THE DEVELOPMENT OF EXTRACTION AND ANALYSIS TECHNIQUES FOR THE QUANTIFICATION OF PESTICIDES AND PESTICIDE METABOLTES IN HUMAN URINE AND BLOOD

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Introduction

Blood and urine have been extensively used to test for the presence of drugs of abuse and to monitor exposure to toxicologically relevant compounds in forensic investigations. Over the last couple of years blood and urine have been evaluated as bio-monitoring tools for persistent organic pollutants (POPs) as well as current use pesticides such as organophosphate pesticides. Exposure to POPs has been associated with a myriad of negative health effects leading to the implementation of stringent regulations on the use of these chemicals. Although emerging pollutants, including current use pesticides are not yet governed by regulations, they may pose a threat to human and environmental health at environmentally relevant concentrations, and therefore require continued monitoring. In addition, current-use pesticides, such as organochlorine pesticides, have been linked to suicides, poisonings and other forms of misuse increasing their forensic importance[1].

Traditional tissue monitoring techniques included the collection of adipose tissue or muscle, which was highly invasive. In comparison, blood and urine collection is rapid, cheap and non-invasive. The extraction and analysis of these matrices is less challenging compared to high fat tissue samples. An additional advantage of using blood or urine sampling, over environmental media, is that these matrices link concentrations directly to internal dose, which can in turn be used to estimate integrated exposure and evaluate health risk[2]. This information is particularly valuable for the assessment of occupational exposure.

Although POPs are relatively recalcitrant and parent compounds can act as indicators of internal dose, the measurement and data interpretation of current use pesticides is more challenging. These pesticides have relatively short half-lives in the human body and are metabolised and excreted rapidly[3]. This paper will discuss the development of a single extraction method per matrix and dual gas chromatography coupled to time of flight mass spectrometry (GC×GC-TOFMS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis methods to determine the presence and concentration of pyrethroid, carbamate, organophosphorus and organochlorine pesticides and their associated metabolites in urine and blood samples.

Materials and methods

Extraction of urine samples: Urine samples were diluted with deionized water, spiked with labelled standard, aldicarb-(N-methyl-^{13}C,d_3, carbamoyl-^{13}C) sulfone, and enzymatically digested with β-glucoronidase type H-1 from *Helix promatia*[4]. After enzymatic digestion, samples were acidified, and centrifuged before solid phase extraction (SPE) using reverse phase C18 cartridges. The cartridges were rinsed and eluted with methanol and dichloromethane (DCM). The eluent was evaporated, filtered and reconstituted in 100 µl isooctane for the GC×GC-TOFMS and in aqueous acetonitrile for LC-MS/MS analysis.

Urine extracts –screening by GC×GC-TOFMS: A LECO Pegasus GC×GC-TOFMS was used to determine the presence of organochlorine, pyrethroid and organophosposphate pesticides. A non-polar, 30 m Rxi-5SilMS column in the first dimension was used in combination with a more polar 2 m Rtx-200 column using helium gas as a carrier. Due to possible retention time shifts in the presence of matrix, retention times were confirmed by spiking analytes into extracted urine samples. The list of analytes screened together with the limits of detection (LOD) and limits of quantification (LOQs) for each analyte, are summarized in **Table 1**.

