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AN IMPROVED METHOD FOR DETERMINING TOTAL URINARY PHENOL

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SUMMARY

A simple and easily manipulated method is described for the determination of total urinary phenol. This modification of earlier procedures incorporates the enzymatic hydrolysis of conjugated phenol, extraction of the hydrolysis products into isopropyl ether and final quantitation by gas–liquid chromatography–flame ionization detection. The method employs benzyl alcohol as internal standard.

INTRODUCTION

Prolonged inhalation of benzene at 100 ppm can cause toxic symptoms, while acute exposure due to ingestion or inhalation, can cause irritation of mucous membranes, restlessness, convulsions, excitement and depression. Death from respiratory failure may follow acute exposure. Chronic exposure can cause bone-marrow aplasia and, rarely, leukemia. The level of benzene exposure may be directly related to the level of conjugated phenol that is excreted by the kidneys. The mechanism of this relationship is that the liver oxidizes benzene to phenol and subsequently detoxifies the phenol by conjugation with either sulfate or glucuronide.

Exposure to benzene may not be the only source of urinary phenol. Several drugs commonly available, such as phenyl salicylate, zinc phenyl sulfonate (both in Pepto-Bismol), phenol itself (from throat soothing preparations) and phenobarbital, are metabolized by the liver and are excreted as conjugated phenol. Intestinal flora especially in patients with gastro-intestinal disorders may also contribute to the urinary phenol level through their degradation of tyrosine[1].

The quantitation of total urinary phenol has only been practical from a clinical standpoint for about fifty years. Early methods of urinary phenol analysis had been limited to colorimetric procedures which lacked specificity, could not guarantee the total hydrolysis of conjugated phenol or required precise manipulation and excessive time to complete. Although the early method of Theis and Benedict[2] using diazotized *p*-nitroaniline was straightforward, results were arbitrarily high since *p*-cresol, usually found in urine in higher concentrations than phenol itself, interfered with the determination. Later methods of Gibbs[3] and Walkley *et al.*[4] were either difficult to manipulate or may also have been plagued by *p*-cresol interference.

In 1965, Van Haaften and Sie[5] developed a gas chromatographic technique

for determining total urinary phenol. The method used an on-column phosphoric acid hydrolysis of the conjugated phenol. An internal standard was not used and chromatograms showed a relatively high background making quick chromatographic analysis difficult. Duran *et al.*[1] have described a gas–liquid chromatographic (GLC) technique using hydrochloric acid hydrolysis of conjugated phenol at 100° with *n*-valeric acid as internal standard. The method does not lend itself to simple manipulation and the authors indicate that *n*-valeric acid and crotonic acid are not separated by the technique. Crotonic acid can be formed from β-hydroxybutyric acid during acid hydrolysis. Fell and Lee[6] have also described a gas chromatographic method that will separate monohydric and dihydric phenols as acetate or trimethylsilyl (TMS) derivatives. The hydrolysis of the conjugated phenols was done enzymatically using a mixture of arylsulfatase and β-glucuronidase. The method of Fell and Lee will separate many phenolic compounds but, in that capacity, the assay may be too sensitive for use as a method for quantitating phenol alone. Early attempts in our laboratory to duplicate the perchloric acid hydrolysis method of Sherwood and Carter[7] yielded disappointing results. The method did not use an internal standard and the results showed poor replication. It was found that benzyl alcohol could be used as an internal standard with an improved standard deviation, but the method still gave disappointing results.

The method described here is a modification of the gas chromatographic (GC) technique of Van Haaften and Sie[5] as modified by Sherwood and Carter[7]. Along with benzyl alcohol as an internal standard an enzymatic hydrolysis technique was used. This technique was a modification of the procedure recommended by Endo Labs (Garden City, N.Y., U.S.A.)[8].

