Toxicology Letters 292 (2018) 46–54

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

A toolbox for microbore liquid chromatography tandem-high-resolution mass spectrometry analysis of albumin-adducts as novel biomarkers of organophosphorus pesticide poisoning

Jens von der Wellen[a], Peter Winterhalter[b], Markus Siegert[c], Florian Eyer[d], Horst Thiermann[a], Harald John[a,*]

[a] Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstraße 11, 80937 Munich, Germany
[b] TU Braunschweig, Institute of Food Chemistry, Schleinitzstraße 20, 38106 Brunswick, Germany
[c] Department of Chemistry, Humboldt-Universität zu Berlin, Brook-Taylor-Straße 2, 12489 Berlin, Germany
[d] Department of Clinical Toxicology, TUM School of Medicine, Technical University of Munich, Ismaninger Strasse 22, 81675 Munich, Germany

ARTICLE INFO

Keywords:
Biomonitoring
Human serum albumin
μLC-ESI MS/HR MS
Organophosphate
Pesticide
Protein-adducts
Verification

ABSTRACT

Exposure to toxic organophosphorus pesticides (OPP) represents a serious problem in the public healthcare sector and might be forced in terroristic attacks. Therefore, reliable verification procedures for OPP-intoxications are required for forensic, toxicological and clinical reasons. We developed and optimized a toolbox of methods to detect adducts of human serum albumin (HSA) with OPP considered as long-term biomarkers. Human serum was incubated with diethyl-oxono and diethyl-thiono pesticides for adduct formation used as reference. Afterwards serum was subjected to proteolysis using three proteases separately thus yielding phosphorylated tyrosine residues (Y*) detected as single amino acid (pronase), as hexadecapeptide LVRY*^{411}TKKVPQVSTPTL (pepsin) and as the tripeptide Y*^{411}TK (trypsin), respectively. Adducts were analyzed *via* microbore liquid chromatography coupled to electrospray ionization (μLC-ESI) and tandem-high-resolution mass spectrometry (MS/HR MS).

Using paraoxon-ethyl as model OPP for adduct formation, methods were optimized with respect to MS/HR MS-parameters, protease concentrations and incubation time for proteolysis. HSA-adducts were found to be stable in serum *in vitro* at +37 °C and −30 °C for at least 27 days and resulting biomarkers were stable in the autosampler at 15 °C for at least 24 h. Limits of identification of adducts varied between 0.25 μM and 4.0 μM with respect to the corresponding pesticide concentrations in serum. Applicability of the methods was proven by successful detection of the adducts in samples of OPP-poisoned patients thus demonstrating the methods as a reliable toolbox for forensic and toxicological analysis.

1. Introduction

Intoxications with organophosphorus pesticides (OPP) pose a major problem in the public healthcare sector with approximately 200,000 fatal human poisonings per year worldwide (Mew et al., 2017). Particularly in developing countries like in rural asia a lack of safety precautions and an inadequate medical care system result in a high number of fatal accidental exposures and suicidal attempts (Eddleston and Phillips, 2004; Eddleston et al., 2005; Gunnell et al., 2007; Mew et al., 2017; Pavlic et al., 2002). At present, there is a reasonable threat by OPP deployable in terroristic attacks as they are easily available and could be used in large quantities to compensate for their lower toxicity when compared to organophosphorus nerve agents (Central Intelligence Agency, 2003).

OPP react with acetylcholinesterase (AChE) at the catalytic serine residue of the active site and thus inhibit this pivotal enzyme. Inhibition leads to an accumulation of the neurotransmitter acetylcholine at both nicotinic and muscarinic receptors thus causing the cholinergic crisis with symptoms like miosis, excessive respiratory secretion, paralysis and fasciculation. If not treated appropriately, death may occur due to respiratory failure (Grob, 1956; John et al., 2015a; Kwong, 2002; Lee, 2003; Sikka and Gurbuz, 2006).

