CHROMATOGRAPHIC METHODS FOR DETERMINATION OF CHLOROPHENOLS AS BIOMARKERS OF EXPOSURE TO PESTICIDES AND ORGANOCHLORINE SUBSTANCES

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Abstract. Chromatographic methods for chlorophenols (CPs) determination that can be applied to clinical (blood, urine) and breast milk samples are reviewed. Special emphasis is given to sample storage conditions and to the application of preconcentration techniques. Solid phase extraction, solid phase microextraction, stir-bar sorptive extraction, liquid phase microextraction and steam distillation methods are considered in view of the potential use of CPs as biomarkers of exposure to different pesticides.

Keywords: chlorophenols; chromatography; blood plasma; urine; breast milk

1. Introduction

Chlorophenols (CPs) are highly toxic chemicals, widespread in the environment, mainly used as pesticides, wood and leather impregnation agents and in chemical synthesis. They are derivatives of phenol having different degrees of substitution, with 1 to 5 chlorine atoms: monochlorophenols (MCPs), dichlorophenols (DCPs), trichlorophenols (TCPs), tetrachlorophenols (TeCPs) and pentachlorophenol (PCP). Their determination in liquid biological samples is an indicator of exposure to CPs or other chlorinated organic substances, including polychlorinated dioxins and furans (Collins et al., 2008).

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The CPs in human urine appear as a result of occupational or environmental (food, water, air) exposure to CPs or as metabolites of other chlorine-containing compounds (Lee et al., 1998; Kontsas et al., 1995). The excretion of PCP in the urine of the general population is related to its use, especially in the past, as wood and leather preservation agent (Becker et al., 2003; Hill et al., 1995a). CPs in urine could also potentially be biomarkers of exposure to a wide range of other products, like insecticides, fungicides, herbicides and industrial chemicals (Hill et al., 1995a). However, even if there was a reason for such claim, the concentration of CPs in urine has been related to the exposure to a parent compound in a limited number of cases. For example, lindane is metabolized to 2,4,6-TCP and 2,4-DCP in mouse (Kurihara and Nakajima, 1974) and to TCPs and TeCPs in calf (Marsden et al., 1986). More information is available on the urinary excretion of CPs as metabolites of chlorobenzenes (CBs), used as solvents, lubricants and heat transfer fluids and as intermediates in chemical synthesis. Hexachlorobenzene is applied as fungicide and 1,4-dichlorobenzene (1,4-DCB) has a household use as moth repellent. It is the reason for the presence of 2,5-DCP, a metabolite of 1,4-DCB, in urine samples of professionally non-exposed individuals (Becker et al., 2003; Hill et al., 1995a). Its concentrations have been positively correlated with the levels of 1,4-DCB in blood (Hill et al., 1995b). 2,5-DCP concentrations in urine have been also used as a biomarker for professional exposure to 1,4-DCB (Hsiao et al., 2009). MCPs are excreted in urine as metabolites of monochlorobenzene (Kumagai and Matsunaga, 1994) and 2,3-DCP and 3,4-DCP as metabolites of 1,2-DCB (Kumagai and Matsunaga, 1997). In both cases, the concentrations of CPs in urine were related to the airborne exposure to CBs.

The concentrations of free (non-conjugated) CPs in urine samples are usually less than 1 $\mu g\ L^{-1}$, or less than 0.1 $\mu g\ L^{-1}$ for the case of PCP (Lee et al., 1998). After de-conjugation of CPs in the urine samples, the maximum concentrations, depending on the compound and the level of exposition, were in the order of a few $\mu g\ L^{-1}$ (Bartels et al., 1999), from less than 1 $\mu g\ L^{-1}$ to several tens $\mu g\ L^{-1}$ (Kawaguchi et al., 2005; Crespín et al., 2002; Kontsas et al., 1995; Ito et al., 2008), from a few $\mu g\ L^{-1}$ to hundreds $\mu g\ L^{-1}$ (Becker et al., 2003) and from several tens to several thousands $\mu g\ L^{-1}$ (Hill et al., 1995a). The median concentrations are usually from less than 1 $\mu g\ L^{-1}$ to several tens $\mu g\ L^{-1}$ (Becker et al., 2003; Hill et al., 1995a).

CPs are retained in blood and only to a lesser extent in adipose tissue (Zhou et al., 2007). Direct exposure to CPs can be proved by analyzing blood or blood plasma samples. However, there is a limited number of analytical methods, for example, to determine polychlorinated CPs. The concentrations of PCP found in the blood plasma were in the range <0.02-6.3 $\mu g\ L^{-1}$ (Zhou et al., 2007).

Babies are particularly vulnerable to chemical contamination. In breast milk, CPs were measured with maximum values in the order of tens to a hundred µg kg^{-1} but median concentrations were at or below 1 µg kg^{-1} (Veningerová et al., 1996). The most frequently detected, and with highest levels, were 2,4-DCP, 2,4,5-TCP and PCP. PCP is of particular interest, since it has the highest toxicity of all CPs.

