or dealkylation, oxidative group transfer, cleavage of esters and dehydrogenation reactions [11]. 1-Aminobenzotriazole is considered a general mechanism-based inhibitor of CYPs, initiated by metabolism into benzyne, which irreversibly reacts with the CYP heme [28, 29]. When CYP activity was inhibited by 1-aminobenzotriazole, TATP-OH formation was also inhibited, with only 3.6% formed (**Table 2-2**), suggesting that CYP is involved in the hydroxylation of TATP. To support this evidence and to narrow down the CYP isoform catalyzing TATP hydroxylation, TATP was incubated with rCYPs (**Figure 2-4, Table B-1**). The CYPs selected for testing are responsible for the metabolism of 89% of common xenobiotics [37]. TATP-OH was not observed when TATP was incubated with CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. TATP hydroxylation was performed exclusively by CYP2B6, with $5.6 \pm 0.3 \mu\text{M}$ TATP-OH produced in 10 min. CYP2B6 has been found to metabolize endoperoxides by hydroxylation, as observed for TATP [13, 14]. HLM, which contains CYP2B6, also exhibited TATP hydroxylation ($1.5 \pm 0.1 \mu\text{M}$).

Table 2-2. Average % metabolites formed (triplicates) in 15 min incubations with chemical inhibitors or heat

Metabolite formation in 15 min	Percent found						
	Inhibitor pre-incubated in HLM for 30 min					HLM pre-heated for 5min	
	No inhibitor	1-ABT	MMI	TIC	TATP or BUP	37°C	45°C
TATP-OH (%)	100 ± 5	3.6 ± 0.7	34 ± 2	4.8 ± 0.7	125 ± 21	100 ± 10	69 ± 4
BUP-OH (%)	100 ± 3	23 ± 1	62 ± 2	9.6 ± 0.4	62 ± 2	100 ± 6	99 ± 2
BZD-NO (%)	100 ± 2	97 ± 1	48 ± 1	98 ± 3	N/A	100 ± 2	44 ± 2

I-ABT 1-aminobenzotriazole (CYP inhibitor), *CYP* cytochrome P450, *FMO* flavin monooxygenase, *HLM* human liver microsomes, *MMI* methimazole (FMO inhibitor), *TIC* ticlopidine (CYP2B6 inhibitor) (for other abbreviations, see **Table 2-1**)

Figure 2-4. TATP-OH formation from TATP incubations with recombinant cytochrome P450 (rCYP) and recombinant flavin monooxygenase (rFMO). Experiments with rCYP or rFMO consisted of 10 μ g/mL TATP incubated with 10 mM phosphate buffer (pH 7.4), 2 mM MgCl_2 and 1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH). Incubations were done in triplicate and quenched at 10 min

Since DLM studies indicated that TATP is metabolized by CYP2B11 [15], it was not surprising that another CYP2B subfamily enzyme, CYP2B6, metabolizes TATP in humans. CYP2B6 metabolism of TATP was further investigated by incubating TATP with ticlopidine, a mechanism-based inhibitor of CYP2B6 [32, 33]. When CYP2B6 activity was inhibited by ticlopidine, TATP-OH formation was also inhibited by 95% (**Table 2-2**), further supporting the primary involvement of CYP2B6 in the metabolism of TATP. Bupropion hydroxylation is catalyzed by CYP2B6; therefore, it was chosen as a positive control for CYP and CYP2B6 inhibition tests [28]. Hydroxybupropion formation was inhibited by 77 and 90% when incubated with 1-aminobenzotriazole and ticlopidine, respectively (**Table 2-2**).

FMO catalyzes oxygenation of nucleophilic heteroatoms, such as nitrogen, sulfur, phosphorous and selenium [34, 38]. Although FMO involvement in the hydroxylation of the TATP methyl group was unlikely, it seemed prudent to examine this enzyme class. Addition of methimazole, an FMO competitive inhibitor [30, 31], caused a decrease in TATP-OH formation by 66% (**Table 2-2**), but this is not necessarily direct inhibition of TATP metabolism by FMO since methimazole has been reported to reduce CYP2B6 activity by up to 80% [34, 39]. While this result was inconclusive about the FMO contributions to TATP metabolism, it could be considered further support for the role of CYP2B6. TATP was incubated with available rFMOs: FMO1, FMO3 and FMO5 (**Figure 2-4, Table B-1**). FMO1 is expressed in adults in the kidneys, and it should not contribute to the liver metabolism of TATP [34]; indeed, none appeared to be involved as no TATP-OH was produced (positive control, **Figure B-8**). Since FMO is inactivated by heat [11,

34], TATP was incubated in HLM pre-heated to 45 °C for 5 min. **Table 2-2** compares the formation of TATP-OH at 37 °C to that at 45 °C and to the *N*-oxidation of the positive control, benzydamine. Although, there was a decrease in TATP hydroxylation, the inhibitory effect was not as significant as compared to the decrease in benzydamine *N*-oxidation, which is catalyzed by FMO.

TATP-OH appears to be metabolized by HLM in an NADPH-dependent manner; therefore, TATP-OH was incubated for 10 min with recombinant enzymes (CYP and FMO) to determine which isoform is responsible for this secondary phase I metabolism (**Table 2-3**). Although TATP-OH is not nearly as volatile as TATP, some TATP-OH was lost in all incubations (i.e., 37 °C); however, notable depletion (by 40%) was observed only with CYP2B6. TATP-OH depletion in CYP2B6 was faster in the presence of NADPH than in its absence, supporting metabolism by CYP2B6 (**Figure B-9**). Unfortunately, no subsequent metabolite was identified.

Table 2-3. Average % TATP-OH remaining (triplicates) after 10 min incubation in recombinant enzymes

Incubation matrix	Percent TATP-OH remaining
HLM	69 ± 5
rCYP control	76 ± 4
rCYP1A2	77 ± 2
rCYP2B6	60 ± 3
rCYP2C9	71 ± 8
rCYP2C19	76 ± 5
rCYP2D6	76 ± 5
rCYP2E1	73 ± 5
rCYP3A4	78 ± 6
rFMO control	69 ± 3
rFMO1	78 ± 12
rFMO3	78 ± 6
rFMO5	72 ± 7

rCYP recombinant cytochrome P450, *rFMO* recombinant flavin monooxygenase

To identify which isoform is responsible for TATP glucuronidation, TATP-OH was incubated with the most clinically relevant rUGTs (**Figure 2-5, Table B-2**) [40]. Glucuronidation was not observed with UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9. TATP-*O*-glucuronide was formed only in UGT2B7 incubations with 0.05 ± 0.03 area count relative to IS produced after 2 h of incubation. HLM, which contains UGT2B7, also displayed TATP-*O*-glucuronide (0.26 ± 0.02 area count relative to IS). Relative quantification of TATP-*O*-glucuronide was done by area ratio to the IS because a TATP-*O*-glucuronide standard is not available. Endoperoxide glucuronidation by UGT2B7 has been reported, in which urine analysis of patients treated with artesunate, an artemisinin derivative, found dihydroartemisinin-glucuronide to be the principal metabolite excreted [16].

Figure 2-5. TATP-*O*-glucuronide formation from TATP-OH incubations with recombinant uridine diphosphoglucuronosyltransferase (rUGT). Experiments with rUGT consisted of 10 μ g/mL TATP-OH incubated with 10 mM phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 50 μ g/mL alamethicin, 1 mM NADPH, and 5.5 mM uridine diphosphoglucuronic acid (UDPGA). Glucuronidation done in triplicate and quenched at 2 h. Quantification was done using area ratio TATP-*O*-glucuronide/internal standard 2,4-dichlorophenoxyacetic acid

Enzyme kinetics

Rate of TATP hydroxylation by CYP2B6 was evaluated by plotting concentration of TATP-OH formed over time. The initial rate of TATP hydroxylation at various TATP concentrations was used to estimate enzyme kinetics using the Michaelis–Menten model: $v = V_{\max} \times [S] / (K_m + [S])$. Here, $[S]$ is the substrate (TATP) concentration, $V_{\max}$ is the maximum formation rate, K_m is the substrate concentration at half of $V_{\max}$, and k_{cat} is the turnover rate of an enzyme-substrate complex to product and enzyme [41]. The kinetic constants were obtained using nonlinear regression analysis on GraphPad Prism software (version 8.2.1). The Michaelis–Menten evaluation for TATP hydroxylation by CYP2B6 (**Figure 2-6**) yielded K_m of 1.4 μM ; $V_{\max}$ of 8.7 nmol/min/nmol CYP2B6; and k_{cat} of 174 min^{-1} . Linearized models, such as Lineweaver–Burk (**Figure B-10**), Eadie–Hofstee (**Figure B-11**) and Hanes–Woelf (**Figure B-12**), give similar values.

Figure 2-6. Rate of TATP hydroxylation by CYP2B6 versus TATP concentration. Incubations of various TATP concentrations consisted of 50 pmol rCYP2B6/mL with 10 mM phosphate buffer (pH 7.4), 2 mM MgCl₂ and 1 mM NADPH. Incubations were done in triplicate and quenched every min up to 5 min

The low K_m indicates TATP has a high affinity for CYP2B6 [42]. **Table 2-2** shows that TATP inhibits bupropion hydroxylation by CYP2B6, with only 62% hydroxybupropion formation in 15 min. However, in the presence of bupropion, TATP-OH formation was enhanced, with 125% formed compared to the reaction uninhibited by bupropion (**Table 2-2**). CYP2B6 preference for TATP affects the metabolism of bupropion, but further testing is needed to establish the specific type of inhibition.

Using the Michaelis–Menten parameters, in vitro intrinsic clearance ($Cl_{int} = V_{max} / K_m$) was calculated to be 6.13 mL/min/nmol CYP2B6 [11, 43, 44]. Scale-up of the Cl_{int} to yield intrinsic clearance on a per kilogram body weight was done using values of 0.088 nmol CYP2B6/mg microsomal protein, 45 mg microsomal protein/g liver wet weight and 20 g liver wet weight/kg human body weight [43]. Taking that into account, the scale-up Cl_{int} was calculated to be 485 mL/min/kg.

In vivo intrinsic clearance (Cl) is the ability of the liver to metabolize and remove a xenobiotic, assuming normal hepatic blood flow ($Q = 21$ mL/min/kg [43, 45]) and no protein binding [43]. Cl can be extrapolated using the well-stirred model excluding all protein binding as $Cl = Q \times Cl_{int} / Q + Cl_{int}$ [43]. The in vivo intrinsic clearance of TATP was estimated as 20 mL/min/kg. Compared to common drugs, TATP has a moderate clearance [46].

TATP-OH kinetics were also investigated by substrate depletion. Substrate depletion was plotted as the natural log of substrate percent remaining over time (**Figure 2-7**). Half-life ($t_{1/2}$) was calculated to be 16 min, as the natural log 2 divided by the negative slope of the substrate depletion plot. Cl_{int} can also be estimated using half-life as $Cl_{int} =$

$(0.693 / t_{1/2}) \times (\text{incubation volume} / \text{mg microsomal protein})$ [47, 48]. The in vitro intrinsic clearance of TATP-OH was estimated as 0.042 mL/min/mg. Even though we identified two metabolic pathways for TATP-OH, it appears to be cleared slower than TATP.