A wide-spread group of pesticides comprises derivatives of phosphorus acid esters being substituted by two ethylester-groups (diethyl-oxono phosphate, *dep*). In agriculture, their thiono-analogs are typically applied, which contain a P = S double bond (diethyl-thiono phosphate, *desp*) that undergoes desulfuration by CYP450 enzymes in the liver yielding their oxono-biotransformation products (P = O double bond)

* Corresponding author.
E-mail address: haraldjohn@bundeswehr.org (H. John).

https://doi.org/10.1016/j.toxlet.2018.04.025
Received 6 March 2018; Received in revised form 13 April 2018; Accepted 23 April 2018
Available online 25 April 2018
0378-4274/

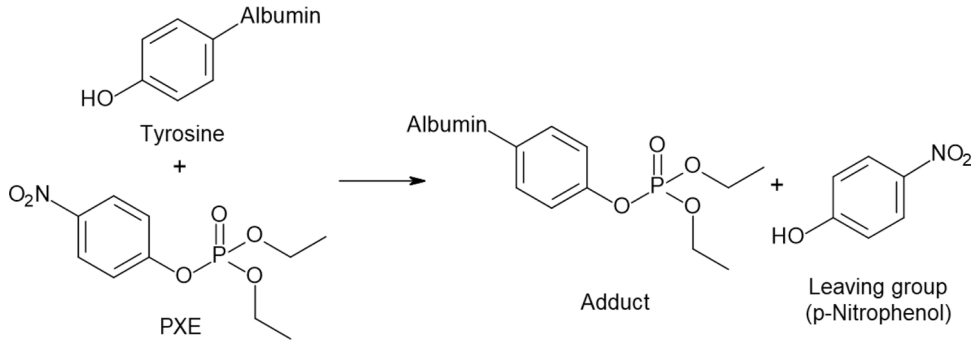

Fig. 1. Adduct formation at a tyrosine residue of HSA with the pesticide paraoxon-ethyl (PXE).

(Buratti et al., 2003; Sams et al., 2000; Sikka and Gurbuz, 2006). These molecules possess high reactivity with diverse esterases and are thus more toxic than the thiono-compounds.

Bioanalytical verification of acute OPP-exposure *in vivo* might be quite challenging as these compounds may be rapidly biotransformed and cleared from the body. Products of biotransformation can be detected in blood and urine for a very limited period of time of some days after incorporation (John et al., 2008). Merely in cases of suicide attempts characterized by ingestion of huge amounts of OPP, the poison itself might be detectable for a longer period (Eyer et al., 2009; Thiermann et al., 2009).

Current research focuses on OPP-adducts with butyrylcholinesterase (BChE) and human serum albumin (HSA) as these adducts possess a longer half-life and are discussed to be detectable for days or even weeks after exposure (Black and Read, 2013; John et al., 2015a; Kranawetvogl et al., 2016, 2017, 2018). HSA exhibits a half-life of approximately 20 days and is known to be phosphorylated by OPP particularly at the hydroxyl-group of tyrosine residue 411 (Fig. 1), even though other tyrosine residues can also be affected with distinctly lower reactivity (Noort et al., 2009; Ding et al., 2008; John et al., 2010a, 2015a, 2018; Noort et al., 2009). In the following a tyrosine residue modified by phosphorylation is referred to as Y*. In contrast to BChE-adducts, HSA-adducts of nerve agents, that are structurally related to OPP, do not undergo the well-known time-dependent dealkylation process of the phosphorylester-moiety (“aging”) thereby causing higher stability (Andersen et al., 2014; Black and Read, 2013; Li et al., 2013; Noort et al., 2009). In contrast to BChE-adducts, HSA-adducts are not degraded by oxime antidotes administered for therapy thus showing higher stability which is beneficial for bioanalytical verification (Williams et al., 2007).

For bioanalytical evidence HSA-adducts may be proteolyzed by an appropriate enzyme to produce specific phosphorylated peptides and amino acids. These biomarkers can be detected *via* liquid chromatography (LC) on-line coupled to tandem-mass spectrometry (MS/MS).

