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GAS CHROMATOGRAPHIC ANALYSIS OF CHLOROPHENYLMERCAPTURIC ACID LINDANE METABOLITES

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SUMMARY

An analytical method for phenols has been adapted for the analysis of chlorophenylmercapturic acids in rat urine. Chlorothiophenols were produced from the mercapturic acids by hydrolytic cleavage with sodium hydroxide. Acetate esters of the chlorothiophenols were formed by addition of acetic anhydride to the aqueous alkaline solution. After acylation, the acetate derivatives were extracted into hexane. Forming the acetate esters of the chlorothiophenols prevented their oxidation to disulfides and significantly improved their chromatographic properties. Electron-capture gas chromatographic analysis of the stable acetate esters was performed on a mixed phase column, 4% SE-30 + 6% OV-210. Recoveries of four chlorothiophenols ranged from 82 to 93%. This method required no sample transfer steps; therefore, sample loss and analysis time were minimized.

INTRODUCTION

The formation of mercapturic acids (N-acetyl-cysteine conjugates) is an important detoxification mechanism. The conjugation of glutathione (a tripeptide: glutamic acid, cysteine, glycine) with a xenobiotic is the first step in mercapturic acid biosynthesis. Glutamic acid and glycine are catabolically elminated from the glutathione conjugate. The resulting cysteine moiety is then acetylated to form the mercapturic acid. A wide variety of potentially harmful electrophilic compounds is detoxified by this mechanism.

When lindane (γ-hexachlorocyclohexane) was administered intraperitoneally to rats, approximately 15% of the dose was recovered in the mercapturic acid fraction[1]. In 1965, Grover and Sims[2] were the first to document the existence of a chlorophenylmercapturic acid as a mammalian metabolite of lindane. They presented chromatographic evidence for the existence of a small amount of 2,4-dichlorophenylmercapturic acid in urine from lindane-treated rats. Koransky *et al.*[3], studying α-hexachlorocyclohexane in rats, identified several isomers of dichlorothiophenol in urine following alkaline hydrolysis. More recently, Kurihara, *et al.*[4] reported the presence of mono- and polychlorophenylmercapturic acids as lindane metabolites.

The chlorophenylmercapturic acids have been analyzed intact and as chloro-

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thiophenols (Fig. 1). Kurihara *et al.*[4] analyzed intact chlorophenylmercapturic acids in urine. The chlorophenylmercapturic acids were butylated following separation from the netural compounds, free chlorophenols, and conjugated chlorophenols. Butyl esters of the chlorophenylmercapturic acids were then acetylated with trifluoroacetic anhydride and examined by gas chromatography–flame photometric detection. Koransky *et al.*'s[3] analysis of chlorophenylmercapturic acids was performed on chlorothiophenols. The chlorothiophenols were generated from the chlorophenylmercapturic acids by alkaline hydrolysis, derivatized with diazomethane, and analyzed as the methyl ethers by gas chromatography–electron-capture detection.