EXPERIMENTAL

Apparatus

The apparatus used for analysis was a Hewlett-Packard 7620A gas chromatograph equipped with a hydrogen flame ionization detector (FID) and interfaced with a Hewlett-Packard 3380A reporting integrator. Separation was effected on a borosilicate glass column (4 ft. × 4 mm I.D.) packed with 10% polyethylene glycol 6000 on Anakrom ABS (100–110 mesh). The column was conditioned overnight at 190° with a helium flow-rate of 15 ml/min. Final conditioning was achieved by multiple solvent injections on to the column under assay conditions. Chromatograms were run at an oven temperature of 175° with the injector at 210° and the FID at 250°. Helium was the carrier with a flow-rate of 35–40 ml/min. A sample volume of 5 μl was injected using a standard 10-μl Hamilton syringe or a 25-μl Hamilton syringe fitted with a 26-gauge needle.

Calculations

Results were calculated by determining the peak area ratio of the sample peak area to the internal standard peak area, comparing this ratio with an extracted four point standard curve and finally all results were normalized (as suggested by Fishbeck *et al.*[15]) to urine with a specific gravity of 1.024 by the following formula[4]:

$$\text{total corrected urinary phenol} = \text{mg\% phenol} \cdot \frac{24}{\text{digits}}$$

Digits in the formula referred to the last two digits of the sample's specific gravity. Twenty four referred to the last digits of the assumed normal urine specific gravity as determined by Levine and Fahy[14] and recommended by the Occupational Safety and Health Administration (O.S.H.A.)[9].

Procedures

Sample collection. Urine specimens were collected at random intervals from workers normally exposed to low levels of benzene and from those who were not exposed. Samples were frozen upon collection and maintained at —20° until they were thawed for analysis.

Extraction. The specific gravity of the urine samples was determined by refractometry and those of less than 1.010 or more than 1.030 were excluded from analysis. Phenol standards ranging from 0.5 mg% to 10 mg% were prepared in pooled urine. Volumes of 5 ml of standard urine blank, standards or samples were pipetted into 15-ml glass-stoppered centrifuge tubes to which was added 1.0 ml of pH 5.0, 2 *M* acetate buffer which contained 1 mg benzyl alcohol as internal standard and 0.1 mg amoxycillin as preservative. After thorough mixing, 50 μl of Glusulase (Endo Tabs: *ca.* 1500 β-glucuronidase units and 400 arylsulfatase units per ml of urine) were added. The tubes were stoppered, thoroughly mixed and incubated in a water bath at 37° for 24 h. After cooling, 0.5 ml of concentrated HCl and 1.0 ml of diisopropyl ether were added. The stoppered tubes were slowly shaken on a reciprocating mechanical shaker for 20 min. Centrifugation separated the organic and aqueous phases. The diisopropyl ether layer was transferred to a clean set of 15-ml glass-stoppered centrifuge tubes, stoppered and kept at room temperature until it could be analyzed.

Thin layer chromatography (TLC). The presence of *p*-cresol in the urines was confirmed through a modification of the TLC procedure of Crump[10,11] using a modified extraction scheme in which the Glusulase hydrolysis technique was scaled-down five fold and the incubation conditions were left unchanged. After incubation, the samples were acidified and extracted into 2 ml of diisopropyl ether. The organic phase was then back extracted with 1 ml of 1 *N* NaOH. The alkaline phase was transferred to 40-ml screw-cap centrifuge tubes placed on ice and then reacted for 1 min with 1 ml of diazotized *o*-nitroaniline (0.75 ml 10% $NaNO_3$ + 25 ml 0.15% *o*-nitroaniline) which was kept at 4°. After adding 2 ml of 20% Na_2CO_3, the reaction mixture was extracted with 10 ml of diethyl ether. The ether phase was evaporated to dryness in a 10 × 75 mm tube and the residue was dissolved in 100 μl of chloroform. 5-μl aliquots were spotted on silica gel plates slurried from 0.5 *N* NaOH. The chromatogram was developed in an equilibrated chromatography tank using chloroform–acetone (9:1).