Diverse methods for sample preparation and detection, different proteases used for albumin degradation and several organophosphorus compounds applied for phosphylation (denominating both phosphorylation by pesticides and phosphonylation by nerve agents) have been introduced so far (Black and Read, 2013; Ding et al., 2008; John et al., 2010a; Li et al., 2013; Noort et al., 2009; Williams et al., 2007).

Based on these findings, we developed a novel toolbox of optimized methods for systematic identification and detection of diverse OPP-HSA-adducts focusing on diethyl-pesticides like paraoxon-ethyl (PXE, Fig. 1) and parathion-ethyl (PTE). These methods make use of the proteases pronase, pepsin and trypsin applied in a simplified sample preparation procedure and of an analytical setup, which could easily be transferred to other OPP and nerve agents. Fig. 2 illustrates the overall strategy, that was successfully applied to *in vivo* samples of patients intoxicated by OPP and thus proved its applicability.

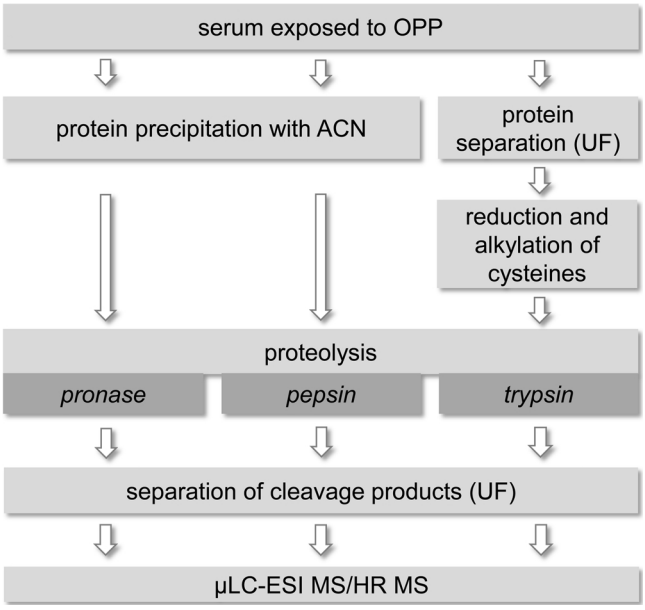

Fig. 2. Workflow of the toolbox for HSA-OPP-adduct detection.
HSA-OPP-adducts are proteolyzed by three enzymes separately (pronase, pepsin, trypsin) to produce phosphorylated tyrosine residues as well as two different peptides containing the phosphorylated Tyr^{411} residue that are detectable in µLC-ESI MS/HR MS analysis. Pronase produces phosphorylated tyrosine (Y*), pepsin yields the nonapeptide LVRY*TKKVPQVSTPTL and use of trypsin results in the tripeptide Y*TK.
ACN, acetonitrile; OPP, organophosphorus pesticide; UF ultrafiltration.

2. Materials and methods

2.1. Materials

HSA (≥99%), pepsin (from porcine gastric mucosa), trypsin, dithiothreitol (DTT, ≥99%), iodoacetamide (IAA, ≥99%) and phosphate-saline-buffer-tabs (PBS) were purchased from Sigma-Aldrich (Steinheim, Germany). Human serum and EDTA-plasma obtained from individual donors were provided by Sonnen-Gesundheitszentrum (Munich, Germany). *In vivo* samples of OPP-intoxicated patients were made available by the School of Medicine (Technical University, Munich, Germany). Informed consent was obtained from close relatives of both anesthetized and artificially ventilated patients (Eyer et al., 2009). The pesticides PXE, PTE, chlorpyrifos-oxon, chlorpyrifos, chlorfenvinphos, bromfenvinphos, terbufos and diazinon (all > 92%) were purchased from LGC Group (Wesel, Germany). Pesticide stock solutions (50 mM) were prepared in acetonitrile (ACN) and only stored for a short time of some hours at 4 °C before use. Pronase from *Streptomyces griseus* was delivered by Roche (Mannheim, Germany), ammonium hydrogencarbonate (hyper-grade) by Fluka (Bucks,