Figure 2-7. Natural log of TATP-OH percent remaining in HLM versus time. TATP-OH (1 μ M) incubated in 1 mg/mL HLM with pre-oxygenated 10 mM phosphate buffer (pH 7.4), 2 mM MgCl_2 and 1 mM NADPH. Incubations were done in closed vials, in triplicate and quenched every 10 min up to 1 h

Lung metabolism

Inhalation is the most probable pathway for systemic exposure since TATP is both volatile and lipophilic. With passive diffusion into the bloodstream being very possible, TATP metabolism in the lung was also investigated. TATP was incubated in lung and liver microsomes for comparison of metabolic rate. The results, shown in **Table 2-4**, indicated that TATP hydroxylation in the lungs was negligible. Though CYP2B6 gene and protein are expressed in the lungs, enzyme activity in lungs is minimal as compared to the liver, limiting TATP metabolism [49, 50]. This suggests that TATP is most likely distributed through the blood to the liver for metabolism. News reported that traces of TATP was found in the blood samples extracted from the 2016 Brussels suicide bombers [51]. This indicates the possibility of using blood tests as forensic evidence for TATP exposure.

Table 2-4. Rate of TATP hydroxylation (triplicates) in human liver microsomes versus human lung microsomes

Human microsomes	Rate of TATP-OH formation (nmol/min/mg)
Liver	0.425 ± 0.06
Lung lot1710142	< LOQ
Lung lot1410246	< LOQ
<LOQ lower than the limit of quantification	

In vivo human urine analysis

Laboratory workers, who normally work with TATP on a daily basis, performing tasks like explosive sensitivity testing, were screened for TATP exposure. These laboratory workers volunteered to collect their urine before and after exposure to TATP vapor. Since the health effects of TATP exposure are unknown, to minimize any additional risks to these workers, this pilot study was performed in duplicates using only three volunteers to establish some reproducibility. TATP and TATP-OH were not observed in the urine of any of the workers. However, TATP-*O*-glucuronide was present in all urine samples collected 2 h after TATP exposure (**Figure 2-8**). Two out of the three volunteers still showed TATP-*O*-glucuronide in the urine collected the next day (**Table 2-5**). TATP-*O*-glucuronide was identified in human urine samples as both $[\text{TATP-}O\text{-glucuronide} - \text{H}]^-$ and $[\text{TATP-}O\text{-glucuronide} + \text{NH}_4]^+$. The presence of TATP-*O*-glucuronide in the urine of all three volunteers is summarized in **Table 2-5**.

Figure 2-8. Extracted ion chromatogram of [TATP-*O*-glucuronide - H]⁻ (m/z 413.1301) in HLM 2 h after incubation with TATP, and in human urine, before TATP exposure and 2 h after TATP exposure

Table 2-5. Summary of TATP-*O*-glucuronide presence in human urine (duplicates) in vivo

Human	m/z 413.1301			m/z 432.1712		
	#1	#2	#3	#1	#2	#3
Before TATP exposure	-	-	-	-	-	-
Two hours after TATP exposure	+	+	+	+	+	+
One day after TATP exposure	+	+	-	+	-	-

TATP glucuronide is observed as [TATP-*O*-glucuronide – H][–] (m/z 413.1301) and [TATP-*O*-glucuronide + NH₄]⁺ (m/z 432.1712, less sensitive). Only one trial performed on next day samples

Discussion

As described before, TATP is the explosive of choice by terrorists because it is easily synthesized from household items [1, 2]. In our previous study, we have clarified that TATP-OH is produced as an in vitro metabolite from TATP in dogs [15], because canines are currently one of the most reliable detection techniques used to find an explosive [52]. In the present article, the study has been conducted in continuation of our previous findings on both in vitro and in vivo metabolism of TATP in humans (**Figure 2-2**).

Because TATP has high volatility, it is likely to be absorbed into the body by inhalation; however, no appreciable metabolism in the lung was observed in either dog [15] or human microsomes (**Table 2-4**). Therefore, systemic exposure and subsequent liver metabolic clearance was presumed. Across three species, dog, rat, and human, TATP was metabolized in liver microsomes by CYP to TATP-OH (**Figure B-5**). Using recombinant enzymes, we have previously established that CYP2B11 is responsible for this metabolism in dogs [15]. Interestingly, human CYP2B6 appears to be the major phase I enzyme responsible for the same metabolism (**Figure 2-4**). TATP hydroxylation by CYP2B6 kinetics determined K_m and V_{max} as 1.4 μ M and 8.7 nmol/min/nmol CYP2B6, respectively (**Figure 2-6**). Though heat inactivation and chemical inhibition of FMO appeared to affect TATP hydroxylation (**Table 2-2**), incubations with recombinant FMO suggest that FMO was not forming TATP-OH (**Figure 2-4**). Methimazole, an FMO inhibitor, inhibited TATP-OH formation (**Table 2-2**); but, inhibition of bupropion by this chemical inhibitor suggests that methimazole also inhibits CYP2B6 activity [39].

When incubated together, TATP and bupropion, appear to compete for CYP2B6 metabolism, with bupropion hydroxylation being inhibited by 38% in the presence of TATP (**Table 2-2**). Considering CYP2B6 expression in the liver is low and exhibits broad genetic polymorphisms, CYP2B6 activity can be widely affected if TATP affects the metabolism of other compounds, like bupropion [53, 54]. TATP may be a serious perpetrator for drug-drug interactions for compounds cleared by CYP2B6 [55, 56].

In vitro clearance of TATP was calculated as 0.54 mL/min/mg protein with hepatic in vivo extrapolation to 20 mL/min/kg. In vitro clearance of TATP-OH was estimated, using substrate depletion, as 0.038 mL/min/mg protein (**Figure 2-7**). We also established the clearance of TATP in dogs as 0.36 mL/min/mg protein in our previous study in canine microsomes [15], which is significantly relevant to K9 units, where the dog and human handler are both exposed to the explosive.

Investigation into the next step on the metabolic pathway indicated that TATP-OH is further metabolized by CYP2B6 (**Table 2-3**), but a secondary phase I metabolite was not identified. No glutathione adducts of any TATP metabolism products were observed in the microsomal incubations with GSH (**Figure B-6**). However, glucuronidation converted TATP-OH to TATP-*O*-glucuronide in HLM with UGT2B7 specifically catalyzing this reaction (**Figure 2-5**). Considering glucuronides are often observed as urinary metabolites, the presence of TATP-*O*-glucuronide in urine can be exploited as an absolute marker of exposure to TATP, which can be used as forensic evidence of TATP illegal use.

Urine from scientists working to prevent terrorist attacks by synthesizing, characterizing and detecting TATP, who are inevitably exposed to this volatile compound

were negatively tested for TATP and TATP-OH, but TATP-*O*-glucuronide was present at high levels in their fresh urine (**Figure 2-8**). In one out of the three volunteers, TATP-*O*-glucuronide was not observed in the urine collected the day after TATP exposure (**Table 2-5**), suggesting TATP to TATP-*O*-glucuronide in vivo clearance occurs within about a day depending on the exposure level. TATP-*O*-glucuronide presence in the urine of all three volunteers shows good in vivo correlation to in vitro data.

Like TATP (hydrophilicity expressed as TPSA = 55.38 and lipophilicity expressed as cLogP = 3.01, calculated using PerkinElmer ChemDraw Professional version 16.0.1.4), TATP-OH is lipophilic with TPSA and cLogP of 75.61 and 1.72, respectively. TATP-*O*-glucuronide, on the other hand, is hydrophilic with TPSA and cLogP of 171.83 and 0.32, respectively. The increase in TPSA and decrease in cLogP from TATP to TATP-*O*-glucuronide accounts for the glucuronide greater water solubility and facilitated excretion [11]; thus explaining the presence of only TATP-*O*-glucuronide in urine.

Even though working with TATP falls under the protection of several standard operating procedures to handling explosives, considering the breathing exposure that these laboratory workers revealed, implementation of precautionary measures to absorption by inhalation, such as the use of respirators, should be considered. Such detection of TATP-*O*-glucuronide is also useful for judicial authorities to raise a scientific evidence for exposure to TATP of terrorists and/or related individuals.

This paper is the first to examine some aspects of TATP human ADMET, elucidating the exposure, metabolism and excretion of TATP in humans. However, the detailed pharmacological and toxicological studies remain to be explored.

Conclusions

This article dealt with in vitro and in vivo studies of TATP metabolism in humans. TATP is highly volatile and easily introduced into human body via aspiration. By this study, TATP was found to be metabolized into TATP-OH by the action of CYP2B6, followed by glucuronidation of TATP-OH catalyzed by UGT2B7; the resulting TATP-*O*-glucuronide was found to be excreted into urine in live humans. After extracting the TATP conjugate from urine specimens, it can be analyzed by HPLC–MS/MS, which gives scientific evidence for exposure to TATP. This evidence can be useful to prove exposure of persons, such as terrorists, to TATP for judicial authorities. Although this study includes the metabolism of TATP and also an analytical method to detect the TATP-*O*-glucuronide, the toxicology of TATP remains to be explored.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval This study was approved by the University of Rhode Island Institutional Review Board (IRB) for Human Subjects Research (approval number 1920-206).

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MANUSCRIPT 3**Paper spray ionization – high resolution mass spectrometry (PSI-
HRMS) of peroxide explosives in biological matrices**

by

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Abstract

Mitigation of the peroxide explosive threat, specifically triacetone triperoxide (TATP) and hexamethylene triperoxide diamine (HMTD), is a priority among the law enforcement community, as scientists and canine (K9) units are constantly working to improve detection. We propose the use of paper spray ionization – high resolution mass spectrometry (PSI-HRMS) for detection of peroxide explosives in biological matrices. Occurrence of peroxide explosives and/or their metabolites in biological samples, obtained from urine or blood tests, give scientific evidence of peroxide explosives exposure. PSI-HRMS promote analysis of samples *in situ* by eliminating laborious sample preparation steps. However, it increases matrix background issues, which were overcome by the formation of multiple alkali metal adducts with the peroxide explosives. Multiple ion formation increases confidence when identifying these peroxide explosives in direct sample analysis. Our previous work examined aspects of TATP metabolism. Herein, we investigate the excretion of a TATP glucuronide conjugate in the urine of bomb-sniffing dogs and demonstrate its detection using PSI from the *in vivo* sample.

Keywords

Explosives detection, Triacetone triperoxide (TATP), Hexamethylene triperoxide diamine (HMTD), Bomb-sniffing dogs, Urine, Blood

Introduction

Triacetone triperoxide (3,3,6,6,9,9-hexamethyl-1,2,4,5,7,8-hexoxonane, TATP) and hexamethylene triperoxide diamine (3,4,8,9,12,13-hexaoxa-1,6-diazabicyclo[4.4.4]tetradecane, HMTD) (**Figure 3-1**) are primary explosives, highly sensitive to initiation when exposed to friction, shock or static. TATP and HMTD are often called homemade explosives (HME) because they are easily synthesized from household items, an attractive feature to extremists that can legally procure their reagents when planning terrorist attacks [1–4]. To mitigate this threat, certain law enforcement specialists, e.g. bomb squads, and canine (K9) units, as well as scientists developing detection devices must be trained to recognize these peroxides [5]. However, there is no literature on the short or long term effects of TATP exposure in bomb-sniffing dogs.

