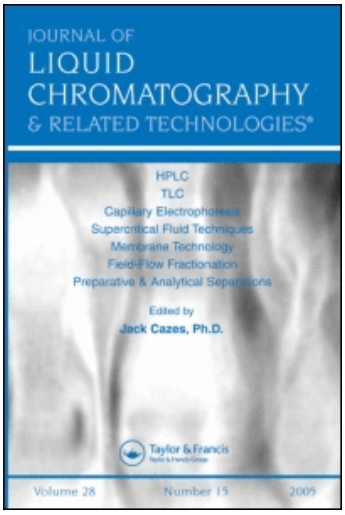

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Reig, F. Bosch , Falcó, P. Campíns and Andrés, J. Verdü(1995) 'Preconcentration, Sample Clean-Up, and HPLC Determination of Phenol and its Chloro, Methyl, and Nitro Derivatives in Biological Samples. A Review', Journal of Liquid Chromatography & Related Technologies, 18: 11, 2229 — 2241
To link to this Article: DOI: 10.1080/10826079508010267
URL: http://dx.doi.org/10.1080/10826079508010267

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(11), 2229-2241 (1995)

PRECONCENTRATION, SAMPLE CLEAN-UP, AND HPLC DETERMINATION OF PHENOL AND ITS CHLORO, METHYL, AND NITRO DERIVATIVES IN BIOLOGICAL SAMPLES. A REVIEW

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ABSTRACT

This critical review shows the different high performance liquid chromatography methods proposed for phenol and its chloro, methyl and nitroderivatives determination in biological samples, with special attention to the sample treatment.

INTRODUCTION

Phenol denatures and precipitates cellular proteins, and thus may rapidly cause poisoning [1]. It is metabolized by conjugation to yield phenyl glucuronide and phenyl sulphate [2]. In addition, phenol (and *p*-cresol and 4-ethylphenol) is produced in the gut by microbial degradation of tyrosine [3]. So, for urine samples obtained from healthy, unexposed men, concentration values from 4.5 to 10 ppm were obtained [4]. Acid-labile *phenol conjugates* are present endogenously in serum at concentrations of about 0.1 μg/mL. Niwa et *al* [5] quantified serum phenol and *p*-cresol in uremic patients on hemodialysis. Concentrations of phenol and *p*-cresol in uremic serum were significantly higher than those in normal serum. Reduction rates of phenol and *p*-cresol by hemodialysis were lower than those of urea and creatinine, suggesting a protein-binding property of phenol and *p*-cresol. Phenol and its derivatives are among the most toxic and widely spread pollutants in industrial

effluent and natural waters. Due to their toxicity, measurement in biological materials (expired air, blood, serum and urine) would be of interest. In addition, they are also metabolites of other toxic compounds (*e.g.* benzene), and their measurement would indicate exposure. An excess of respiratory cancer has been associated with phenol in Finnish woodworkers [6]. Moreover, phenol is a metabolite of benzene. Benzene is considered as a group I carcinogen [7]. It is also a constituent of engine emissions and tobacco smoke. The most commonly used method for biological monitoring of benzene exposure is measurement of phenol in urine. In the body, benzene is metabolized by the microsomal cytochrome P-450 monooxygenase system into benzene epoxide [8]. This benzene epoxide is metabolized in three different pathways which end in excretion of *trans,trans*-muconic acid, phenylmercapturic acid and different phenols. By using enzymes it is possible to differentiate between conjugated (glucuronides and sulphate esters) and unconjugated metabolites. About 30% of retained benzene gives rise to phenol *in vivo*. Therefore, the measurement of excreted urinary phenol has applications in the evaluation of exposure [9], but urinary phenolic compounds are not useful biological markers for exposure to benzene below 1 ppm. No significant increase in phenolic compounds was observed after exposure to benzene (0.01-0.63 ppm) for both smoking and non smoking workers [10].

For cresols, only *p*-cresol is endogenously produced in normal subjects, and may be present in urine at concentrations of 20 to 200 μg/mL (mainly in conjugated form) [1]. *o*- and *m*-cresol are reported to occur in the urine as a result of exposure of an individual to toluene [11] and not as a result of the microbial degradation of tyrosine. Consequently, the occurrence of *o*- and *m*-cresol would be expected to be rare, except in those individuals subjected to toluene exposure. However, only a small fraction of the inhaled vapour is oxidized at the aromatic ring with the production of cresols.

The urinary concentration of *p*-nitrophenol has been proposed for assessing the exposure to nitrobenzene [12], which is used in many industrial processes. Skin is the principal route of exposure to nitrobenzene solution [13].

