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GAS CHROMATOGRAPHY OF SIMPLE PHENOLS IN BIOLOGICAL FLUIDS

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SUMMARY

Acid hydrolysis of phenol conjugates in urine by concentrated H_3PO_4 followed by extraction of phenols with *n*-hexane and their acetylation before gas chromatography on columns packed with OV-1 or OV-17 is described. The sensitivity of the method is sufficient to monitor normal levels of phenol and *p*-cresol or phenol and *o*-cresol after exposure to benzene or toluene vapours. The detection limit is 1 mg/l. The method can also be used to diagnose acute oral intoxication by phenol or cresols and to estimate its significance in clinical or forensic toxicology. Normal urine levels of phenols and those in different cases of human intoxication are evaluated.

INTRODUCTION

Determination of phenolic compounds in serum or urine is important in clinical chemistry, occupational medicine and clinical and forensic toxicology. Some simple phenols, such as phenol, *p*-cresol, *p*-ethylphenol, catechol and hydroquinone in free and conjugated forms, are commonly found in normal urine [1]. The presence of guajacol has also been described [2]. The variety and amount of phenols excreted into urine is quite individual and varies with nutrition, smoking habit, etc. Abnormal excretion of phenols may be caused by changes in intestinal flora caused by the intake of antibiotics, gastrointestinal disorders and liver and kidney diseases [3–5]. The levels of catechol and guajacol are much lower than those of phenol and *p*-cresol [2,3,5].

The occurrence of *o*-cresol and *m*-cresol in urine is considered to be of exogeneous origin [3,5]. After exposure to benzene or toluene, the normal levels

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of phenol and *p*-cresol are increased. The excreted amount of *o*-cresol correlated well with the extent of exposure to toluene [6], but the level of *p*-cresol was ca. 200 times higher than those of *meta*- and *ortho*-isomers [7].

Even if oral ingestion of phenol or cresols (soap solutions called Lysol) is rare, a diagnostic method should be available in the emergency toxicological laboratory. A method based on gas chromatography (GC) with packed columns of universal use (OV-1 or OV-17) appears to be most convenient.

The direct GC determination of phenols can cause problems with reproducibility owing to their polarity and volatility. A special packing material for acid compounds is necessary [8], unless derivatization is used. Further, phenols in biological material must be released from conjugates and isolated [8–12]. There is as yet no generally accepted opinion on the reliability of total hydrolysis of phenol conjugates. Phenol sulphate is considered to be more susceptible to hydrolysis than glucuronide [1,13]. Enzymic hydrolysis is too lengthy for an emergency toxicological method, and hydrolysis by concentrated HCl takes 90 min [11]. However, some authors [4] suspected that hydrolysis of glucuronides was minimal. Surprisingly, Needham et al. [8] hydrolysed phenol conjugates by the action of concentrated H_2SO_4 alone for 30 min at ambient temperature. Šedivec and Flek [12] compared the efficiency of hydrolysis by concentrated H_2SO_4 and H_3PO_4 at 100°C. Somewhat better results were achieved by boiling with H_3PO_4.

In this report we describe the procedure of acid hydrolysis by concentrated H_3PO_4 [12] followed by extraction of phenols with *n*-hexane and their acetylation before GC. Normal levels of phenols in urine and those in various instances of human intoxications are evaluated.

EXPERIMENTAL

Chemicals

n-Hexane, analytical grade, was from Park (Northampton, U.K.). Analytical-grade H_3PO_4, K_2CO_3 and acetic acid anhydride were from Lachema (Brno, Czechoslovakia). 2-Chlorophenol was supplied by Janssen Chimica (Beerse, Belgium), analytical-grade phenol, *o*-cresol, *p*-cresol and catechol by Lachema and analytical-grade *m*-cresol and guajacol by Fluka (Buchs, Switzerland). The GC stationary phase was 3% SP 2250 Supelcoport, 80–100 mesh, from Supelco (Gland, Switzerland).

Stock solutions

The internal standard solution was 2-chlorophenol (5 g/l in distilled water). A standard mixture of phenol, *o*- and *p*-cresol (1 g/l each) and a standard solution of *m*-cresol (1 g/l) were prepared with distilled water.