Knowledge of the absorption, distribution, metabolism, excretion and toxicity (ADMET) of peroxide explosives could be used to expand their detection capabilities. Once absorbed, chemical compounds are either excreted unchanged or are distributed to the liver for metabolism. In the liver, phase I oxidative reaction, usually catalyzed by cytochrome P450 (CYP), and/or phase II conjugation reactions, occasionally catalyzed by uridine diphosphoglucuronosyl-transferase (UGT) occur. These metabolic processes produce species that can be effectively excreted from the body [6]. Our previous work described the hepatic metabolism of TATP in humans in which TATP undergoes hydroxylation by CYP2B6 into TATP-OH, followed by glucuronidation into TATP-*O*-glucuronide by UGT2B7 [7]. Even though the blood distribution of TATP to the liver was not inspected, news outlets have reported that traces of TATP were found in the blood

samples extracted from the 2016 Brussels suicide bombers [8]. Glucuronides often progress via urinary excretion [6], and this was supported by our findings that TATP-*O*-glucuronide was present in the urine of laboratory personnel working with TATP [7]. Information about the blood distribution and urinary excretion of TATP can be used to develop blood and urine tests to be used as forensic evidence of TATP and/or HMTD exposure.

TATP and HMTD are not detected by ultraviolet and fluorescence spectroscopy, techniques commonly used for toxicological assays; this precluded biological analysis [9, 10]. Detection of these peroxide explosives relies heavily on infrared or Raman spectroscopy [11], gas or liquid chromatography mass spectrometry (MS) [5, 12], using laboratory-grade and field-portable devices. Airport security, for example, depends on trace techniques such as ion mobility spectrometry (IMS) and bomb-sniffing dogs [13, 14]. Currently, detection of compounds, such as explosives and drugs, from biological matrices requires exhaustive sample preparation including extraction, purification and chromatography before MS analysis [15, 16]. An analytical method is needed that allows rapid analysis, without sample preparation, with high selectivity to avoid false positives and high sensitivity to allow trace detection [14]. Ambient ionization techniques which allow generation of analyte ions directly from complex mixtures and require minimal sample preparation include desorption electrospray ionization (DESI) [17], direct analysis in real time (DART) [18] and paper spray ionization (PSI) [19, 20].

In PSI, the sample is deposited on paper, and the analytes are extracted by addition of a solvent, which transports the dissolved compounds, by capillary action, to the tip of

the paper. This is followed by the application of high-voltage to the paper, which generates charged droplets and, consequently, ionizes the analytes [19, 20]. PSI has been extensively used to detect drugs and metabolites from dried blood [15, 16, 21] and has demonstrated its capability of detecting *in vivo* glucuronides from urine [22]. Recently PSI has been used to analyze military explosives [23–25], but its use in detection of HME in biological samples, e.g. blood and urine, has not previously been shown.

Cyclic peroxide explosives are readily ionized by atmospheric pressure chemical ionization (APCI), with TATP often observed as the ammonium adduct, and HMTD as a protonated ion [26, 27]. The formation of peroxide explosives complexes with alkali metal ions using DESI has also been described [9, 28]. Detection of TATP and HMTD in human skin has been demonstrated using extractive electrospray ionization (EESI) coupled with a desorption device [29]; yet, there remains a need for a consistent method of detection of peroxide explosives in biological matrices. This work demonstrates that paper spray ionization - high resolution mass spectrometry (PSI-HRMS) provides a simple, quick and efficient method for reliably detecting TATP and HMTD via adduct formation in biological samples, such as blood and urine.

Trace detection of explosives relies on extracting residues from hair, clothing and/or other personal items [30]. Although hair studies indicate that explosives, such as TATP, trinitrotoluene (TNT) and ethylene glycol dinitrate (EGDN), remain on human hair for days after exposure [31], fixated hygiene may prevent explosive residues to endure. Thus, detection of a metabolite from biological matrices (e.g. blood or urine) could be useful to authorities, providing scientific evidence of exposure to these peroxide

explosives. Also, the detection of TATP-*O*-glucuronide in the urine of bomb-sniffing dogs was explored as forensic marker of TATP exposure.

Figure 3-1. Chemical structure of TATP, TATP metabolites (TATP-OH and TATP-O-glucuronide), and HMTD

Materials and Methods

Chemicals

Optima HPLC grade methanol (MeOH), Optima HPLC grade water (H₂O), Optima HPLC grade acetonitrile (ACN), ACS grade acetone, ACS grade methanol, ACS grade pentane, citric acid anhydrous, ammonium acetate (NH₄OAc), dipotassium phosphate (K₂HPO₄), monopotassium phosphate (KH₂PO₄), magnesium chloride (MgCl₂), sodium carbonate (Na₂CO₃), and potassium carbonate (K₂CO₃) were purchased from Fisher Chemical (Fair Lawn, NJ, USA). Reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1-naphthol, hexamethylenetetramine, hydroxyacetone, formic acid (FA), lithium carbonate (Li₂CO₃) and cesium carbonate (Cs₂CO₃) were purchased from Acros Organics (Morris Plain, NJ, USA). Uridine-5'-diphosphoglucuronic acid (UDPGA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Alamethicin was purchased from Alfa Aesar (Ward Hill, MA, USA). Silver acetate (AgOAc) was purchased from Mallinckrodt Specialty Chemicals (Paris, KY, USA). Deuterated acetone (D₆-acetone) and ¹³C-acetone were purchased from Cambridge Isotope Labs (Cambridge, MA, USA). Hydrogen peroxide (50 %) was purchased from Univar (Redmond, WA, USA). Dog liver microsomes (DLM) were purchased from Sekisui XenoTech (Kansas City, KS, USA). Dog whole blood with anticoagulant was purchased from Innovative Research (Novi, MI, USA).

TATP was synthesized according to the literature [1]. Deuterated TATP (D₁₈-TATP), carbon-13 labeled TATP (¹³C₃-TATP) and hydroxy-TATP (TATP-OH) were

synthesized as above using D₆-acetone, ¹³C-acetone and a mixture of acetone and hydroxyacetone, respectively. HMTD was synthesized according to the literature [32].

HPLC–HRMS Analysis

Metabolite identification was performed by high-performance liquid chromatography coupled to a Thermo Scientific Exactive high-resolution mass spectrometer (HPLC–HRMS) (Thermo Scientific, Waltham, MA, USA). A CTC Analytics PAL autosampler (CTC Analytics, Zwinger, Switzerland) was used for LC injections, solvent delivery was performed using a Thermo Scientific Accela 1200 quaternary pump, and data collection/analysis was done using Xcalibur software (Thermo Scientific, ver. 2.1). The HPLC gradient and MS tune conditions for all TATP derivatives were previously described [7].

In Vitro Glucuronidation from Dog Liver Microsomes

To examine metabolism by canine uridine diphosphoglucuronosyl-transferase (UGT), 1mg/mL DLM, 10mM phosphate buffer (pH 7.4), 2mM MgCl₂ and 50 µg/mL alamethicin were equilibrated on ice for 15 minutes before 1mM NADPH (CYP co-factor [6]) was added, and the mixture was warmed to 37 °C and shaken at 800 rpm using a Heating Shaking Drybath (Thermo Scientific, Waltham, MA, USA). After 3 minutes of subsequent equilibration, the substrate (10 µg/mL TATP or ¹³C₃-TATP) was added; and 2 minutes later, the reaction was initiated by addition of 5.5 mM UDPGA (UGT co-factor [6]) [33, 34]. At different time points, an aliquot was transferred to a vial containing an

equal volume of ice cold ACN and immediately vortex-mixed to quench the reaction. The sample was centrifuged for 5 minutes at 14,000 rpm; and the supernatant analyzed by HPLC–HRMS. Negative control incubations excluded the UGT co-factor, UDPGA, to prevent glucuronidation. Positive control incubations used a known UGT substrate, 1-naphthol, to promote glucuronidation [35].

In Vivo Glucuronidation from Bomb-Sniffing Dogs

K9 units are trained on explosive odor by “operant conditioning” on a daily basis [36, 37]. Briefly, small containers with explosives are hidden in multiple locations, e.g. inside a cabinet, behind a trash can, under a drain. The handler slowly walks the dog around the area, allowing it to sniff and sit when it recognizes the odor it was trained to, expecting a positive reward.

Multiple vials of solid TATP (0.5 g) were used for canine training. To abide with the police schedule, this pilot study was performed using only three bomb-sniffing labradors; each dog, tested twice. Urine from these canine subjects was collected in intervals of 1, 2, 3 hours or overnight after training exposure and, within 4 hour of collection, tested for the presence of TATP and its metabolites. TATP and its metabolites were extracted and concentrated from the urine using a RDX solid-phase extraction (SPE) cartridge (Restek, Bellefonte, PA, USA) with the following procedure: the sorbent was conditioned with 6 mL of MeOH, equilibrated with 6 mL of H₂O, followed by sample introduction (10-150 mL of urine, depending on the availability), washed with two 3 mL

aliquots of MeOH/H₂O (1:1, v/v), and finally, the analytes were extracted with 1 mL of ACN prior to HPLC–HRMS analysis.

Paper Spray Ionization

Direct analysis of peroxide explosives in biological matrices was done using paper spray ionization on a Thermo Scientific LTQ-Orbitrap XL HRMS (Thermo Scientific, Waltham, MA, USA). The paper swabs were cut as an equilateral triangle with 1 cm side (60° tip angle). A copper clip was used to attach an external power supply set to a specific voltage and to hold the paper about 3 mm from the MS inlet. The positive mode source parameters were as follows: paper spray voltage, 4.5 kV; capillary temperature, 275 °C; capillary voltage, 35 V; and tube-lens voltage, 90 V. The negative mode source parameters were: paper spray voltage, -4.5 kV; capillary temperature, 275 °C; capillary voltage, -15 V; and tube-lens voltage, -84 V.

Performance of our in-house PSI setup was determined by depositing a constant volume of 200 µL of a TATP standard curve made in MeOH/H₂O (1:1, v/v) with d₁₈-TATP as an internal standard on a paper, and ionizing it using the PSI procedure described above. Peroxide explosive selectivity via metal adduct formation was achieved by dissolving TATP in MeOH/aqueous salt solution (1:1, v/v) and HMTD in ACN/MeOH/aqueous salt solution (1:1:2, v/v/v), which contained approximately 50 µM of Li⁺, Na⁺, K⁺ and NH₄⁺ or 100 µM of Cs⁺ and Ag⁺. Urine and blood samples, fortified with 1mM of TATP or HMTD, were diluted in MeOH to promote desolvation and/or aqueous salt solutions to stimulate

adduct formation. The swab type usually used was High Efficiency sampling swipes (FLIR Systems, Wilsonville, OR, USA, part no. FS-03-E).

To optimize silver adduct formation using PSI, a variety of paper materials were tested: High Efficiency sampling swipes, Shark Skin swabs (Smiths Detection, London, UK, part no. 2811791-H), sample traps multi-purpose made with PTFE-coated fiberglass (DSA Detection, North Andover, MA, USA, part no. ST1318P), parchment specialty paper (Southworth, Atlanta, GA, USA, part no. P874CK), phase separator 1 treated with silicone (Whatman, Maidstone, UK, part no. 2200 125) and Flagship copy paper (W.B. Mason, Brockton, MA, USA, part no. WBM21200).