An improved analytical method was developed in our laboratory for the de-

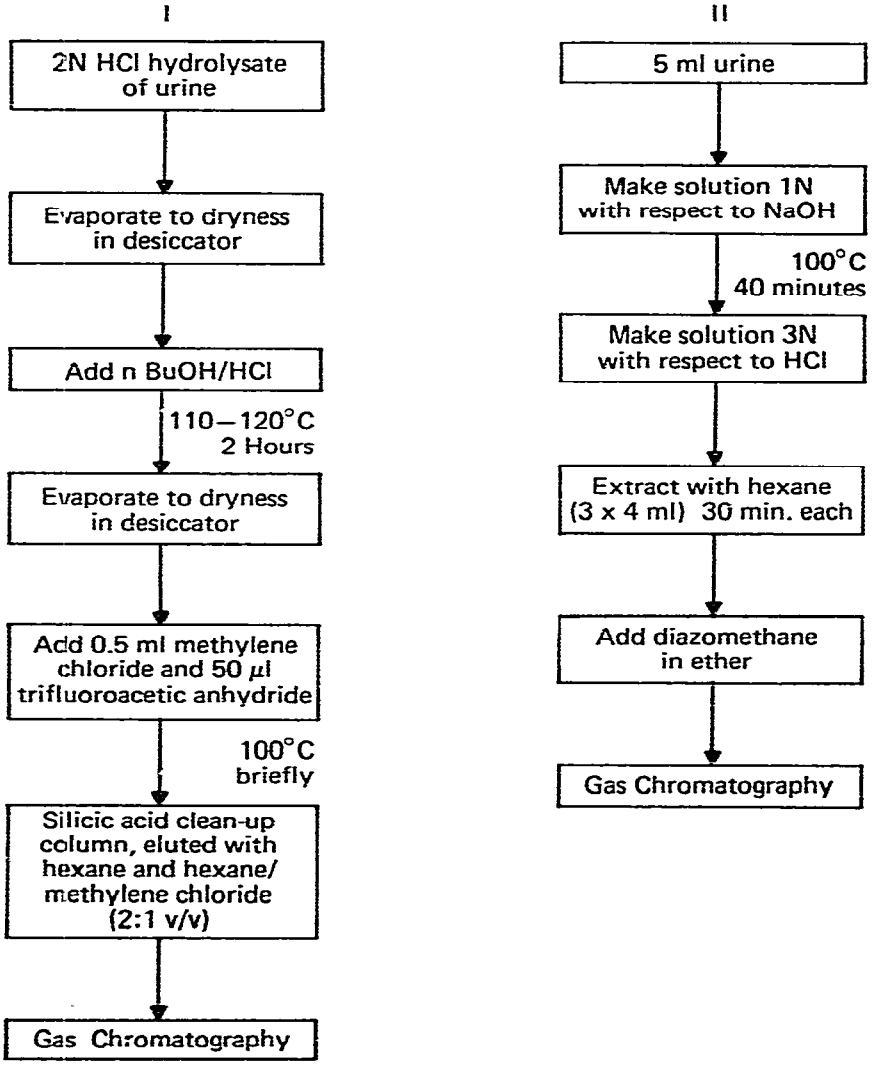


Fig. 1. (I) Analysis of mercapturic acids intact by Kurihara *et al.*[4]. (II) Analysis of mercapturic acids as thiophenols by Koransky *et al.*[3]. n BuOH = *n*-Butanol.

termination of chlorophenylmercapturic acids in urine. The analysis of chlorothiophenols derived from chlorophenylmercapturic acids was preferred because analysis of the intact chlorophenylmercapturic acids was time-consuming and tedious. Analysis of the derivatized chlorothiophenols had two significant advantages compared to the analysis of the underivatized compounds: greatly improved chromatographic properties and increased stability against autooxidation. Due to the extremely hazardous properties of diazomethane, several other derivatizing reagents were examined.

The similarities in the reactivity of phenols and thiophenols suggested that the literature concerning derivatization of phenols also should be reviewed. Various reagents have been used in the derivatization of phenols including: diazomethane[5], diazoethane[6], heptafluorobutyrylimidazole[7], and silanizing reagents[8]. However, all of these reagents required that the phenol be in an organic solvent prior to derivatization. The solvent extraction of phenols from aqueous solution often resulted in sample loss; therefore, a new derivatization procedure was investigated.

Coutts *et al.*[9] omitted the solvent extraction step and formed the acetate esters of various phenols in an aqueous sodium bicarbonate solution. This method eliminated several steps necessary in most phenol derivatizations including: acidification of the matrix, extraction of the phenol into an organic solvent, drying, and concentration before derivatization. The direct formation of the acetate esters in aqueous alkaline solution aided in the quantitative extraction of the phenols.

The derivatization method of Coutts was very effective for phenols; however, chlorothiophenols required the stronger base sodium hydroxide to provide consistently reproducible yields. It was also necessary to increase the volume of acetic anhydride from 500 μl, used by Coutts, to 5 ml possibly due to the biological matrix in the extract.