***Table 1**: LODs (ng/ml) and LOQs (ng/ml) for GCxGC-TOFMS screening of urine samples*

Analyte	LOD	LOQ	Analyte	LOD	LOQ	Analyte	LOD	LOQ
Atrazine	7	25	Fenthion	0.1	0.3	p,p'-DDT	1	2
Bendiocarb	9	31	Fenvalerate	0.5	2	Paraoxone	3	10
Bifenox	2	7	Flamprop-isopropyl	6	19	Parathion	0.4	1
Carbaril	1	3	Fluometuron	0.3	1	Paroxan	3	10
Carbofurane	0.3	1	Fluvalinate	4	14	Permethrin	0.1	0.2
Chlorbromuron	0.3	1	Formothion	2	8	Phenothrin	1	3
Chlorpyrifos	1	2	Lindane	0.3	1	Phosphamidon	4	15
Cyanophos	0.2	1	Linuron	0.2	1	Pirimicarb	0.1	0.2
Cyfluthrin	0.3	1	Malathion	1	4	Pirimiphos ethyl	0.3	1
Cypermethrin	1	4	Methiocarb	0.5	2	Pirimiphos methyl	0.2	1
Dichlofenthion	0.1	0.5	Methomyl	1	3	Procymidone	0.3	1
Diflufenican	0.1	0.3	Methyl chlorpyriphos	0.2	1	Profam	0.2	1
Dimethoate	2	7	Methyl parathion	0.3	1	Propoxur	0.0	0.1
Dioxothion	2	8	Metobromuron	0.2	1	Quizalofop-p-ethyl	1	2
Disulfoton	0.1	0.2	Norflurazon	1	4	Fenchlorphos	0.1	0.4
Endosulfan	9	29	Nuarimol	0.3	1	Simazine	4	13
Endosulfan sulfate	2	5	Oxadixyl	1	4	Tebuthiuron	0.1	0.5
Ethiofencarb	0.1	0.4	Oxamyl	2	8	Tetramethrin	0.2	1
Fenitrothion	0.2	1	p,p'-DDD	0.2	1	Tolclofos-methyl	0.1	0.2
Fenpropathrin	0.2	1	p,p'-DDE	3	9	Trichloronat	0.1	0.5

Urine extracts – screening and quantification by LC-MS/MS: A Waters Quattro Premier triple quadrupole Mass spectrometer coupled to an ultra performance liquid chromatograph (UPLC) and photodiode array (PDA) detector was used together with a Acquity UPLC BEH C18 column (1.7 um, 2.1 x 100 mm). A generic LC method was employed and optimized using commercially available authentic standards for the compounds described in Table 2. The MS method contained two multiple reaction monitoring (MRM) transitions for the analytes listed in Table 2[4, 5]. A six point matrix matched calibration curve, spiked with native pesticide parents/ metabolites after extraction, was used for quantification. The calibration curve areas were used in a linear regression, where S_0 was defined as the x-intercept. The LOD was defined as three times the standard deviation at S_0 and the LOQ was defined as ten times the standard deviation at S_0[5]. The LOD and LOQs for compounds that were quantified are summarized in **Table 2**. With each set of extractions a blank urine sample, solvent blank and non-matrix matched standards were run to ensure the quality of analytical runs.

***Table 2**: LODs (ng/ml) and LOQs (ng/ml) forLC-MS/MS quantification of selected pesticide and pesticide metabolites in human urine*

Analyte	LOD	LOQ	Analyte	LOD	LOQ
1,2,3 Benzo triazin-4(3H)-one (BTA)	17.24	57.47	Coumaphos	9.89	32.89
2-isopropyl-6-mehtly-4-pyrimidinol (IMPY)	17.63	8.77	2-Diethylamino-6-methylpyrimidin-4-ol (DEAMPY)	12.51	41.71
3,5,6-Trichloro-2-pyridinol (TCPY)	13.53	45.10	N,N-diethyl-m-toluamide (DEET)	22.84	76.11
4-Nitrophenol (PNP);	18.14	60.47	Isazophos	16.39	54.64
Acetochlor oxanillic acid	70.50	235	Oxamyl	10.96	36.54
Acetochlor ESA sodium Salt	83.25	277	Methamidophos	23.83	79.44
Acephate	56.15	187	Pirimiphos-ethyl	20.26	67.54
Aldicarbsulfone	18.84	62.79	Pirimiphos-methyl	22.86	76.21
Chlorpyrifos-methyl	62.69	200.80			

Blood plasma extraction: A general extraction procedure was adopted[6,7]. In short, 1 ml of plasma was spiked with internal standard, mixed with 2 ml of formic acid and extracted using 2 ml of dichloromethane and 8 ml of 50:50 toluene: hexane (v/v). The mixture was centrifuged and underwent florisil/ silica open column clean-up[8]. Thereafter extracts were evaporated and reconstituted in 200 µl of iso-octane.