Instrumentation

The gas chromatograph, CHROM 61 (Laboratorní Přistroje Prague, Czechoslovakia), was equipped with a flame ionization detector and a silanized glass column (1.95 m×2 mm I.D.). The output of the detector electrometer was connected to the TZ 4601 chart recorder (10 mV full scale).

The nitrogen carrier gas flow-rate was 20 ml/min, that of hydrogen 18 ml/min and that of air 400 ml/min. The injection and detector port temperatures were 140°C, and the column was maintained at 110°C.

Procedures

A 2-ml volume of standard solution (for calibration) or 2 ml of urine or serum, with the addition of 0.1 ml of the internal standard solution and 2 ml of concentrated H_3PO_4, were boiled in ground-glass stoppered glass tubes in a water-bath for 30 min. After cooling, the hydrolysate was extracted with 6 ml of *n*-hexane for 10 min, and 3 ml of the hexane layer were transferred to another tube and briefly shaken with 2 ml of aqueous 0.1 *M* K_2CO_3. Then 1 ml of the alkaline layer was taken for acetylation with 10 μl of acetic anhydride and immediately mixed with 0.5 ml of *n*-hexane. The mixture was shaken for 20 min at ambient temperature, and 1 μl of the upper hexane layer containing acetyl esters of phenols was injected into the gas chromatograph.

RESULTS

Acetylation of phenols

The acetylation of simple phenols in water samples was previously reported [14]. We tried to adapt this procedure to the limited amounts of biological material in which phenols can be conjugated.

As expected, the pH value of the reaction mixture affected the reproducibility of acetylation of phenols (Table I). Aqueous 0.1 *M* K_2CO_3 was optimal for stability of the derivatives and completeness of the reaction. The acetyl deriv-

TABLE I

REPRODUCIBILITY OF ACETYLATION OF *m*-CRESOL

m-Cresol (200 μg) was mixed with 2-chlorophenol (100 μg) in solutions of K_2CO_3 of different concentrations.

Concentration of K_2CO_3 (*M*)	Peak-height ratio of *m*-cresol to 2-chlorophenol (mean ± S.D., $n=5$)
0.1	3.727 ± 0.016
0.2	3.751 ± 0.070
0.5	5.681 ± 0.560

TABLE II

EXTRACTION RECOVERY OF PHENOLS FROM ACIDIFIED WATER INTO *n*-HEXANE

Phenol	Recovery (%)	
	50 mg/l ($n=3$)	500 mg/l ($n=3$)
Phenol	49.1	45.6
o-Cresol	69.4	59.9
p-Cresol	73.3	65.8

TABLE III

REPRODUCIBILITY OF DETERMINATION OF *p*-CRESOL IN HYDROLYSED NORMAL URINE

Internal standard, 2-chlorophenol (250 μg/ml), was boiled with concentrated H_3PO_4.

Time of hydrolysis (min)	*n*	Peak-height ratio (mean ± S.D.)
30	9	0.681 ± 0.022
60	3	0.670

TABLE IV

RELATIVE RETENTION TIMES OF PHENOLIC ACETYL ESTERS

Stationary phase, 3% SP 2250 Supelcoport, 80–100 mesh; retention time of internal standard, 2-chlorophenol, 7.5 min.

Compound	Relative retention time
Phenol	0.42
o-Cresol	0.63
m-Cresol	0.73
p-Cresol	0.77
2-Chlorophenol	1.00
Guajacol	1.77
Catechol	3.62

atives were stable for at least 24 h without observable changes. After 48 h the decrease in phenol and cresol derivatives was more evident than that of 2-chlorophenol.

A greater excess of acetic anhydride (50 μl per ml of 0.1 *M* K_2CO_3) provided a more acid medium for acetylation and unfavourably shifted the equilibrium of the reaction. Free phenol and cresols then remained in the reaction mixture.