Results

In Vitro Glucuronidation from Dog Liver Microsomes

In vitro incubations established the presence of TATP-*O*-glucuronide in dog liver microsomes. When TATP was incubated with UDPGA in DLM, formation of TATP-*O*-glucuronide was monitored as an intensity increase of m/z 413.1301 for the deprotonated ion using negative mode electrospray ionization (ESI-) or m/z 432.1712 for the ammonium adduct using APCI+ mode. TATP-*O*-glucuronide was not observed in the negative controls, i.e. excluding UDPGA or NADPH, indicating that TATP-OH must first be formed by phase I enzymes in the presence of NADPH and then further metabolized into TATP-*O*-glucuronide in the presence of UDPGA. The use of carbon-13 labeled TATP in the incubation mixture confirmed formation of both metabolites, TATP-OH and TATP-*O*-glucuronide (**Figure 3-2**).

Figure 3-2. Extracted ion chromatogram using APCI+ of **a)** $[^{13}\text{C}_3\text{-TATP} + \text{NH}_4]^+$ (m/z 243.1542), **b)** $[^{13}\text{C}_3\text{-TATP-OH} + \text{NH}_4]^+$ (m/z 259.1491), and **c)** $[^{13}\text{C}_3\text{-TATP-O-glucuronide} + \text{NH}_4]^+$ (m/z 435.1812), from $^{13}\text{C}_3\text{-TATP}$ incubated in DLM

In Vivo Glucuronidation from Bomb-Sniffing Dogs

A SPE procedure for TATP and TATP-OH was developed using TATP and TATP-OH spiked urine. Experiments indicated that MeOH/H₂O was a suitable wash solution, with the lipophilic compounds being extracted with ACN. However, the hydrophilic TATP-*O*-glucuronide was washed away in the aqueous phase, failing to be retained by the sorbent material under generally accepted conditions. This observation advocates for an analytical technique, such as PSI, that does not require sample preparation prior to MS analysis.

TATP, TATP-OH and TATP-*O*-glucuronide were not observed in the analysis of the extracted dog urine collected 1, 2 or 3 h after canine training with TATP. In contrast, during human studies, TATP-*O*-glucuronide was observed in urine as early as 2 h after TATP exposure [7], but since dog metabolism is different [38, 39], urine was also collected over a longer period of time. TATP excretion in dogs was only observed in the urine samples collected approximately 12 h (overnight) after training. At that time TATP-*O*-glucuronide was present in the urine of all three dogs, providing good *in vivo* correlation to *in vitro* data (**Figure 3-3**).

Figure 3-3. Extracted ion chromatogram using ESI- of [TATP-*O*-glucuronide - H]- (m/z 413.1301) from **a)** complete incubation mixture in DLM 2 h after TATP addition, **b)** dog urine before TATP exposure, and dog urine 12 h after TATP exposure **c)** trial, **d)** trial 2

Even though the amount of TATP was standardized, quantification of TATP-*O*-glucuronide in the dog urine was deemed unnecessary because the training was unstandardized and each of the dogs' handlers controlled the duration of training, testing location, and urine collection. Since the amount of TATP (0.5g) used in our experiment was significantly less than the minimum amount of explosive used for the bomb-sniffing dog certification testing ($\frac{1}{4}$ lbs or 113.5 g) [37], it is evident that both dog and handler are absorbing TATP.

Paper Spray Ionization

Analysis of Pure Peroxides

Peroxide explosives analysis was done using a homemade PSI setup in conjunction with HRMS (**Figure 3-4**). The successful operation of the PSI setup was evaluated by using a standard calibration curve [40]. TATP linearity was observed over the range of 10 to 10,000 ng (**Figure 3-5**). Limit of detection (LOD) was determined to be 1 ng of TATP based on $3 \times S/N$ ratio, and limit of quantification (LOQ) was taken to be $10 \times LOD$, which is comparable to other more laborious analytical techniques [13, 41].

Figure 3-4. Schematic of PSI setup

Figure 3-5. TATP calibration curve (run in triplicates) using PSI+ mode on Thermo LTQ-Orbitrap XL. Intensity ratio of $[\text{TATP} + \text{Na}]^+ / [\text{d}_{18}\text{-TATP} + \text{Na}]^+$ was plotted versus amount of TATP (10-10,000 ng)

Formation of multiple adducts, such as multiple alkali metals adducts, increases selectivity when identifying an analyte. Solutions of TATP and d_{18} -TATP were made with alkali metal ions (Li^+ , Na^+ , K^+) and ionized using the homemade PSI source. TATP alkali metal complexes were observed in all cases with confirmation from deuterated TATP. The collision induced dissociation (CID) fragmentation of these TATP complexes follows the literature [9] pattern consisting of the retention of the metal and loss of ethane (**Figure 3-6**). The precursor ion, $[TATP + H]^+$ (m/z 223.1176), was not observed; and the abundance of the normally observed ammonia adduct, $[TATP + NH_4]^+$ (m/z 240.1442), was greatly reduced when TATP was dissolved with alkali metal solutions.

Figure 3-6. TATP and d18-TATP metal adducts fragmentation pattern [9] formed using PSI

Although previously reported at low levels by ESI [42], the TATP cesium complex was not observed in PSI. Reported density functional theory (DFT) calculations estimated that the metal ion is centered equidistant from the oxygen atoms of TATP, but slightly above the cavity, due to steric effects related to the size of the metal ion [9]. This suggested that the period 6 alkali metal was too bulky to effectively interact with TATP.

Silver was tested as a potential adducting agent since the formation of diacyl peroxide silver complexes by ESI have been proposed [43]. Silver has two naturally occurring isotopes, with ^{107}Ag and ^{109}Ag having composition abundance of 52% and 48%, respectively [44], which increase mass assignment confidence when considering their specific isotopic ratio. TATP interacted with silver to create the $[\text{TATP} + ^{107}\text{Ag}]^+$ and $[\text{TATP} + ^{109}\text{Ag}]^+$ with m/z 329.0149 and 331.0145, respectively (**Figure 3-7**). Solutions of deuterated TATP with silver were also used to confirm the adduct ion formation as $[\text{d}_{18}\text{-TATP} + ^{107}\text{Ag}]^+$ (m/z 347.1279) and $[\text{d}_{18}\text{-TATP} + ^{109}\text{Ag}]^+$ (m/z 349.1275). Unlike the ammonia adduct, commonly observed by APCI+, the silver adduct is stable enough to be isolated and activated by high-energy collision dissociation (HCD), yielding the same pattern in both ESI and PSI. Similarly, as with the alkali metal complexes, the silver adduct was not observed in APCI, and the primary fragment consisted of the loss of ethane with retention of the metal (**Figure 3-7**). Acetone silver clusters can be easily formed [45], and they appear to be present in the mass spectrum of TATP (**Figure 3-7**). Considering acetone is an intrinsic part of the TATP structure, acetone silver clusters further supports the identification of TATP and may be used to corroborate its detection. TATP detection can also be improved by identification of its metabolites, such as TATP-OH, which with silver

dopant easily produced m/z 345.0098 $[\text{TATP-OH} + {}^{107}\text{Ag}]^+$ and m/z 347.0095 $[\text{TATP-OH} + {}^{109}\text{Ag}]^+$.

Figure 3-7. Averaged full scan mass spectra of 1 mM TATP with 100 μM Ag^+ dopant using PSI+ mode on Thermo LTQ-Orbitrap XL. Sample was deposited on paper, desolvated with MeOH, ionized using 4.5 kV. TATP silver adducts, m/z 329.0149 and 331.0145 with isolation width of m/z 1.6, were fragmented by collision induced dissociation (CID) at 7 eV. Proposed structures are shown

Since TATP detection is often done by portable IMS, which are available in most ports of entry (e. g. airports), the swabs, already employed by these instruments were tested for PSI use [46]. TATP silver adducts formation by PSI varied with the paper used; of the six different types of paper tested, the TATP-silver adduct was only observed with the three IMS swab materials (FLIR Systems High Efficiency sampling swipes, Smiths Detection Shark Skin swabs and DSA Detection sample traps multi-purpose made with PTFE-coated fiberglass). On the other hand, m/z 245.0996 $[\text{TATP} + \text{Na}]^+$ was formed by all tested materials, indicating that the larger, heavier metal ions were not easily desorbed from some materials, unlike sodium, which was the most stable adduct for TATP.

HMTD detection by PSI was challenging. HMTD has low solubility in most common solvents, and solutions of HMTD in pure ACN were not used because it has been shown that ACN suppresses HMTD and TATP ion formation [47]. In PSI, the HMTD principle ion (m/z 209.0768) was assigned to $[\text{HMTD} + \text{H}]^+$, with less intense alkali metal complexes, $[\text{HMTD} + \text{Li}]^+$, $[\text{HMTD} + \text{Na}]^+$ and $[\text{HMTD} + \text{K}]^+$, observed as m/z 215.0850, 231.0588 and 247.0327, respectively. Isotopically labeled d_{12} -HMTD with m/z 227.1603 $[\text{d}_{12}\text{-HMTD} + \text{Li}]^+$, m/z 243.1341 $[\text{d}_{12}\text{-HMTD} + \text{Na}]^+$ and m/z 259.1080 $[\text{d}_{12}\text{-HMTD} + \text{K}]^+$ confirmed alkali metal complex formation. Although the formation of silver complexes would be advantageous for the detection of HMTD because of the easily recognizable silver isotopic pattern, HMTD silver adducts were not observed by either ESI or PSI.

Analysis of Peroxides in Biological Matrices

The peroxide explosive complexes with different alkali metals contribute to their confirmation in biological matrices via multiple ion identification. In addition, the high levels of sodium and potassium normally present in both urine and blood [48, 49] were leveraged for *in situ* analysis. When TATP (**Figure 3-8**) or HMTD (**Figure 3-9**) were spiked in both urine and blood without the addition of any dopant, the sodium and potassium adducts of the peroxide explosives were detected.

Figure 3-8. Averaged full scan mass spectra of **a)** urine and **b)** blood, fortified with 1 mM TATP using PSI+ mode on Thermo LTQ-Orbitrap XL. Samples were deposited on paper, desolvated with MeOH and ionized using 4.5 kV

Figure 3-9. Averaged full scan mass spectra of **a)** urine and **b)** blood, fortified with 1 mM HMTD using PSI+ mode on Thermo LTQ-Orbitrap XL. Samples were deposited on paper, desolvated with MeOH and ionized using 4.5 kV

It was thought that the capability of TATP to form silver complexes, the presence of acetone silver clusters in TATP samples, and the silver characteristic isotopic ratio would also serve as confirmatory tools in deconvolution of mass spectra from biological matrices. However, urine or blood solutions of TATP doped with silver did not result in the observation of the TATP silver adduct; the sodium and potassium adducts were the only TATP species present in the mass spectrum.

Bomb-sniffing dogs were exposed to TATP, and several hours later their urine was collected, deposited on paper and analyzed by PSI with no further sample preparation (**Figure 3-10**). The relative abundance of [TATP-*O*-glucuronide – H]⁻, *m/z* 413.1301, in the full scan mode was low; nevertheless, it could be identified from the matrix background without any chromatographic separation. The detection of TATP-*O*-glucuronide from *in vivo* urine samples demonstrate the practical application of this technique.