2. Methods for CPs Determination

2.1. SAMPLE STORAGE

Care must be taken when storing samples before the analysis of CPs. There are discrepancies in the literature about the stability of CPs. The CPs were found to be stable in frozen urine samples (Kontsas et al., 1995; Marsden et al., 1986). However, other authors previously reported decomposition of PCP at the same conditions (Rick et al., 1982).

Urine samples have been collected in sterilized containers and stored at -20 °C (Crespín et al., 2002; Lee et al., 1998; Kontsas et al., 1995; Hiao et al., 2009) or -30 °C (Kumagai and Matsunaga, 1994) or even at -80 °C (Kawaguchi et al., 2005) prior to use. If acetic acid (1% v, v) is used for the preservation of urine, it can be stored at 4 °C (Bartels et al., 1999). Human plasma should be immediately frozen and stored up to one month (Norberg et al., 1997). Blood samples have been stabilized with ethylenediamine tetraacetic acid (EDTA), stored at 4 °C and analyzed within 24 hours of collection (Röhrig et al. 1998). Milk samples have been stored frozen at -20 °C (Röhrig and Meisch, 2000; Veningerová et al., 1996).

2.2. SAMPLE PRETREATMENT AND DE-CONJUGATION

CPs in urine are presented mainly as glucuronide and sulfate conjugates but free forms also exist and were found to be up to 20-30% of the total (Crespín et al., 2002). When methods with low limits of detection (LODs) are going to be used, it is possible to proceed directly to the analysis of free forms of the CPs (Lee et al., 1998; Hiao et al., 2009). Sometimes, it is not possible to measure the CPs without previous de-conjugation due to the low concentrations of free CPs in the urine of non-exposed individuals, especially when using methods with relatively high LODs. Different techniques for hydrolysis of the conjugates have been developed. Most of the researchers used acid hydrolysis with sulfuric (Kontsas et al., 1995; Bartels et al., 1999) or hydrochloric acid (Wada et al., 1999; Guidotti et al., 1999; Crespín et al., 2002; Kumagai and Matsunaga,

1994). Conditions (acid concentration, temperature and time) for acid hydrolysis were optimized (Crespín et al., 2002). Enzymatic hydrolysis was carried out with β-glucuronidase and sulfatase at 37 °C for 3 hours (Kawaguchi et al., 2005; Ito et al., 2008; Hill et al., 1995a). The choice of hydrolysis procedure could depend sometimes on the determination method used. For example, alkaline hydrolysis for the urine samples could be preferred if the acid and enzymatic treatments show interferences with the analytical procedure (Navarro-Villoslada et al., 1999). Alkaline hydrolysis was also used for the case of breast milk samples (Veningerová et al., 1996) but the lack of variety of analytical methods for the determination of CPs in milk does not allow drawing general conclusions.

Blood plasma can be analyzed without any sample pretreatment (Norberg et al., 1997). However, sometimes long pretreatment procedures could be necessary. Precipitation of plasma proteins and the release of bound analytes from the matrix could be achieved by adding water-miscible solvents, like methanol and acetone (Zhou et al., 2007). That procedure required subsequent evaporation of the organic solvents.

2.3. DETERMINATION OF CHLOROPHENOLS

Some of the methods used for water analysis, reviewed by de Morais et al., 2012, have been applied for CPs determination in biological fluids. The analytical methods for CPs determination in human milk, urine and blood plasma, the degree of chlorine substitution in the analytes and the LODs are summarized in Table 1. From the concentrations of CPs found in real samples (see the introduction), target LODs for CPs can be established. The LODs of the analytical methods for CPs determination in urine (after de-conjugation) must be set to target values in the order of 1-10 $\mu g\ L^{-1}$. Similarly, for blood plasma and breast milk samples, the target LODs have to be less than 1 $\mu g\ L^{-1}$. The use of gas chromatography (GC) with flame ionization detector (FID) or liquid chromatography (LC) with ultraviolet (UV) detection leads to very high LODs (Table 1). Even the use of very efficient microextraction techniques, for example, supported liquid membrane extraction (SLME), could not lead to low LOD owing to the weak detector response to CPs (Norberg et al., 1997). The methods that match the target LODs include either GC with electron-capture detection (ECD), mass spectrometry (MS), tandem mass spectrometry (MS/MS) detection or LC equipped with fluorescence detection (FLD) after fluorescence labeling (FLLabel) of the CPs. Nevertheless, it is always necessary to preconcentrate the analytes.

TABLE 1. Methods for determination of CPs in liquid biological samples.