The respiratory and dermal absorption of chlorophenols results in measurable levels of these compounds in blood and urine of occupationally exposed individuals [14]. Food and drinking water are considered to be the major sources of chlorophenols in serum, urine and adipose tissues of general population. Pentachlorophenol concentration of 10 μg/Kg in food

and of 60 μg/Kg in drinking water have been reported [15]. This can be due to an average daily intake of 1-6 μg pentachlorophenol. A large proportion of pentachlorophenol is excreted in urine, which leads to average levels of 1-6 ppb [16]. The presence of pentachlorophenol in urine has been shown to be a sensitive indicator of human exposure not only to those but also to other organochlorine compounds being metabolized to chlorophenols, *e.g.* hexachlorocyclohexane and chlorobenzenes. Once adsorbed in the body, a significant amount of the chlorophenols may be retained due to the formation of lipophilic conjugates of fatty acids [17]. An accumulation of such conjugates in membranes of tissues and their further metabolism may have deleterious consequences. Selective toxicity of palmitoylpentachlorophenol was found in exocrine pancreas in rats [18]. In urine samples from people working in the wood industry, concentrations of 0.1-10 ppm of pentachlorophenol have been found [19]. About 25% of the pentachlorophenol present in urine is excreted as its glucuronide [20]. Therefore, in order to measure the total amount of pentachlorophenol in urine, hydrolysis is necessary before analysis. Acid hydrolysis has been used, but 70-95% of pentachlorophenol is present in the precipitate formed upon hydrolysis. 2,4-dichlorophenol has been also determined as a common metabolite of the β-blocker B 24/76 [21].

The United States Environmental Protection Agency list eleven substituted phenols, with a variety of substituent such as chloro, methyl and nitro groups, as priority pollutants phenols (PPP) [22], as shown in table I. Analytical procedures are needed to detect and quantify phenolic compounds at very low concentrations. The present study reviews the determination of phenol and its chloro, methyl and nitro derivatives by high performance liquid chromatography (HPLC) in biological samples. This technique is more suitable than gas chromatography for polar analytes with low vapour pressure (as in the case of phenols) in aqueous samples. Our objective is to consider the different HPLC methods proposed in the recent literature (especially referred to the eleven PPP) with attention to all the different kind of biological samples studied, and the preconcentration techniques used. A previous review of 1984 was centered around the determination of priority toxic pollutants in urine, where chlorinated phenols were considered [23]. As it can be seen in figure 1, about 20% of the published works in the last 10 years, as reviewed by Chemical Abstracts, about phenol and its derivatives are directly related with biological samples.

Table 1
The Eleven Priority Pollutants Phenols (PPP) Listed by the United States Environmental Protection Agency.

Phenol
2-chlorophenol
4-chloro-3-methylphenol
2,4-dichlorophenol
2,4-dimethylphenol
2,4-dinitrophenol
2-methyl-4,6-dinitrophenol
2-nitrophenol
4-nitrophenol
Pentachlorophenol
2,4,6-trichlorophenol

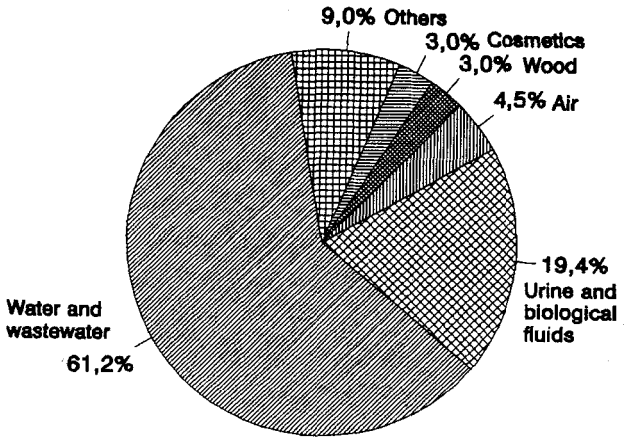

Figure 1 Distribution of the HPLC procedures for the analysis of phenols according to the samples assayed during the last decade.

SAMPLE TREATMENT

Preliminary treatment is often necessary to both separate and preconcentrate the analytes, although direct aqueous sample injection has been also used. Generally, hydrolysis and/or sample clean-up procedures are carried out prior to the chromatographic separation. Direct injection of enzymatic hydrolysed urine samples have been used [24]. After acetonitrile deproteinization, plasma phenol was determined [25]. Diluted urine samples after filtration and hydrolysis were injected [26], and Ogata et *al.* [27] diluted the urine sample and after centrifugation injected the supernatant. Although for phenol determination, enzymatic hydrolysis (Helix Pomatia) was necessary. Automatic HPLC methods, not involving solvent extraction, described [28]. Table 2 summarizes the different methods.