Figure 3-10. Averaged full scan mass spectra of bomb-sniffing dog urine collected 12 h after training with TATP using PSI- mode on Thermo LTQ-Orbitrap XL. In vivo sample was deposited on paper, desolvated with MeOH and ionized using -4.5 kV

Discussion

PSI promotes analysis of samples *in situ* by eliminating the laborious sample preparation step. Direct analysis is specifically important for TATP, because it is so volatile that sample concentration or other routine workup greatly lowers its concentration. However, the lack of sample preparation increases matrix background interferences. These were overcome by the formation of multiple alkali metal adducts with the peroxide explosives. The particular use of sodium and potassium adducts, which are abundant in blood and urine [48, 49], were exploited as an advantage, avoiding the need for an external ionization dopant. The formation of both sodium and potassium adducts with TATP (Fig. 8) or HMTD (Fig. 9) increased the mass assignment confidence when identifying these peroxide explosives in direct analysis of blood and urine samples. Urine and blood tests have been widely used to trace drug abuse [50, 51], and PSI has been shown to easily and quickly analyze dried blood spots [15, 16, 21]. Since PSI can selectively detect peroxide explosives from blood and urine, it is a technique that can also be expanded to trace people involved in the production of illegal explosives.

The advantages of using silver adducts for PSI analysis included the formation of two adducts from the isotopes of silver, the identification of the silver isotopic pattern, the specific fragmentation of TATP metal adducts, and the formation of acetone silver clusters; each helped confirm the identity of the compound. However, silver adducts were not formed with all peroxide explosives, not being observed with HMTD.

Detection of peroxide explosives from biological samples can also be greatly improved by identification of their metabolites. Although the metabolism of HMTD is still

unknown, we have recently described some of the metabolic pathways of TATP identifying that hydroxylation to TATP-OH (Fig. 1) was catalyzed by phase I enzymes, i.e. CYP2B11 in dogs [52] and CYP2B6 in humans [7]. To extend previous findings, we assessed the effects of TATP metabolism *in vitro* and determine that TATP-OH undergoes phase II glucuronidation to TATP-*O*-glucuronide (Fig. 1) in canine liver microsomes. Since glucuronide conjugates are often excreted in urine [6], the potential excretion of TATP after glucuronidation in canines was also investigated. The urine of bomb-sniffing dogs, collected approximately 12 h after training with TATP, underwent the established SPE clean-up procedure, HPLC separation method and HRMS analysis. The results were compared to the *in vitro* data and TATP-*O*-glucuronide was confirmed in the dogs' urine.

In order to quickly detect TATP absorption in the field, PSI is proposed. The same bomb-sniffing dogs' urine samples were also analyzed using PSI- with no sample preparation or chromatographic separation, and TATP-*O*-glucuronide was observed in the full scan (Fig. 10). The detection of TATP-*O*-glucuronide from the urine of bomb-sniffing dogs exposed to TATP provides strong support for the use of PSI in forensic analysis of *in vivo* metabolites [15, 22].

Detection of peroxide explosives by PSI has several advantages. It increases throughput by simplifying or completely eliminating sample preparation steps. It minimizes false positive detection via increased selectivity gained by complex formations with multiple alkali metals. Coupled with mass spectrometry, PSI allows trace detection, with detection limits down to nanogram levels. Furthermore, advances in portable MS

technologies, in combination with PSI for field deployment, could greatly improve on-site analysis and forensic efficiency [14].

Conclusions

This work explored the use of PSI-HRMS as a valuable detection method for TATP, HMTD and their metabolites in blood and urine. Without the need for chromatography or extensive sample clean-up, the presence of TATP or HMTD in biological matrices can be confirmed with PSI-HRMS using one or more of the following: 1) the fragmentation patterns of the peroxide; 2) its adducts with various alkali metals; 3) the isotope patterns resulting for its silver adducts. Although TATP and HMTD form alkali metal adducts, we have found that sodium and potassium, both abundant in blood and urine, can serve as *in situ* dopants to assist in the ionization of peroxide explosives. In this case, the matrix background, which accompanies biological samples, can be used advantageously in promoting adduct formation. We have demonstrated the use of PSI-HRMS to analyze TATP and HMTD in fortified blood and urine samples, and its practical application was validated by successfully detecting TATP-*O*-glucuronide from *in vivo* urine samples.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The used protocol was reviewed and approved by the University of Rhode Island Institutional Animal Care and Use Committee (IACUC) with AN 13-05-023.

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MANUSCRIPT 4**In vitro blood stability and toxicity of peroxide explosives in canines and
humans**

by

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Abstract

Triacetone triperoxide (TATP) and hexamethylene triperoxide diamine (HMTD) are prominent explosive threats. Mitigation of peroxide explosives is a priority among the law enforcement community. Canine (K9) units are trained to recognize the scent of peroxide explosives. Herein, the metabolism, blood distribution and toxicity of peroxide explosives is investigated. HMTD metabolism studies in liver microsomes identified two potential metabolites, tetramethylene diperoxide diamine alcohol aldehyde (TMDDAA) and tetramethylene peroxide diamine dialcohol dialdehyde (TMPDDD). However, blood stability studies in dogs and humans showed that HMTD was rapidly degraded, whereas TATP remained for at least one week. Toxicity studies in dog and human hepatocytes indicated minimum cell death for both TATP and HMTD.

Keywords

Triacetone triperoxide (TATP), hexamethylene triperoxide diamine (HMTD), explosives, metabolism, blood stability, toxicity, hepatocytes, canine (K9) units

Introduction

Energetic materials can be classified by chemical structure; including nitrate esters, nitroaromatics, nitroamines, peroxide and others. The toxicity of most military explosives is well-characterized, with even some therapeutic properties being identified. For example, nitrate ester explosives, such as nitroglycerin and PETN (pentaerythritol tetranitrate), are widely used as vasodilators to treat angina (FDA 2014). Ill side effects have been linked to other explosives. Nitroaromatic explosives, such as TNT (2,4,6-trinitrotoluene), picric acid (2,4,6-trinitrophenol) and tetryl (2,4,6-trinitrophenyl-N-methylnitramine), may cause cytotoxicity, their metabolic pathways include single- or two-electron enzymatic reduction that forms radical species (Nemeikaite-Ceniene *et al.* 2006). Nitroamine explosives, such as RDX (1,3,5-trinitro-1,3,5-triazinane) and HMX (1,3,5,7-tetranitro-1,3,5,7-tetrazoctane), may be carcinogenic, their metabolic pathways promote the formation of N-nitroso species which cause genetic damage (Pan *et al.* 2007). However, the metabolic pathways and toxicity of peroxide explosives, such as TATP (triacetone triperoxide) and HMTD (hexamethylene triperoxide diamine, **Figure 4-1**) has not been thoroughly investigated (Colizza *et al.* 2019, Gonsalves *et al.* 2020).

TATP was used in the 2015 Paris attack, the 2016 airport bombings in Brussels, the 2017 concert bombing in Manchester, UK, the 2018 bombings in Surabaya, Indonesia, and the 2019 assaults in hotels and churches across Colombo, Sri Lanka (Rossi *et al.* 2019). Peroxide explosives are a threat because they are easily initiated and readily synthesized from household items. (Oxley, Smith, and Chen 2002, Oxley, Smith, Chen, *et al.* 2002, Oxley *et al.* 2013, 2016). As a counter to this threat, peroxide explosives are made available

to personnel, such as canine (K9) detection units and scientists developing detection devices (Oxley *et al.* 2015). Understanding the absorption, distribution, metabolism, excretion and toxicity (ADMET) of peroxide explosives is essential to safe handling of these chemicals. Collaborating with K9 units, we established the hepatic metabolism of TATP, in which TATP undergoes hydroxylation into TATP-OH, followed by glucuronidation into TATP-O-glucuronide, which is excreted in the urine of both human and canines exposed to TATP vapours (Gonsalves *et al.* 2020).

TATP is a highly volatile compound with vapour pressure estimated around 5.91×10^{-5} atm at 25 °C (Oxley *et al.* 2005, Damour *et al.* 2010), and HMTD is lipophilic, with partition coefficient (cLogP) of 1.11 (PerkinElmer 2017) indicating inhalation and dermal exposure are the most probable routes of absorption. Both TATP and HMTD contain three peroxide functionality, suggesting potential similarities with the side effects of hydrogen peroxide (H₂O₂) exposure. Risks of hydrogen peroxide absorption by inhalation include coughing and temporary dyspnoea, and by dermal contact include from whitening of the skin to blistering and severe skin damage (Watt *et al.* 2004).

It has been reported that authorities found TATP in the blood of the Brussels 2016 suicide bombers (Goulard 2016). Considering the presence of catalase in blood, which facilitates the cleavage of peroxide bonds in H₂O₂ (Watt *et al.* 2004), and the instability of the TATP cyclic multi-peroxidic structure, this claim seemed unlikely. However, if TATP were stable in blood, the potential forensic evidence is obvious.

Once absorbed, compounds are either excreted unchanged or are distributed to the liver for metabolism, where phase I oxidative reactions, usually catalysed by cytochrome

P450 (CYP), and/or phase II conjugation reactions, catalysed by glutathione S-transferase (GST), uridine diphosphoglucuronosyltransferase (UGT) or others, occur, producing metabolites that can be effectively excreted from the body (Parkinson *et al.* 2018). Some metabolites of TNT, including 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene, have been found in the urine and bound to haemoglobin in the blood of munition workers. The chronic exposure of these workers to TNT caused anaemia, hepatitis and cataracts, exemplifying the importance of biomarkers to track contact and prevent chronic exposure to explosives (Sabbioni *et al.* 2005, Voříšek *et al.* 2005). Even though working with peroxide explosives falls under the protection of several standard operating procedures, information about their blood distribution, metabolism and toxicity is imperative to ensure the safety of K9 units, where both the dog and human handler are constantly exposed to these explosives.

Materials and Methods

Chemicals

Optima HPLC grade methanol (MeOH), Optima HPLC grade water (H₂O), Optima HPLC grade acetonitrile (ACN), ACS grade acetone, ACS grade methanol, ACS grade pentane, citric acid, ammonium acetate (NH₄OAc), dipotassium phosphate (K₂HPO₄), monopotassium phosphate (KH₂PO₄), magnesium chloride (MgCl₂), Tris-base, hydrochloric acid (HCl) and reduced glutathione (GSH) were purchased from Fisher Chemical (Fair Lawn, NJ, USA). Reduced nicotinamide adenine dinucleotide phosphate (NADPH), hexamethylenetetramine, 1-naphthol and hydroxyacetone were purchased from Acros Organics (Morris Plain, NJ, USA). Uridine-5'-diphosphoglucuronic acid (UDPGA), bovine serum albumin (BSA), glycerol and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bupropion, and alamethicin were purchased from Alfa Aesar (Ward Hill, MA, USA). Ticlopidine was purchased from Tokyo Chemical Industry (Tokyo, Japan). Staurosporine was purchased from Cayman Chemical (Ann Arbor, MI, USA). Deuterated acetone (D₆-acetone), deuterated formaldehyde (D₂-formaldehyde), ¹³C-acetone and ¹⁵N-ammonium hydroxide were purchased from Cambridge Isotope Labs (Cambridge, MA, USA). Hydrogen peroxide (50 %) was purchased from Univar (Redmond, WA, USA). Collagen coated 96 well-plates were purchased from Gibco (Gaithersburg, MD, USA). Human liver microsomes (HLM), dog liver microsomes (DLM), cryopreserved human hepatocytes (CryostaX, 5 donor pool), cryopreserved dog hepatocytes, OptiThaw media, OptiPlate media and OptiCulture media were purchased from Sekisui XenoTech (Kansas City, KS, USA). Dog whole blood with

anticoagulant was purchased from Innovative Research (Novi, MI, USA). Human whole blood with anticoagulant was purchased from ZenBio (Research Triangle Park, NC, USA); blood was tested in accordance with FDA regulations prior to shipping.