RESULTS

Three prominent peaks were separated by the GC technique used in this study (Fig. 1). The first was the internal standard, benzyl alcohol, which was given a relative retention time (t_R) of 1.00. The second was the phenol peak with an t_R of 1.55. The third peak was *p*-cresol having an t_R of 2.05. Under normal assay conditions, phenol had an absolute retention time of *ca.* 8 min.

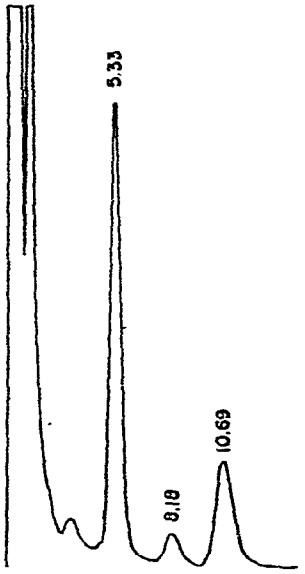

Fig. 1. Gas–liquid chromatogram of an extracted human urine sample (see text). Conditions: 10% polyethylene glycol 6000 on Anakrom ABS (100–110 mesh) packed in a 4 ft. × 4 mm borosilicate glass column; injection port, 210°; column oven, 175°; FID, 250°; helium carrier flow-rate, 35 ml/min (5-μl samples).

Phenol was used as the assay standard because phenyl sulfate and phenyl glucuronide were not commercially available. The complexity of their chemical synthesis and the impossibility of predicting the ratio of sulfate to glucuronide for use as a standard also mitigated for the choice of phenol as standard. In order to show that the procedure was measuring end-point values for the enzymatic hydrolysis of conjugated phenol, the time course for the hydrolysis was studied using urines from workers exposed to benzene. Appropriate phenol standards in pooled urine were also carried through the time course to demonstrate the stability of phenol under assay conditions, as well as *p*-cresol release from the standard pooled urine. The *p*-cresol release was monitored in these experiments as a further indication of complete hydrolysis. The results of two time course experiments shown in Figs. 2 and 3 indicate that the enzymatic hydrolysis of both the conjugated phenol and the conjugated *p*-cresol was complete in 20–24 h. These figures also show that even the hydrolysis of an abnormally high urinary phenol sample is complete in the period stated and that the hydrolysis of the sample *p*-cresol was also complete at the same point. In Fig. 4, *p*-cresol data are presented as peak area ratios (PARs) since *p*-cresol standards were not included. During the same time course experiments, no variation in the net PARs of the standards was noted although, as expected, the PAR for the *p*-cresol in the standard pooled urine increased and plateaued in 20–24 h.

It was found that 0.5 mg% phenol was the convenient limit of sensitivity for the procedure. Reported analysis of a 0.5 mg% standard gave a 4% coefficient of variation for two standard deviations. Concentrations of a less than 0.5 mg% could be determined with a somewhat higher coefficient of variation. Repeated analysis of a random urine specimen from a worker exposed to benzene gave a 7% coefficient

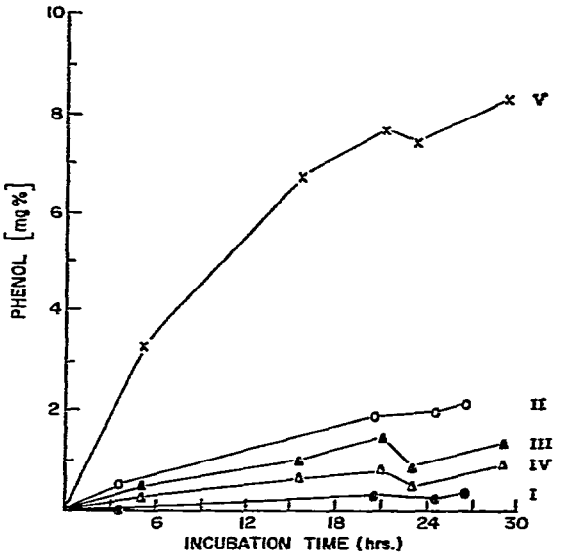

Fig. 2. Plot of phenol peak area ratios *versus* incubation time (h) for five human samples (I–V). 5 ml of urine sample was combined with 1.0 ml of 2 *M* acetate buffer, pH 5.0, containing 1.0 mg benzyl alcohol and 0.1 mg amoxycillin. Samples were incubated at 37° for various periods of time, acidified and extracted with diisopropyl ether.