Steam distillation.

The quantitative performance of steam distillation-extraction for different types of organic substances at concentrations ranging from tens of ppm to ppb has been studied in [29]. A theoretical model is introduced, describing the recovery of different classes of organic compounds as a function of the process time. A 100% recovery is obtained within 20 min. for most substances. The effect of concentration, process time and solvent used were also studied. Norwitz et *al.* [30] studied the distillation of 42 phenolics, and the recoveries are related with the hydrogen-bondings (inter and intra molecular) present. As applications, a small steam-distillation scheme for concentrating phenol, *p*-cresol and 4-ethylphenol from faeces and urine samples was described [31]. Recoveries from 99.4 to 101.9% were obtained for the three analytes for added concentrations ranged 0.1-1000 nmol/g wet weight sample. For urine samples it was found that acid hydrolysis did not result in the loss of free phenols, and the recoveries were 100.1-101.7%. Steam distillation has been also used for the determination of phenol and cresols in urine [32]. After enzymic hydrolysis of urine (β-glucuronidase), a 1 mL aliquot was submitted to steam-distillation in a micro-Kjendahl apparatus for phenol and *p*-cresol extraction [33]. Recoveries were 95 to 107%.

Liquid-liquid extraction

The extraction of 25 monohydric alkylphenols with different solvents (*n*-pentane, tetrachlorometane, trichlorometane, dichlorometane, benzene and diethyl ether) from water

Table 2
Analytical Properties of the Different Methods Proposed for the Analysis of Phenols by HPLC

Analyte	Sample	Hydrolysis	Sample clean-up	Stationary phase	Mobile phase	Detector	Detection limit	Notes	Reference
26DCP PCP 235TCP 2345TeCP	Urine	HCl 1 h. 100°C	on-line trace enrichment on a styrene-divinylbenzene polymer	LiChrosorb Si-60 (5μm)	*n*-hexane-toluene-glacial acetic acid (74:25:1)	ECD	1 ppb of PCP	0.4 mL sample Normal phase LC	[41]
PCP 2346TeCP	Urine	HCl 1 h. 100°C	on-line post-column extraction (LiChrosorb RP18)	LiChrosorb RP18 (5 μm) Spherisorb C1 (5μm)	MeOH-1 mM H_3PO_4 (80:20)	UV ECD	40 ppb of PCP (UV) 1 ppb of PCP (ECD)	2.3 mL of sample 2,3,4,6-TeCP not found	[15]
Phenol 4-nitrophenol	Urine	Enzymic (Helix Pomatia Juice) 37°C Overnight(6 h.)	Filtered (0.22 μm)	ODS-2 (5 μm)	MeCN-1% H_3PO_4 10:90 (Screening) 30:70 (simultaneous specific determination of phenol and 4-nitrophenol)	UV (265, 280 nm)		Other compounds assayed	[26]
Phenol	Mice urine	Enzymic (β-glucuronidase and/or arylsulphatase) 37°C 48 h.	Anion exchanger and diethyl ether extraction	Nucleosil ODS (5 μm)	Phosphate (pH=3.4)-MeOH (70:30)	UV (270 nm)	36 mg/L	Other compounds assayed	[8]
Phenol *p*-cresol 4-ethylphenol	Faeces and urine	H_2SO_4 100°C 30 min.	Steam distillation	RP-8 (5 μm)	H_2O-MeCN-HAc (89:9:1)	Fluorescence λ_{exc} = 275 λ_{ems} = 300	<0.1 pg/μL		[31]