Synthesis of peroxide explosives

TATP was synthesized according to the literature (Oxley *et al.* 2013). Deuterated TATP (d₁₈-TATP), carbon-13 labelled TATP (¹³C₃-TATP) and hydroxy-TATP (TATP-OH) were synthesized as above using d₆-acetone, ¹³C-acetone and a mixture of acetone and hydroxyacetone, respectively. HMTD was synthesized according to the literature (Wierzbicki and Cioffi 1999). A similar reported procedure was used to synthesize deuterated HMTD (d₁₂-HMTD) and nitrogen-15 labelled HMTD (¹⁵N₂-HMTD) using d₂-formaldehyde and ¹⁵N-ammonium hydroxide, respectively (Nielsen *et al.* 1979).

Instrumental analysis

Metabolite identification was performed by high-performance liquid chromatography coupled to a Thermo Scientific Exactive high-resolution mass spectrometer (HPLC–HRMS) (Thermo Scientific, Waltham, MA, USA). A CTC Analytics PAL autosampler (CTC Analytics, Zwingen, Switzerland) was used for LC injections, solvent delivery was performed using a Thermo Scientific Accela 1200 quaternary pump, and data collection/analysis was done using Xcalibur software (Thermo Scientific, ver. 2.1).

Quantification of blood incubations was performed by high-performance liquid chromatography coupled to an AB Sciex Q-Trap 5500 triple quadrupole mass spectrometer (HPLC–MS/MS) (AB Sciex, Toronto, Canada). A CTC Analytics PAL autosampler was used for LC injections, solvent delivery was performed using a Thermo Scientific Accela 1200 quaternary pump and data collection/analysis was done with Analyst software (AB Sciex, ver. 1.6.2).

The HPLC method for metabolite identification of all HMTD derivatives was as follow: sample of 40 μ L was injected into LC flow at 250 μ L/min of 5 % MeOH (A) and 95 % aqueous 10 mM NH_4OAc (B) in positive ionization mode or aqueous 200 μ M NH_4OAc , 200 μ M NH_4Cl and 0.1 % FA (C) in negative ionization mode for introduction onto an Analytical Advantage FluroPhase PFP column (100 $\times$ 2.1 mm i.d., particle size 5 μ m, Analytical Sales and Services, Flanders, NJ, USA). Initial conditions were held for 2 min before ramping to 2%A/98%B or C over 1 min. This ratio was held for 2 min before reverting to initial conditions over 30 s for additional 2 min re-equilibration. The HPLC method for quantification of HMTD and d_{12} -HMTD was the same as above, except the sample volume was reduced to 20 μ L and the gradient was quickly ramped over the course of only 3 s instead 1 min.

The HPLC gradient and MS tune conditions for all TATP derivatives were previously described (Gonsalves *et al.* 2020). The MS tune conditions for all HMTD metabolite identification and quantification analysis are shown in **Table 4-1**. The Q-trap 5500 MS multiple reaction monitoring (MRM) conditions are shown in **Table 4-2**. TATP and HMTD quantification was done in the range of 10 – 8,000 ng/mL using d_{18} -TATP and

d₁₂-HMTD as internal standards, respectively. The relative standard deviation (RSD) for all quality control (QC) samples was lower than 15 %.

Table 4-1. Mass spectrometer tune conditions.

Parameters	Exactive				Q-trap 5500
	ESI+	APCI+	ESI-	APCI-	APCI+
Spray voltage (kV)	4	N/A	-3.4	N/A	N/A
Discharge or nebulizer current (μ A)	N/A	6	N/A	10	3
Vaporizer temperature ($^{\circ}$ C)	N/A	250	N/A	200	250
Sheath gas or ion source gas 1 (AU)	30	25	30	35	15
Auxiliary gas or ion source gas 2 (AU)	15	15	15	17	3
Capillary voltage or declustering potential (V)	28	35	-53	-25	26
Capillary temperature ($^{\circ}$ C)	245	275	275	150	N/A
Tube lens voltage or entrance potential (V)	90	35	-110	-100	10
Skimmer voltage (V)	18	14	-36	-18	N/A
Curtain gas (psi)	N/A	N/A	N/A	N/A	15
Collision gas (psi)	N/A	N/A	N/A	N/A	5

AU, arbitrary units

Table 4-2. Triple quadrupole mass spectrometer (Q-trap 5500) MRM conditions.

Parameters	TATP		HMTD	
Deuterated IS MRM transitions (m/z)	258 →	80, 46	221 →	154, 94
Collision energy (V)		11, 27		5, 13
Collision cell exit potential (V)		14, 20		54, 10
Analyte MRM transition (m/z)	240 →	74, 43	209 →	145, 88
Collision energy (V)		11, 28		7, 13
Collision cell exit potential (V)		10, 11		4, 14

Metabolism of HMTD

The metabolism of HMTD was investigated via metabolite identification and substrate depletion in liver microsomes. The 1 mL incubation mixture contained 10 mM phosphate buffer (pH 7.4), 2 mM MgCl₂, 1 mM NADPH (CYP cofactor (Parkinson *et al.* 2018)) and 1mg/mL HLM or 0.5 mg/mL DLM. After 3 min of equilibration in a Heating Shaking Drybath (Thermo Scientific, Waltham, MA, USA) set to body temperature, 37 °C, and 800 rpm, the reaction was started with the addition of 1 mg/mL of each HMTD (or 1 µM for substrate depletion experiment), d₁₂-HMTD or ¹⁵N₂-HMTD, maintaining the organic concentration at less than 1 % (Chauret *et al.* 1998). Negative controls consisted of the incubation mixture excluding either microsomes or NADPH. Positive control used 100 µM bupropion as the substrate (Faucette *et al.* 2000). At different time points, an aliquot was transferred to a vial containing equal volume of ice cold ACN and immediately vortex-mixed to quench the reaction. The sample was centrifuged for 5 min at 14,000 rpm, and the supernatant analysed by HPLC–HRMS. The substrate depletion experiment was quantified by HPLC–MS/MS.

Phase II metabolism by glutathione S-transferase (GST) was probed by equilibrating 5 mM GSH (GST cofactor (Parkinson *et al.* 2018)) for 5 min in the incubation mixture including HLM (1 mg/mL) or human liver cytosol (2 mg/mL) before the substrate was added. Positive control used 10 µM ticlopidine, an GST substrate (Ruan and Zhu 2010). To examine phase II metabolism by uridine diphosphoglucuronosyltransferase (UGT), HLM (1 mg/mL), buffer, MgCl₂ and alamethicin (50 µg/mL in MeOH/H₂O) were equilibrated on ice for 15 min before NADPH was added, and the mixture was warmed to

37 °C and shaken at 800 rpm. After 3 min of subsequent equilibration, the substrate was added; and 2 min later, the reaction was started by the addition of 5.5 mM UDPGA (UGT co-factor (Parkinson *et al.* 2018)). Positive control used 100 µM 1-naphthol, an UGT substrate (Di Marco *et al.* 2005). Phase II metabolite samples were analysed as above using HPLC–HRMS.

Blood stability of peroxide explosives

The stability of peroxide explosives in blood was examined. Human and canine whole blood samples from multiple donors, or water, were transferred to vacutainers containing heparin, and fortified with TATP or HMTD (less than 1 % organic). The 1 mL mixture was incubated in closed vials at 37 °C and 800 rpm in a heated shaker for up to 1 week for TATP and for up to 1 h for HMTD. To stop the reaction, 1.5 mL of cold MeOH and 2.5 mL of cold ACN, containing the internal standard solution (d₁₈-TATP or d₁₂-HMTD), were added to the vial and immediately vortexed. The samples were centrifuged twice, first at 4,400 rpm for 10 min, then at 14,000 rpm for 5 min, prior to quantification by HPLC–MS/MS.

Toxicity of peroxide explosives

Cryopreserved human and dog hepatocytes were prepared according to manufacturer protocols. Briefly, a cryotube was thawed in a 37 °C water bath for approximately 60 seconds, re-suspended in pre-warmed (37 ± 1 °C) OptiThaw media, and centrifuged at 100 × g for 5 min at room temperature. The supernatant fluid was gently

aspirated and discarded, and the cell pellet was re-suspended with OptiPlate media (15 mL). The hepatocytes were seeded into a collagen coated 96-well plate at the recommended seeding density (0.75×10^6 cells/ mL, 75 μ L/ well). The plate was placed in a 37 °C humidified CO₂ static incubator for 3 h to allow the cells to attach and adhere to the plate. The plate was then removed from the incubator; media containing non-attached cells was removed; and the cells were overlaid with 75 μ L of 2-8°C of OptiCulture media, and incubated for 24 h. The cells were then dosed with incubation media (75 μ L/well) containing various concentrations of TATP, positive control staurosporine (2 μ M) (Feng and Kaplowitz 2002) or vehicle control 0.1% DMSO with media change after 12 h for a total treatment of 24 h (Amaeze *et al.* 2019).

Cell death was assessed using the LDH-Glo™ Cytotoxicity Assay (Promega, Madison, WI, USA) according to manufacturer protocol. The LDH reagent was prepared by mixing LDH Detection Enzyme Mix and Reductase Substrate 1:0.005 (v/v). Following the 24 h incubation period, the plate was removed from the incubator and 2 μ L of the media of each well was transferred to another 96 well-plate well containing 48 μ L of LDH storage buffer (200mM Tris-HCl, 10% glycerol and 1% BSA). The LDH reagent (50 μ L) was added to the diluted media in each well and allowed to sit at room temperature for 1 h prior to luminescence measurement.

Cell viability was assessed using the CellTiter-Glo® 2.0 Assay (Promega, Madison, WI, USA) according to manufacturer protocol. Following the 24 h incubation period and preparation for the cell death assay, the Cell Titer Glo 2.0 reagent (75 μ L) was added to the remaining media in each well and mixed for two min on an orbital shaker to induce cell

lysis, and after 10 min luminescence was measured using a GloMax Discover (Promega, Madison, WI, USA).

Results

Metabolism of HMTD

Data mining uncovered the possibility of two metabolites with the presence of m/z 231.0588 (HMTD M1) and 247.0327 (HMTD M2), supported by formation of their labelled counterparts in incubations with labelled HMTD - m/z 242.1278 (d_{12} HMTD M1) and 258.1017 (d_{12} HMTD M2), or m/z 233.0528 ($^{15}N_2$ -HMTD M1) and 249.0268 ($^{15}N_2$ -HMTD M2) (**Table 4-3, Figure 4-2**). These masses were not observed in incubation mixtures without microsomal enzymes (**Figure 4-2**) but were observed in incubation mixtures with enzymes but without NADPH, albeit, at significantly lower levels (**Figure 4-2**). This observation indicates the reaction is catalysed primarily by NADPH-dependent enzymes, such as cytochrome P450 (CYP) or flavin monooxygenase (FMO).

Figure 4-2. Extracted ion chromatogram using ESI+ of m/z 231.0588 (HMTD M1) from HMTD **a)** in complete incubation mixture, **b)** incubation mixture without NADPH, **c)** incubation mixture without HLM.

Table 4-3. Observed m/z for HMTD and suspected metabolites under HPLC-ESI-HRMS conditions.