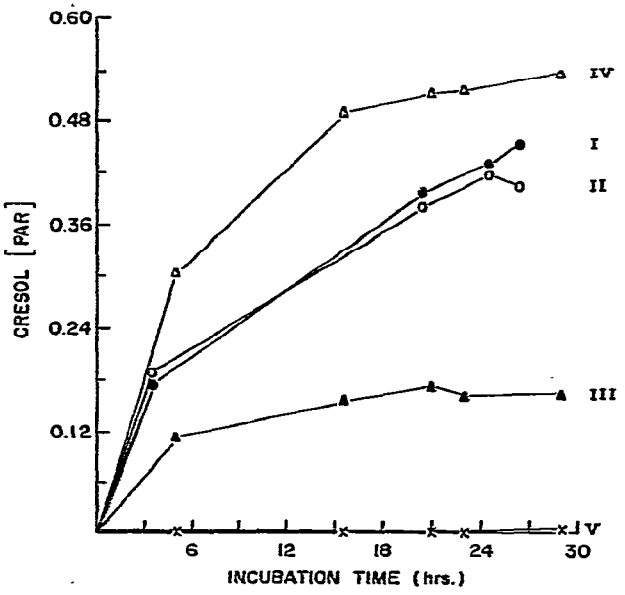

Fig. 3. Plot of *p*-cresol peak area ratios *versus* incubation time for five human samples (I–V). Samples and incubation conditions were identical to those in Fig. 2.

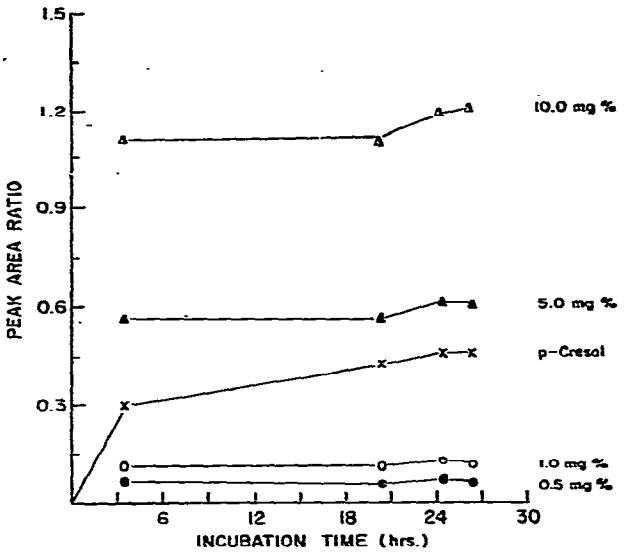

Fig. 4. Stability of added phenol under incubation conditions. Urine samples were spiked with known amounts of phenol. Extractions at various times demonstrate the stability of phenol to assay conditions. The gradual appearance of *p*-cresol results from hydrolysis of conjugated *p*-cresol normally present in urine. Incubation temperature was 37°.

of variation for the phenol results. The coefficients of variation for the *p*-cresol results for the 0.5 mg% sample and the random urine specimen were 3% and 2% respectively.

The plot of the extracted four point standard curve in Fig. 5 passed through zero and was linear over the assay range of 0.5 mg%–10 mg% having a normal coefficient of determination (COD) of 0.999. Earlier work demonstrated linearity out to 300 mg% with a similar COD.