Determination method / sample type	CPs*	LOD $\mu g\ L^{-1}$
LLE-Acetyl-GC-MS / urine[(a)]	**2-5**	**3.1-5.4**
LLE-BackE-ClPropyl-GC-MS/MS / urine[(b)]	**2, 3, 5****	**1-2**
LLE-GC-FID / urine[(c)]	**2**	**500**
LLE-FLLabel-LC-FLD / urine[(d)]	**1-3**	**3.1-9.9**
LLE-LC-UV / urine[(e)]	**1**	**100**
SPE-GC-MS / urine[(f)]	**1-3, 5****	**0.3-0.6**
SteamD-SPE-GC-ECD / urine[(g)]	**3-5**	**0.01-0.03**
SPME-GC-MS / urine[(h)]	**1**	**8**
SPME-GC-MS / urine[(i)]	**1-5**	**0.001-0.04**
HS-SPME-GC-ECD / urine[(j)]	**2**	**0.55**
Acetyl-SBSE-TD-CT-GC-MS / urine[(k)]	**2-5**	**0.01-0.02**
Acetyl-HF-LPME-GC-MS / urine[(l)]	**2-5**	**0.1-0.2**
SLME-LC-biosensor / plasma[(m)]	**1****	**43**
Acetyl-HS-SPME-GC-MS / plasma[(n)]	**5**	**0.02**
HS-SPME-GC-ECD / blood[(o)]	**3-5****	**0.05-0.44**
SteamD-LLE-Benzoyl-GC-ECD / milk[(p)]	**2-5**	**0.1-1.0**
HS-SPME-GC-ECD / milk[(q)]	**3-5****	**0.56-1.01**

*Number of Cl atoms in the analytes; **Other compounds also analyzed; [(a)]Kontsas et al., 1995; [(b)]Hill et al., 1995a; [(c)]Kumagai and Matsunaga, 1997; [(d)]Wada et al., 1999; [(e)]Kumagai and Matsunaga, 1994; [(f)]Crespín et al., 2002; [(g)]Bartels et al., 1999; [(h)]Guidotti et al., 1999; [(i)]Lee et al., 1998; [(j)]Hsiao et al., 2009; [(k)]Kawaguchi et al., 2005; [(l)]Ito et al., 2008; [(m)]Norberg et al., 1997; [(n)]Zhou et al., 2007; [(o)]Röhrig et al., 1998; [(p)]Veningerová et al., 1996; [(q)]Röhrig and Meisch, 2000

The liquid-liquid extraction (LLE) is not currently preferred due to the use of large quantities of organic solvents and tedious, time-consuming procedures with relatively high LODs (Table 1). Lower LODs have been provided (Hill et al., 1995a) when LLE was combined with back extraction (BackE) and the CPs were derivatized by chloropropylation (ClPropyl).

Styrene-divinylbenzene solid phase extraction (SPE) cartridges were used to preconcentrate CPs from acidified urine samples (Crespín et al., 2002) or from the distillate after the removal of the CPs from the matrix by steam distillation (SteamD) (Bartels et al., 1999). Usually, preconcentration by SPE leads to much lower LODs (compared with LLE-based methods) but small quantities of organic solvents are still required. In another method, the SteamD was combined with LLE to extract the CPs from breast milk samples (Veningerová et al., 1996). After derivatization, the pentafluorobenzoyl derivatives (Benzoyl-CPs) were analyzed by GC-ECD obtaining LODs from 0.1 to 1 $\mu g\ L^{-1}$.

Microextraction methods have been mainly applied to determine CPs in liquid samples since they provide the lowest LODs, usually in the order of $ng\ L^{-1}$ to $\mu g\ L^{-1}$ (Table 1). Owing to the low surface tension of the biological liquid samples, leading to drop instability, the use of liquid phase microextraction (LPME) is limited to the hollow fiber (HF) technique. Ito et al., 2008 extracted acetylated derivatives of CPs (Acetyl-CPs) from urine samples by using HF-LPME and analyzed them by GC-MS. Urine samples were also analyzed without derivatization by direct solid phase microextraction (SPME) coupled to GC-MS (Lee et al., 1998) or with headspace (HS) SPME-GC-ECD (Hsiao et al., 2009). The latter method has been also used for analysis of blood (Röhrig et al., 1998) and milk samples (Röhrig and Meisch, 2000). In another method (Kawaguchi et al., 2005), the Acetyl-CPs in urine samples were determined by stir-bar sorptive extraction (SBSE) coupled to thermal desorption (TD) GC-MS with cryogenic trapping (CT). The matrix effects were overcome using isotope-labeled surrogate standards.

3. Conclusion

Not all analytical methods used to determine CPs in liquid biological samples have sufficiently low LODs to study the behaviour of CPs and other chlorinated organic compounds in individuals non-exposed professionally to CPs. The choice of the detector and, sometimes, the application of derivatization procedure seem to be important for the development of sufficiently sensitive analytical methods. Microextraction techniques are preferable, because they are either solvent-free or require much lower amounts of organic solvents. Furthermore, they are very sensitive and require a small sample volume, which is very important in the analysis of liquid biological samples. The potential use

of CPs as biomarkers of exposure to different pesticides is still a field that needs research.

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