Compounds	Molecular formula	Expected ion	Expected m/z	Observed m/z	Δ PPM	RT (min)
HMTD	C ₆ H ₁₂ N ₂ O ₆	[HMTD + H] ⁺	209.0768	209.0771	0.307	5.09
d ₁₂ -HMTD	C ₆ D ₁₂ N ₂ O ₆	[d ₁₂ -HMTD + H] ⁺	221.1521	221.1524	0.306	5.01
¹⁵ N ₂ -HMTD	C ₆ H ₁₂ ¹⁵ N ₂ O ₆	[¹⁵ N ₂ -HMTD + H] ⁺	211.0709	211.0714	0.468	5.09
TMDDD	C ₆ H ₁₀ N ₂ O ₆	[TMDDD + H] ⁺	207.0612	207.0615	0.327	1.66
Metabolites	Proposed molecular formula	Proposed ion	Expected m/z	Observed m/z	Δ PPM	RT (min)
HMTD M1	C ₆ H ₁₂ N ₂ O ₆ Na	[C ₆ H ₁₂ N ₂ O ₆ + Na] ⁺	231.0588	231.0590	0.273	1.61
HMTD M2	C ₆ H ₁₂ N ₂ O ₆ K	[C ₆ H ₁₂ N ₂ O ₆ + K] ⁺	247.0327	247.0330	0.276	1.66
d ₁₂ -HMTD M1	C ₆ HD ₁₁ N ₂ O ₆ N	[C ₆ HD ₁₁ N ₂ O ₆ + Na] ⁺	242.1278	242.1280	0.198	1.67
	a					
d ₁₂ -HMTD M2	C ₆ HD ₁₁ N ₂ O ₆ K	[C ₆ HD ₁₁ N ₂ O ₆ + K] ⁺	258.1017	258.1020	0.281	1.69
¹⁵ N ₂ -HMTD M1	C ₆ H ₁₂ ¹⁵ N ₂ O ₆ N	[C ₆ H ₁₂ ¹⁵ N ₂ O ₆ + Na] ⁺	233.0528	233.0533	0.443	1.66
	a					
¹⁵ N ₂ -HMTD M2	C ₆ H ₁₂ ¹⁵ N ₂ O ₆ K	[C ₆ H ₁₂ ¹⁵ N ₂ O ₆ + K] ⁺	249.0268	249.0272	0.416	1.70

Detection of m/z 231.0588 and 247.0327 at the very onset of the incubation procedure, 30 seconds from the start, prevented traceability of their formations over time. Enzymatic homolytic cleavage of the peroxide bond could be rapidly catalysed by microsomal enzymes (Yeh *et al.* 2007). For HMTD and $^{15}\text{N}_2$ -HMTD, this argument would fit observations; however, for d_{12} -HMTD such a reaction would result in the formation of m/z 243.1341 $[\text{C}_6\text{D}_{12}\text{N}_2\text{O}_6 + \text{Na}]^+$ as shown in the bottom structure in **Figure 4-3**. This metabolite was not observed; instead, the ion m/z 242.1278 was observed, suggesting a concomitant loss of deuterium and gain of hydrogen (Yokota and Fujii 2018).

Figure 4-3. Proposed d12-HMTD metabolites.

The mass-to-charge ratio of the sodium or potassium adduct of HMTD and its suspect metabolites are shown in **Table 4-3**. These m/z values appear to be the sodium and potassium salts of HMTD, but these adducts were not observed via HPLC-HRMS described conditions, even when these cations were present in excess. As **Figure 4-3** suggests homolytic cleavage and concomitant proton exchange would form new species with the same elemental composition as HMTD with the addition of sodium ($\text{C}_6\text{H}_{12}\text{N}_2\text{O}_6\text{Na}^+$) or potassium ($\text{C}_6\text{H}_{12}\text{N}_2\text{O}_6\text{K}^+$). However, the small offset in retention times may indicate two different metabolites are formed with each having their sodium and potassium adducts (**Figure 4-4**).

Figure 4-4. Extracted ion chromatogram using ESI+ of **a)** m/z 231.0588 (HMTD M1), and **b)** m/z 247.0327 (HMTD M2) from HMTD incubated in HLM.

HMTD, similar to TATP, is best ionized by APCI (Colizza *et al.* 2014). On the other hand, tetramethylene diperoxide diamine dialdehyde (1,2,6,7,4,9-tetraoxadiazecane-4,9-dicarbaldehyde, TMDDD, **Figure 4-1**), a known decomposition product of HMTD favours ESI ionization (Colizza *et al.* 2018). We have previously observed that under described HPLC conditions TMDDD elutes earlier than HMTD, suggesting that the open-ring structures would have less retention than the cyclic HMTD. The elemental composition of HMTD metabolites and their similar retention times to TMDDD has led us to speculate on their structures (**Figure 4-1**).

Investigations into the decomposition products of HMTD using density functional theory (DFT) calculations proposed tetramethylene diperoxide diamine alcohol aldehyde (9-(hydroxymethyl)-1,2,6,7,4,9-tetraoxadiazecane-4-carbaldehyde, TMDDAA, **Figure 4-1**) as the first intermediate, formed with energy barrier of 30.2 kcal/mol, and tetramethylene peroxide diamine dialcohol dialdehyde (N,N'-(peroxybis(methylene))bis(N-(hydroxymethyl)formamide), TMPDDD, **Figure 4-1**) as the second intermediate, formed with energy barrier of 26.8 kcal/mol (Oxley *et al.* 2016). However, experimental data only detected the known di-aldehyde product, TMDDD (Oxley *et al.* 2016). This suggests that catalytic activity is required to overcome the activation barrier and start the degradation process of HMTD. Thus, the sodium or potassium adducts of either TMDDAA and TMPDDD are reasonable enzymatic products of HMTD.

HMTD was investigated for phase II metabolism routes of glutathione and glucuronide conjugation. However, no metabolites were observed when HMTD was

incubated with the glutathione-S-transferase (GST) co-factor, GSH, or the uridine diphosphoglucuronosyltransferase (UGT) co-factor, UDPGA. If the peroxide bonds of HMTD were cleaved, it would not be surprising that HMTD would degrade to its synthetic precursors - ammonia, formaldehyde and hydrogen peroxide, which were below the limit of our mass detector.

HMTD was incubated in human and canine liver microsomes since both members of the K9 detection team are exposed. Depletion of HMTD as first-order kinetics HMTD (Słoczyńska *et al.* 2019) shown by linearity of natural log of percent remaining substrate was plotted over time (**Figure 4-5**) and half-life was determined as follows:

$$t_{\frac{1}{2}} = \frac{\ln(2)}{-k_{dep}} \quad t_{1/2} \text{ is the half-life and } k_{dep} \text{ is the slope of the substrate depletion plot.}$$

The half-life of HMTD in liver microsomes was estimated to be 62 and 58 min in humans and dogs, respectively.

Figure 4-5. HMTD percent remaining (ln-transformed) in human (HLM, ●) and dog liver microsomes (DLM, ◆). Substrate depletion experiments were done in triplicates by incubating HMTD (1 μ M) in 1 mg/mL HLM or 0.5 mg/mL DLM with 10 mM phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 1 mM NADPH and quenching every 10 min up to 1 h.

Blood stability of peroxide explosives

Blood is responsible for compound distribution in the body, e.g. transport to the liver for hepatic metabolism. Blood stability experiments were accomplished by incubating HMTD in human and canine whole blood at body temperature, and sampling it in intervals of 10 min for 1 h for analysis. Because it is known that HMTD undergoes decomposition in humid environments (Oxley *et al.* 2016), the results in blood were compared to experiments done in pure water. Most of the analyte was gone after 10 min, and the rate of its depletion in pure aqueous media was insignificant compared to that of the blood, strongly suggesting enzymatic degradation in the latter media (**Figure 4-6**).

In vitro and *in vivo* metabolism data suggest TATP is stable in the body. To further support this observation, the stability of TATP in blood compared to that of pure water was examined with measurements taken every day for 1 week. Approximately 40 and 54 % TATP was still present in human and canine whole blood, respectively, after 1 week incubation at 37 °C (**Figure 4-6**), indicating that TATP was not degraded by blood enzymes. TATP volatility in warm aqueous solution was evident (**Figure 4-6**) as only about 23% TATP remained in closed vials after 7 days (Colizza *et al.* 2019). However, TATP depletion from both human and canine blood was not as extensive as from water (about 50 % difference), suggesting some stable binding to blood proteins, to prevent TATP sublimation.

Figure 4-6. Percent remaining of **a)** TATP and **b)** HMTD in human blood (■), dog blood (■) and water (■). Blood stability experiments were done in triplicates by incubating TATP or HMTD (10 µg/mL) in human whole blood, canine whole blood and water (1 mL) at 37 °C and quenching every day up to 1 week for TATP or every 10 min up to 1 h for HMTD.

Toxicity of peroxide explosives

The toxicity of TATP and HMTD was assessed by treating dog and human hepatocytes with TATP or HMTD and comparing their cellular viability and cellular death as two complementary assays (**Figure 4-7**). Cell viability was determined by measuring adenosine triphosphate (ATP), the principal molecule for storing and transferring energy, therefore indicating the presence of metabolically active cells (Maehara *et al.* 1987). Cell death was determined by measuring lactate dehydrogenase (LDH), a cytosolic enzyme that is released upon disruption of the plasma membrane, therefore, widely used as a cytotoxicity marker (Kumar *et al.* 2018). TATP appears to maintain a consistent ATP production and LDH release regardless of the TATP concentration in humans and dogs. Similarly, HMTD does not appear to indicate a dose response, with constant cell viability and cell death observed even after 24 h exposure in both species.

Figure 4-7. Percent luminescence of ATP production (■) and LDH release (■) relative to untreated control in **a)** human hepatocytes treated with TATP, **b)** dog hepatocytes treated with TATP, **c)** human hepatocytes treated with HMTD and **d)** dog hepatocytes treated with HMTD. Toxicity experiments were done in eight trials by incubating (0.1 to 200 μM) TATP or HMTD in human and dog hepatocytes for 24 h. Assays measured ATP production for cell viability and LDH release for cell death.

Discussion

Peroxide explosives, such as TATP and HMTD, are the compounds of choice by terrorists because it is easily synthesized from household items (Rossi *et al.* 2019). Law enforcement K9 units, handlers and dogs, are regularly exposed to various explosives as part of the routine, since canines are currently one of the most reliable detection techniques used to find an explosive (Harper and Furton 2007). Training includes peroxide explosives even though little is known about their toxicity. Our previous studies of TATP indicate similar ADMET in canine and human; we established its absorption by inhalation, its hepatic metabolism, comprising of hydroxylation followed by glucuronidation, and its excretion in urine as TATP-*O*-glucuronide (Colizza *et al.* 2019, Gonsalves *et al.* 2020). Herein, the microsomal metabolism of HMTD was investigated, in addition to the blood stability and hepatocyte toxicity of both TATP and HMTD in dogs and humans.

HMTD is inherently unstable, its cyclic structure has three peroxide bonds and a very strained ring configuration due to a planar 3-fold coordination on the two bridgehead nitrogen atoms (Schaefer *et al.* 1985). The metabolism of this peculiar compound was investigated in human liver microsomes. Metabolite identification, using HPLC-HRMS, as m/z 231.0588 ($C_6H_{12}N_2O_6Na^+$) and 247.0327 ($C_6H_{12}N_2O_6K^+$) was supported by the formation of their labelled counterparts in incubations with labelled HMTD (d_{12} -HMTD and $^{15}N_2$ -HMTD).