Blood plasma screening analysis by GC×GC -TOFMS: The GC×GC-TOFMS columns used for the screening of plasma samples were a non-polar Rxi®-5SilMS (30 m, 0.25 mm ID, 0.25µm df) as the primary column and a mid-polar Rxi®-17SilMS (1 m, 0.25 mm ID, 0.25 µm df) as the secondary column. The orthogonality provided by this column combination allowed the simultaneous chromatographic separation of over 80 pollutants, with increased resolution compared to the method originally developed for the screening of urine samples as described above.

Blood plasma quantification of organochlorine pesticides (OCPs) by GC-TOFMS: The instrumental analysis was performed on a LECO GC-TOFMS instrument using a Stx-CLPesticides2 (30 m, 0.25 mm ID, 0.20 µm) column. This GC column is an application-specific column designed for the optimum separation of -chlorinated pesticides. The instrumental analysis method was developed using native and labelled standards in the presence of matrix to provide the best separation of the analytes of interest from each other and from matrix associated interferences. The LOD and LOQ was calculated using non-matrix matched standards, and is described as three times signal to noise and ten times signal to noise respectively, using a 95% confidence interval. Recovery was assessed by gravimetrically spiking pre-determined blank samples.

Blood plasma: quantification and quality control: A ten point calibration curve between the ranges of 0 – 1000 ng/ml was constructed for each of the analytes, requiring a R^2 greater than 0.99. With each set of extractions, a blank plasma sample, a spiked plasma sample and solvent blank were run to ensure the quality of analytical runs. Recovery was assessed by analysing gravimetrically spiked plasma. The expanded measurement uncertainty (k = 2) was below 30% for each of the analytes as determined by repeat measurements of spiked plasma.

Results and discussion

The developed methods were successfully used in the analysis of human blood and urine. The variability in the matrix, as the exact composition of blood and urine varies from individual to individual, highlighted the need to use compound specific internal standards for quantification (**Figure 1**). The use of compound matched labeled compounds leads to increased linearity as the labelled and native are matched chemically and undergo exactly the same process with the same fragmentation formation during ionisation. When standards cannot be matched there can be deferential losses or variations in ionization that leads to a decreased linear response and a lower R^2 value. Due to the lack of commercially available native and labeled standards this is not always possible and is one of the greatest challenges in this analysis.

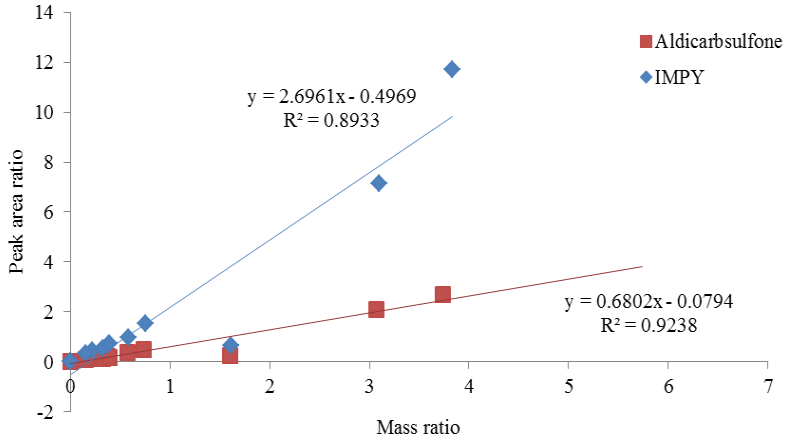

Figure 1: The increase in linearity when a compound is matched with its labelled compound (aldicarbsulfone) against a compound that is quantified with a non-matched labelled compound (IMPY)

The LODs varied depending on the compound of interest with recoveries for quantification purposes ranging between 10 – 120%. The extraction efficiency from urine and plasma could not be accurately assessed as commercially available matrix certified reference material could not be obtained for current-use parent pesticides or their corresponding metabolites. The use of a single extraction method to extract multiple compound classes and chemical properties was a compromise resulting in lower recoveries for certain compounds that require specialised extraction. Nonetheless, these complications did not hinder successful quantification of target compounds in human urine and plasma. The developed methodologies do not only provide a high through-put screening tool for human exposure to current and historical pesticides, but also enables accurate quantification of the compounds identified in the initial screening process.

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