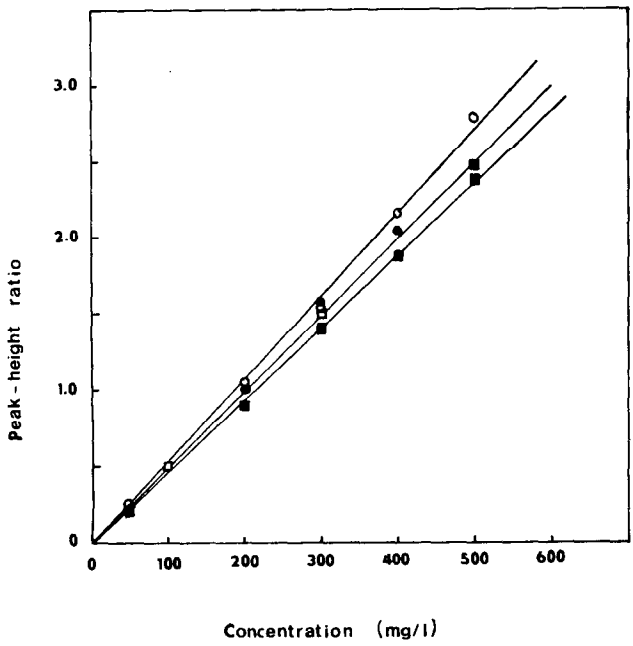

Fig. 1. Linearity of calibration of phenol and cresols as acetyl esters. Internal standard, 2-chlorophenol (250 μg/ml). For GC conditions see text. (○) Phenol; (●) *o*-cresol; (□) *m*-cresol; (■) *p*-cresol.

Extraction recovery

Different solvents have been compared according to how efficiently they extract phenols from acidified water, and also according to their suitability as a medium for acetylation. The extraction with *n*-hexane, benzene and cyclohexane gave similar results. The efficiency was lower with diethyl ether, diisopropyl ether and carbon disulphide. Problems with acetylation arose when ethyl acetate was used, perhaps due to the content of acetic acid.

The efficiency of the extraction of phenol and cresols by *n*-hexane was calculated by comparing the analytical results obtained by the procedure given in Experimental (except that the internal standard was dissolved in 0.1 *M* K_2CO_3) with the results achieved by acetylation of appropriate standards dissolved directly in 0.1 *M* K_2CO_3. The measurement was performed three times (Table II). The recovery of phenol and cresols was lower than that of 2-chlorophenol. There was little difference in recovery if the internal standard was added to the analysed sample before extraction with *n*-hexane. This implies that the recovery of 2-chlorophenol is practically complete.

Acid hydrolysis and overall reproducibility

The alkaline hydrolysis of urine to prevent loss of chlorinated phenols [5,6] is not efficient in the case of cresols, so acid hydrolysis must be used. Boiling

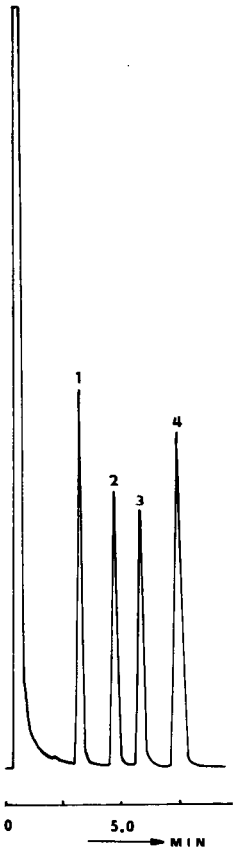

Fig. 2. GC of acetyl esters of phenolic standards (200 mg/l) extracted from acidified water into *n*-hexane. Injection volume, 1 μl; AT, 640/10 mV. Peaks: 1=phenol; 2=*o*-cresol; 3=*p*-cresol; 4=2-chlorophenol.

under reflux was not safe, but boiling in stoppered or tightly sealed glass tubes gave acceptable results. Hydrolysis of conjugates with concentrated H_3PO_4 has been completed in 30 min (Table III). The efficiency of hydrolysis by boiling with concentrated H_2SO_4 seemed to be nearly the same. Further, we verified that action of this acid only at ambient temperature was not sufficient to complete hydrolysis, even the method was previously described [8].