The optimized analytical method used to detect the metabolite indicated a similar retention time and ionization technique (ESI) to TMDDD, rather than HMTD, suggesting an open-ring structure metabolite. Furthermore, the retention times offset between the

sodium and potassium species suggests the formation of different metabolites. Possibly one and two peroxide cleavages occur, forming two metabolites with the same elemental composition with each having either a sodium or potassium adduct. Previous studies into the decomposition of HMTD proposed TMDDAA and TMPDDD as its degradation products (Oxley *et al.* 2016), instead, these could be its enzymatic products, as their mass-to-charge matched the observed metabolites.

No metabolites from incubations with GSH or UDPGA for phase II metabolism were observed. Nonetheless, if the peroxide bonds of HMTD were cleaved, and smaller hydrophilic compounds such as ammonia, formaldehyde and hydrogen peroxide, the precursors of HMTD, were formed, conjugation reactions would not be necessary to promote excretion.

Blood stability studies in dogs and humans showed that HMTD was rapidly degraded, whereas 54 and 40 % TATP remained after 1 week in dog and human, respectively. Catalase, an enzyme found in red blood and liver cells, is known to decompose hydrogen peroxide into water and oxygen (Miller 1958, Watt *et al.* 2004). Here, it might be responsible for the cleavage of HMTD peroxide bonds and promoting its rapid degradation. Unlike HMTD, TATP possesses methyl groups that may create enough steric hindrance to prevent catalase from reaching its peroxide bonds preserving its cyclic structure. Our previous studies on TATP established its hepatic metabolic pathway consisting of hydroxylation of its accessible methyl group (Colizza *et al.* 2019, Gonsalves *et al.* 2020). HMTD, however, might not survive blood transport to the liver for microsomal enzyme metabolism.

A recent mass spectrometric study, in which explosives were screened for recovery efficiency from various matrices, claimed that HMTD was not observed in dried blood samples because it did not form gas phase ions easily (Irlam *et al.* 2019); our observation of HMTD degradation in blood may better explain that observation. We also observed decrease in ionization efficiency of HMTD and d_{12} -HMTD (IS) in blood samples, which is indicative of matrix interferences; however, the use of deuterated internal standard considerably improved the quantification of HMTD in whole blood and may assist in other challenging matrices.

As previously mentioned, the Brussels authorities found TATP in the blood of suicide bombers (Goulard 2016). Our data, which shows the stability of TATP in blood over time, clearly supports their findings. In addition, the presence of TATP in dried blood spots aged 1 week has been reported (Ezoe *et al.* 2015), but the stability of TATP in liquid blood at body temperature over time had not, until now, been established. The stability of TATP in whole blood advocates for the use of blood tests as a forensic tool to screen those suspected to be involved in explosive terrorist attacks.

Toxicity studies, which involved treating dog and human hepatocytes with TATP and HMTD, indicated minimum cell death, suggesting these peroxide explosives are not toxic. Comparably, H_2O_2 , one of their reactants, is harmless at 3 %, being used even by veterinarians to induce emesis in dogs; but at higher concentrations, it can cause irritation to the dog's stomach, leading to more severe medical conditions (Khan *et al.* 2012). However, the larger TATP depletion from sublimation in water than in both human and canine blood, suggests TATP is binding to blood proteins, such as serum albumin and

haemoglobin. This observation suggests possible toxicity in cell lines other than hepatocytes and implies a health concern, since some protein binding are linked to toxicity, as in the case of TNT, where nitroso metabolites covalently react with cysteine residues of haemoglobin (Leung *et al.* 1995, Sabbioni *et al.* 2005).

This paper is the first to examine some aspects of HMTD metabolism and toxicity of peroxide explosives. It also elucidates the stability of peroxide explosives in blood, and proposes blood tests as evidence of TATP exposure. Further investigation into the ADMET of peroxide explosives is necessary in order to understand the long term side effects of working with these energetic materials.

Conclusions

HMTD metabolism was initially probed in liver microsomes, and with the assistance of HMTD derivatives, deuterated-HMTD and $^{15}\text{N}_2$ -HMTD, potential metabolites were identified as TMDDAA and TMPDDD. However, subsequent blood studies in dogs and humans showed that HMTD was rapidly degraded in blood, possibly by the blood enzyme catalase, which is known to cleave peroxide bonds. Thus, hepatic metabolism of HMTD may not be of importance. In contrast, TATP remained in blood for at least one week. Blood tests have been widely used to trace drug abuse, and as suggested herein, it can be used as viable evidence of exposure to this illicit explosive. TATP and HMTD did not indicate toxicity in dog and human hepatocytes. However, the possible binding of TATP to blood proteins suggests possible toxicity in other cell lines.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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APPENDIX A

Characterization of Encapsulated Energetic Materials for Trace

Explosives Aids for Scent (TEAS)

Supplemental Material

Figure A-1. TGA thermograms of polymers to determine T_d

Figure A-2. DSC thermograms of polymers to determine T_d

Figure A-3. Overlaid IR spectra of the vapor released from pure ETN compared with the vapor released from ETN encapsulated with various polymers (corresponding to the largest mass loss from the thermograms illustrated on **Figure A-4**)

Figure A-4. TGA thermograms of ETN encapsulated with various polymers

Figure A-5. DSC thermograms of polymers to determine T_g

Figure A-6. TGA thermograms of TNT encapsulated with various polymers

Figure A-7. Overlaid GC chromatogram, with magnification around the baseline, of the vapor released from pure TATP compared with the vapor released from TATP encapsulated with various polymers at 150 °C for 1 min

Figure A-8. Overlaid GC chromatogram, with magnification around the baseline, of the vapor released from pure TNT compared with the vapor released from TNT encapsulated with various polymers at 150 °C for 1 min

Figure A-9. GC chromatogram of the vapor release from PS-blank and PEI-blank at 150 °C for 1 min using the ETN chromatography method

Figure A-10. Full scan mass spectrum of TATP (from chromatogram at RT 3.0min), ETN (from chromatogram at RT 7.9min) and TNT (from chromatogram at RT 11.6 min) using EI ionization on an Agilent 5973N MS

Figure A-11. DSC thermogram of pure TATP compared with TATP encapsulated with various polymers

Figure A-12. DSC thermogram of pure ETN compared with ETN encapsulated with various polymers

Figure A-13. TGA thermogram of PC-HMTD made under the same solvent evaporation conditions

Figure A-14. DSC thermogram of pure AN compared with AN coated with EC stored at 67% humidity for 1 month

APPENDIX B**In vitro and in vivo studies of triacetone triperoxide (TATP) metabolism
in humans****Supplementary Material**

Figure B-1. Hydroxy-triacetone triperoxide (TATP-OH) standard curve from 10 to 500 ng/mL

Figure B-2. Extracted ion chromatogram of ticlopidine glutathione metabolite from ticlopidine metabolized by glutathione S-transferase (GST) after 1h incubation, presented as a GST positive control

Figure B-3. Extracted ion chromatogram of naphthol glucuronide metabolite from 1-naphthol metabolized by uridine diphosphoglucuronosyltransferase (UGT) after 15 min incubation, presented as a UGT positive control

Figure B-4. Extracted ion chromatograms of triacetone triperoxide (TATP), hydroxy-triacetone triperoxide (TATP-OH) and dihydroxy-triacetone triperoxide (TATP-(OH)₂) from TATP-OH standard. No impurities were observed

Figure B-5. Extracted ion chromatogram of TATP-OH from TATP incubations in human liver microsomes (HLM), dog liver microsomes (DLM) and rat liver microsomes (RLM). Different species exhibit the same metabolite, TATP-OH

Figure B-6. Extracted ion chromatograms using atmospheric pressure chemical ionization (APCI+) of possible TATP reduced glutathione (GSH) metabolites after 2h incubation. $[M+H]^+$ and $[M+NH_4]^+$ are illustrated, though other adducts were searched for. Other ionization methods, such as electrospray ionization (ESI) in positive and negative mode and APCI in negative mode, were also tested (not shown). No TATP-GSH metabolites were identified

Figure B-7. Extracted ion chromatogram of $[\text{TATP-O-glucuronide} + \text{NH}_4]^+$ (m/z 432.1712) using APCI+, showing formation of TATP-O-glucuronide at 4.26 min as incubation of TATP progressed. Glucuronidation samples were concentrated prior to high-performance liquid chromatography–high-resolution mass spectrometry (HPLC–HRMS) analysis

Figure B-8. Extracted ion chromatogram of benzydamine N-oxide metabolite from benzydamine metabolized by recombinant flavin monooxygenase 3 (rFMO3) after 10 min incubation, presented as a rFMO positive control

Figure B-9. TATP-OH depletion from incubations in CYP2B6 with (w/) or without (w/o) reduced nicotinamide adenine dinucleotide phosphate (NADPH). Performed in triplicate

Figure B-10. Lineweaver-Burk plot, used to fit the equation: $1/v = (K_M/V_{max} \times 1/[S]) + 1/V_{max}$ to yield $K_M = 3.1 \mu\text{M}$ and $V_{max} = 11.7 \text{ nmol/min/nmol CYP2B6}$

Figure B-11. Eadie-Hofstee plot, used to fit the equation: $v = (-K_M \times v/[S]) + V_{max}$ to yield

$K_M = 0.54 \mu\text{M}$ and $V_{max} = 4.9 \text{ nmol/min/nmol CYP2B6}$

Figure B-12. Hanes-Woolf plot, used to fit the equation: $[S]/v = [S]/V_{max} + K_M/V_{max}$ to yield

$K_M = 1.2 \mu\text{M}$ and $V_{max} = 8.0 \text{ nmol/min/nmol CYP2B6}$

Table B-1. Hydroxy-triacetone triperoxide (TATP-OH) formation from triacetone triperoxide (TATP) incubations with recombinant cytochrome P450 (rCYP) and recombinant flavin monooxygenase (rFMO)

Incubation matrix	[TATP-OH] (μ M)
HLM	1.47 ± 0.07
rCYP control	not observed
rCYP1A2	not observed
rCYP2B6	5.59 ± 0.3
rCYP2C9	not observed
rCYP2C19	not observed
rCYP2D6	not observed
rCYP2E1	not observed
rCYP3A4	not observed
rFMO control	not observed
rFMO1	not observed
rFMO3	not observed
rFMO5	not observed

Experiments with rCYP (100 pmol CYP/mL) or rFMO (100 μ g protein/mL) consisted of 10 μ g/mL TATP incubated with 10 mM phosphate buffer (pH 7.4), 2 mM MgCl_2 and 1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH). Incubations were done in triplicate and quenched at 10 min.

HLM human liver microsomes

Table B-2. TATP-*O*-glucuronide formation from TATP-OH incubations with recombinant uridine diphosphoglucuronosyltransferase (rUGT)

Incubation matrix	TATP- <i>O</i> -glucuronide/ <i>IS</i> area count
HLM	0.26 ± 0.02
rUGT control	not observed
rUGT1A1	not observed
rUGT1A3	not observed
rUGT1A4	not observed
rUGT1A6	not observed
rUGT1A9	not observed
rUGT2B7	0.05 ± 0.03

Experiments with rUGT (500 µg protein/mL) consisted of 10 µg/mL TATP-OH incubated with 10 mM phosphate buffer (pH 7.4), 2 mM MgCl₂, 50 µg/mL alamethicin, 1 mM NADPH, and 5.5 mM uridine diphosphoglucuronic acid (UDPGA). Glucuronidation done in triplicate and quenched at 2 h. Quantification was done using area ratio TATP-*O*-glucuronide/internal standard.

IS internal standard