Phenol	Urine	HCl 90°C 90 min.	Diethylether extraction after sodium sulphate addition	Partisphere 5 C18 (5 μm)	10 mM sodium acetate buffer (pH = 3.4 or 3.8)-MeCN Gradient	Fluorescence λ_{exc} = 274 λ_{ems} = 298 UV (280 nm.)	0.3 mg/L	Other compounds assayed	[10]
Phenol *o*-,*m*-,*p*-cresol *p*-nitrophenol	Urine	Enzymic (β-glucuronidase-arylsulphatase) 37°C 12 h.	CH_2Cl_2 extraction and sodium hydroxide back extraction	Pecosphere 3x3 C18 (3 μm)	MeOH-H_2O-H_3PO_4 (30:70:0.1)	UV (215 nm)	0.5 mg/L (phenol and cresols) 1 mg/L (*p*-nitrophenol)	*p*-aminophenol also assayed	[9]
Phenol *p*-cresol	Urine	Enzymic (β-glucuronidase) 37°C 20-24 h.	Steam-distillation	RSil C18LL	MeOH-20mM NaH_2PO_4 (pH=4) (12:13)	UV (285 and 295 nm)		Phenol concentration in some urine samples decreased during storage	[33]
Fatty acid (C_{16} and C_{18}) conjugates of 24DCP, 245 and 246TCP, 2346TeCP and PCP				Reversed phase C18 (5 μm)	MeOH-H_2O (39:1)	UV (210 nm)			[17]
Phenol 4-nitrophenol				LC-SAX (5 μm)	0.05 M ammonium formate buffer (pH=4.5)-MeCN (60:40)	UV (254)	0.9 ng/μL	Glucuronide and sulfate conjugates Anion exchange LC	[2]

at mg/L concentration was compared [34]. The recoveries were obtained relative to *n*-octadecane, being the lowest for phenol and its lower alkyl derivatives. A simple extraction by ethyl acetate was used to measure phenol and *p*-cresol in uremic serum [5]. *p*-nitrophenol was extracted from urine samples with dichloromethane, after hydrolysis with hydrochloric acid [35]. Enzymatic hydrolysis of phenol, cresols and *p*-nitrophenol with β-glucuronidase-arylsulphatase for 12 h. at 37°C was made [9]. After hydrolysis, the analytes were extracted with dichloromethane, and then the organic phase was extracted with 0.2 M sodium hydroxide before injection. The recovery was about 95% for phenol and cresols and 90% for *p*-nitrophenol. Urine samples, after acid hydrolysis, were saturated with sodium sulphate and extracted by diethyl ether. The recovery for phenol was over 90% [10]. n-hexane extraction was used in [14]. The recoveries ranged from 79 (2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol and 2,3,4,5-tetrachlorophenol) to 87% (2,4-dichlorophenol) when 1 mL of sample was used, and from 72 (pentachlorophenol) to 90% (2,4-dichlorophenol, 2,4,6-trichlorophenol and 2,4,5-trichlorophenol) when 5 mL of sample was used. Ethyl ether was used for determination of pentachlorophenol in human urine [36]. Mice urine hydrolysed with β-D-glucuronidase was extracted with ethyl ether for unconjugated metabolites of benzene, including phenol [38].

Solid-liquid extraction

A global study of some chemically modified resins for phenolic extraction has been made in [37, 39]. C_{18} reversed phase extraction used Sep-Pak C_{18} cartridges [14]. The recoveries ranged from 59 (2,3,4,5-tetrachlorophenol) to 89% (pentachlorophenol) when 1 mL of sample was used, and from 69 (2,4,6-trichlorophenol) to 96% (pentachlorophenol) when 5 mL of sample was used. Pentachlorophenol in urine was extracted in a Baker-1 C18 extraction column and eluted with the mobile phase used in the HPLC separation. The recovery was 89 to 96% [40]. 4 mL of the hydrolysed mice urine (with β-glucuronidase and/or arylsulphatase) was purified and separated by solid-phase extraction with an anion exchanger, followed by extraction with diethyl ether [8].

Column switching

On-line post column liquid extraction was used, before electron-capture detection [15]. After hydrolysis of urine samples, the preconcentration was made. Recovery for

pentachlorophenol at 10 ppb level was 70% and at 100 ppb level was 75%. For 2,3,4,6-tetrachlorophenol, the recovery was 85%. Same authors, after hydrolysis of the urine, made on-line trace enrichment of 100 ppb of 2,6-dichlorophenol, and 10 ppb of 2,3,5-trichlorophenol, 2,3,4,5-tetrachlorophenol and pentachlorophenol using a pre-column packed with a styrene-divinylbenzene copolymer. For the preconcentration of 0.4 mL of sample, recoveries of the chlorophenols at the 10 ppb levels varied from 60 to 80% [41].

DETECTION

Figure 2 summarized the detectors usually employed in the HPLC phenol determination. When biological samples are studied, electrochemistry detectors are used less frequently.