Analysis of nearly 200 specimens composed of individuals exposed to low levels of benzene and individuals with no known exposure showed no statistical difference. The control group showed a mean phenol level of 1.0 mg% while the exposed group had a mean phenol level of 0.7 mg%. A Student's T comparison gave a p value of greater than 0.05 indicating a low probability of group difference. The mean corrected total urinary phenol concentration for 422 specimens was 0.8 mg% with a normal range from 0–3.5 mg% for two standard deviations. The mean specific gravity for some 480 urine specimens in our laboratory was 1.019.

DISCUSSION

The linearity of the standards even beyond the assay range and the sensitivity as well as the small standard deviation at 0.5 mg% are all indications that the results are reliable. Early results using the perchloric acid hydrolysis method of Sherwood and Carter gave coefficients of variation of 50–100% compared with the 4% as seen in this modified method. Additional sensitivity appears to be unnecessary because the total urinary phenol level is reported to be from 0.45 mg% to 1 mg% for indi-

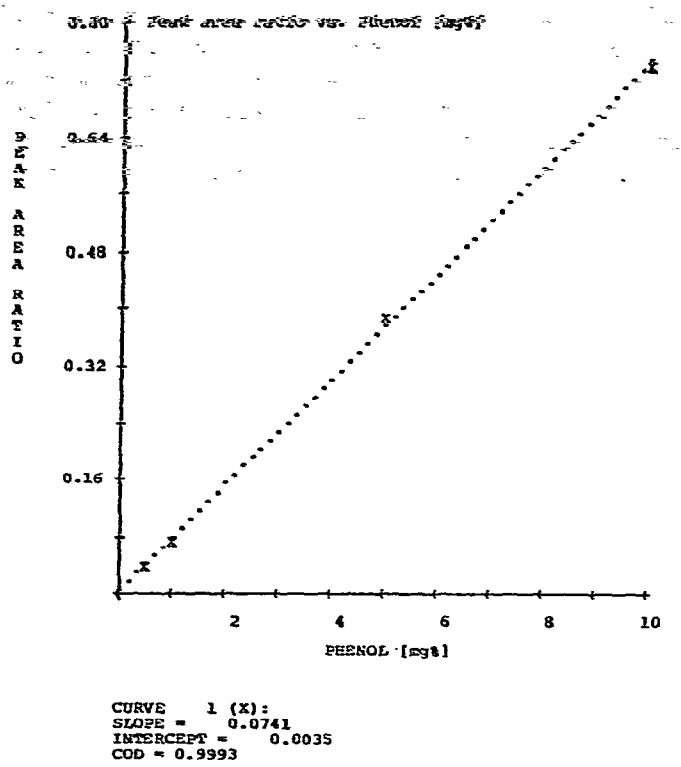

Fig. 5. Plot of peak area ratios *versus* standard phenol concentrations (mg%). GLC conditions were as indicated in the text.

viduals not exposed to benzene[5,7,8,12,13]. A phenol level of more than 1 mg% indicates benzene exposure and a limit of 20 mg% might be considered medically safe[5]. These levels of detection were easily attained with this procedure. Values reported by the other investigators mentioned above compare well with the mean of 0.8 mg% phenol generated in this study.

The time course studies showed that the hydrolysis of the conjugated phenols was completed in 20–24 h. Completion of the reaction was also implied by the apparent completion of the hydrolysis of *p*-cresol. Since no variation in the peak area ratios for the standards included during the time course was observed, stability of the benzyl alcohol (internal standard) and phenol could be inferred. Therefore, phenol and benzyl alcohol were considered the standard and internal standard of choice, respectively.

When data were analyzed to determine whether control samples contained statistically less phenol than those urines from workers exposed to low levels of benzene, no difference was demonstrated. The method, however, is certainly capable of easily detecting any phenol level that might fall outside of the normal range.