GC of phenolic acetyl esters

The acetyl derivatives of phenols exhibited good chromatographic properties on a packed column with the phenylmethylsilicone stationary phase. Acetyl esters of *m*- and *p*-cresol are poorly separated, but for our purposes this was of no relevance (Table IV).

The temperatures of injector and detector were maintained at 140°C to give

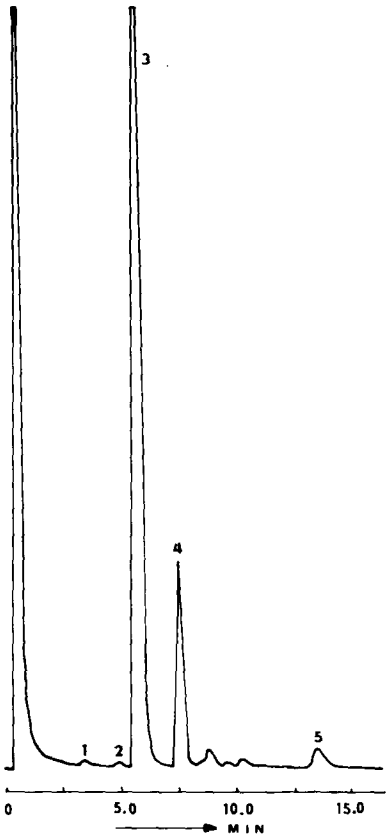

Fig. 3. GC of phenolic acetyl esters extracted from hydrolysed human urine 14 h after oral ingestion of Lysol. Peaks: 1 = phenol; 2 = o-cresol; 3 = *p*/*m*-cresol; 4 = 2-chlorophenol; 5 = guajacol.

the best reproducibility. The linearity of the calibration curve was verified in the range 50–500 mg/l (Fig. 1). Quantification of higher levels was performed after diluting the sample with distilled water. Quantification of lower levels was performed with more diluted internal standard. The concentrations were calculated according to the peak heights related to the internal standard 2-chlorophenol.

Fig. 2 presents a chromatogram of the extract of standards from aqueous solution. Fig. 3 is a chromatogram of hydrolysed human urine collected 14 h after oral ingestion of Lysol (the main component of which is *m*-cresol). Serum from this poisoned patient was diluted with distilled water (1:1) and handled in the same way as urine. The levels of phenol and cresols are included in Table V. In this case of heavy oral intoxication *o*-cresol and guajacol were found, and the total concentration of *m*- and *p*-cresol was significantly higher than normal values obtained by this method. The values for persons exposed to toluene

TABLE V

LEVELS OF SOME PHENOLS IN HUMAN URINE OR SERUM

Detection limit, 1 mg/l.

Sample	*n*	Concentration (mean ± S.D.) (mg/l)			
		Phenol	*o*-Cresol	*m*/*p*-Cresol	Guajacol
Normal urine of non-smokers	19	4.41 ± 4.05	Negative	46.78 ± 33.25	Negative
Normal urine of smokers	11	8.15 ± 11.07	Negative	108.91 ± 80.50	Negative
Urine after occupational exposure to toluene vapours	5	16.66 ± 9.02	8.5 ± 6.79	199.30 ± 61.49	Negative
Urine (A.K.) after toluene sniffing	1	Negative	17.79	131.85	Negative
Urine (V.F.) after toluene sniffing	1	4.06	2.68	76.32	Negative
Urine (K.M.) after ingestion of Lysol	1	2.50	5.30	950.50	Positive
Serum (K.M.) after ingestion of Lysol	1	2.00	Negative	72.00	Negative

vapours (occupationally or by abuse) are also included in Table V. The specific marker of the exposure to toluene, its metabolites *o*-cresol, was found in these cases.

CONCLUSION

The sensitivity of the method described is sufficient to monitor normal levels of phenol and *p*-cresol, or phenol and *o*-cresol, after exposure to benzene or toluene vapours. The method can be also used to diagnose acute oral intoxication by phenol or cresols and estimate its significance in clinical or forensic toxicology.

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