UV detector is the most employed because of its universality. Phenol and chlorinated phenols show absorbance maxima at 220 and 250/320 nm. Direct determination of phenol and chlorophenols is possible if the preconcentration step leads to a higher concentration level. Nitrophenols can be detected in the visible spectra (405 nm.). PCP was determined by UV detection (254 and 313 nm.), with detection limit of 0.4 mg/L [36]. Absorbance at 270nm. was used for determination of benzene metabolites in urine of mice. The detection limit for phenol was 36 mg/L [8]. A wavelength of 210 nm. was used for monitoring fatty acid conjugates of chlorinated phenols [17], and of 215 nm. was used for the determination of phenol, *o*-, *m*- and *p*-cresol, *p*-aminophenol and *p*-nitrophenol in urine [9]. The detections limit were 0.5 mg/l for phenol and cresols and 1 mg/L for the others. 265 and 280 nm. were used for determination of urinary metabolites of benzene, nitrobenzene, toluene, xylene and styrene [26]. 254 nm. has been used with a detection limit of 0.25 mg/L for pentachlorophenol [40], and for phenol determination [38]. 225 nm. for phenol determination [27], with a detection limit of 0.4 ng/μL. 285 nm. for phenol and 295 nm. for *p*-cresol in [33].

The fluorescence detector provides better detection limits than UV detector (table 2). Only phenol and cresols show native fluorescence. Dansyl chloride is used to convert phenols into fluorescent dansyl derivatives. A complete study of a two-phase dansylation procedure for phenolic compounds is described in [42], but we have not found any work which

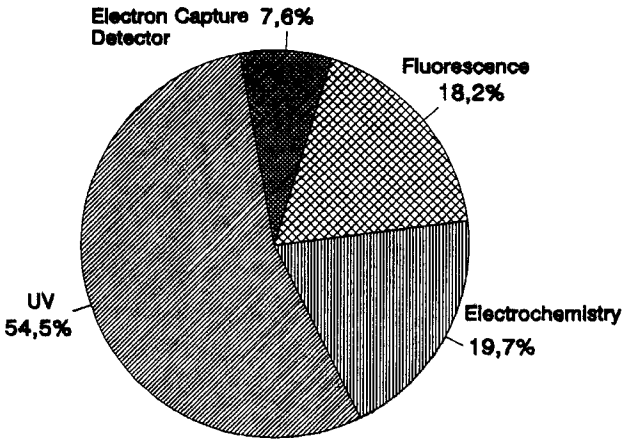

Figure 2 Distribution of the HPLC procedures for the analysis of phenols according to the detection system used during the last decade.

describes an application to biological samples. Fluorescence detection for identification of phenol and *p*-cresol was used [5]. A λ_{exc} = 274 nm. and λ_{ems} = 298 nm. were used, with a detection limit for phenol of 0.3 mg/L [10], and λ_{exc} = 275 nm. and λ_{ems} = 300 nm. for monitoring phenol, *p*-cresol and 4-ethylphenol. The sensibility was of 10 pg. (in 100 μL) [31].

The only described work with electrochemistry detector in biological samples showed electrochemistry detector 30 times more sensitive than UV detection for phenol determination in plasma [25].

Experimental modifications have to be made in order to use electron capture detector (ECD) in liquid chromatography. On-line post-column extraction in column liquid chromatography with a coupled ECD is proposed [15]. Detection limit of 1 ppb for PCP in urine was achieved, when 40 ppb was the detection limit with UV detection. On-line trace enrichment on a reversed phase pre-column for normal phase LC with ECD is described in [41], for low ppb determination of chlorophenols in urine. On-line electron-capture detection was used for determination of ppb levels of PCP in liver [43].

The mass spectrometry has been only used for confirmating phenol and *p*-cresol [5]. The radiochemistry detector has been used for quantification of radiolabelled benzene

metabolites in liver tissue extract or urine of rats exposed to ^{3}H-benzene in air (47.6 ppm) for 6 hours. UV absorbance at 265 nm. was also registered [44].

CONCLUSIONS

A review has been made of the procedures proposed for determination of phenols in biological fluids. Hydrolysis and sample clean-up procedures, and also preconcentration, are generally required for successful determination (table 2). Three types of analytical columns are proposed: normal phase, reversed phase and anion exchange, the second type being most popular. The UV detector is the most used. However, it does not always provide good sensibility, and then other kind of detectors have been tested, as fluorescence and electron capture detectors. The major number of papers referred to urine samples, as can be seen in table 2, where ppb detection limits has been found.

ACKNOWLEDGEMENTS

The authors are grateful to the DGICYT (Project nº PB 91-0651) for financial support. Jorge Verdú Andrés acknowledges a grant from the Conselleria d'Educació i Ciència of the Generalitat Valenciana for carrying out PhD studies.

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Received: August 8, 1994
Accepted: January 24, 1995