In the TLC procedure described, extracted urine samples showed phenol and *p*-cresol as the only detectable spots. Phenol had an R_F of 0.20 and *p*-cresol had an R_F of 0.74. These spots compared well with phenol and *p*-cresol standards.

o-Cresol and *m*-cresol had R_F values of 0.34 and 0.26, respectively. An injection of *p*-cresol into the gas chromatograph showed that it had an R_T identical with the peak that had been assumed to be *p*-cresol ($t_R = 2.05$) (Fig. 6). The presence of *p*-cresol in the urines was, therefore, demonstrated by two independent methods, GLC and TLC. Van Haaften and Sie[5] also mentioned that *p*-cresol was normally present in urine along with phenol while other phenolic compounds appear only in minute quantities.

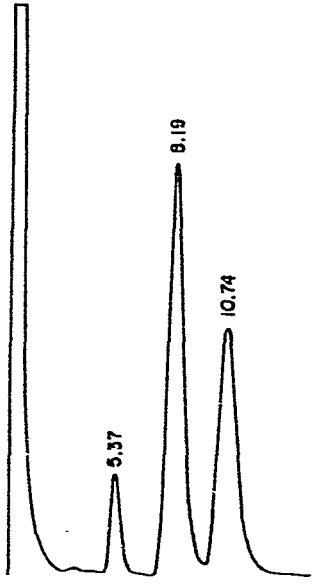

Fig. 6. Gas–liquid chromatogram of benzyl alcohol ($R_T = 5.37$), phenol ($R_T = 8.19$) and *p*-cresol ($R_T = 10.74$). Pure compounds were dissolved in diisopropyl ether and injected into the gas chromatograph. Instrumental conditions as indicated in Fig. 1.

Sherwood and Carter[7] reported a mean specific gravity of 1.018 and also indicated that their findings were essentially confirmed by other workers. The mean observed in this study was 1.019. It appears that the normal specific gravity originally reported by Levine and Fahy[14] in 1945 may no longer be valid and that a somewhat lower value should be considered as normal.

The hydrolysis procedure presented, as well as the extraction procedure, are easily manipulated, requiring no back extraction or derivatization. Use of the internal standard and four-point standard curve as well as the reproducibility of the assay assure reliable results. The separation of phenol and *p*-cresol by the technique removes the *p*-cresol interference which caused difficulties with other methods of analysis of urinary phenol.

REFERENCES

1 M. Duran, D. Ketting, R. K. De Bree, C. Van Der Heiden and S. K. Wadman, *Clin. Chim. Acta*, 45 (1973) 341.
2 R. C. Theis and S. R. Benedict, *J. Biol. Chem.*, 61 (1924) 67.

3 H. D. Gibbs, *J. Biol. Chem.*, 72 (1927) 649.
4 J. E. Walkley, L. D. Ragnotto and H. B. Elkins, *Amer. Ind. Hyg. Ass., J.*, 22 (1961) 362.
5 A. B. Van Haaften and S. T. Sie, *Ind. Hyg., J.*, 26 (1965) 52.
6 V. Fell and C. R. Lee, *J. Chromatogr.*, 121 (1976) 41.
7 R. J. Sherwood and F. W. G. Carter, *Ann. Occup. Hyg.*, 13 (1970) 125.
8 W. H. Fishman, *Methods Enzymol.*, 3 (1955) 55.
9 *Fed. Reg.*, 42 (1977) 22516.
10 G. B. Crump, *J. Chromatogr.*, 10 (1963) 21.
11 G. B. Crump, *Anal. Chem.*, 36 (1964) 2447.
12 J. W. Porteous and R. T. Williams, *Biochem. J.*, 44 (1949) 46.
13 J. Teisinger and V. Fiserova-Bergerova, *Auck. Maladies Profess. Med. Travail Securite Sociale*, 16 (1958) 221.
14 L. Levine and J. Fahy, *J. Ind. Hyg. Toxicol.*, 27 (1945) 217.
15 N. A. Fishbeck *et al.*, *Amer. Ind. Hyg. Assoc., J.*, 36 (1975) 820.