The method described in this paper improved on the methods of Kurihara *et al.* and Koransky *et al.* in several areas (see Figs. 1 and 2). The most significant improvement was the reduction in analysis time. The approximate times required for the analyses were Kurihara *et al.*'s method (> 5 h), Koransky *et al.*'s method (> 2 h), and our method (1.5 h). The method presented here had fewer transfer steps which resulted in higher yields. Utilizing gas chromatography, recoveries obtained by this method ranged from 82–93% which was significantly higher than 75–85% reported by Kurihara *et al.*. The study published by Koransky *et al.* was primarily based on radiolabelled compounds and gave no gas chromatography recovery data.

EXPERIMENTAL

Apparatus

The gas chromatograph used was a Tracor Model MT-222 equipped with a ^{63}Ni electron-capture detector (ECD) operating in the pulsed linearized mode.

Chromatographic conditions

A silanized glass column (75 cm × 4 mm I.D.) was packed with 4% SE-30 + 6% OV-210 on 80–100 mesh Gas-Chrom Q, obtained from Applied Science Labs. (State College, PA, U.S.A.). The gas chromatographic conditions were as follows: column temperature 130°C (4 min initial hold), then programmed to 185°C (2 min hold) at 6°/min; detector temperature 350°C; injection port temperature 220°C;

transfer block temperature 235°C; carrier gas (argon with 5% methane) flow-rate 85 ml/min.

Reagents

Sodium hydroxide, sodium sulfate, and acetic anhydride were obtained from Fisher Scientific (Raleigh, NC, U.S.A.). Nanograde hexane was purchased from Mallinckrodt (Paris, KY, U.S.A.). The chlorothiophenols used in this study were: 4-chlorothiophenol, 2,5-dichlorothiophenol, 3,4-dichlorothiophenol, and 2,4,5-trichlorothiophenol with stated purities of 98%, 98%, 97%, and 90%, respectively. All of the chlorothiophenols were obtained from Aldrich (Metuchen, NJ, U.S.A.).

Hydrolysis, derivatization and extraction

Chadwick *et al.*[10] provided the rat urine which was extracted for the removal of neutrals, free chlorophenols, and conjugated chlorophenols. This urine was obtained from control and lindane-treated rats. The preextracted control urine and reagent blanks were used to determine fortification recoveries.

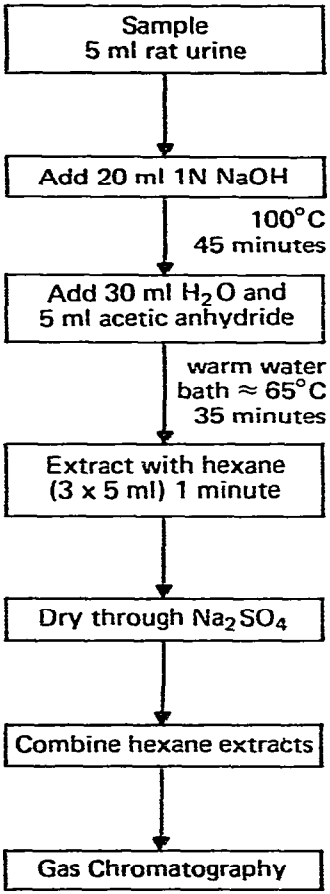
A 5-ml sample of urine was hydrolyzed with 20 ml of 1.0 *N* sodium hydroxide in a screw-capped tube for 45 min at 100°C, a slight modification of Parke's[11] procedure. After hydrolysis, 30 ml of distilled water, followed by 5 ml of acetic anhydride, were added to the sample. The resulting mixture was shaken well and derivatized in a warm water bath of approximately 65°C for 35 min. After derivatization, 5 ml of hexane were added, and the sample was shaken vigorously for 1 min. The hexane extract was dried by filtering through anhydrous sodium sulfate and collected in a concentrator tube. The extraction was repeated twice more, combining the extracts and adjusting the volume (Fig. 2).

Preparation of standards

Stock solutions of standards, 4-chlorothiophenol, 2,5-dichlorothiophenol, 3,4-dichlorothiophenol and 2,4,5-trichlorothiophenol, were prepared at a concentration of 1 mg/ml in acetone and maintained at −15°C. Acetate derivatives of the chlorothiophenols were synthesized using the described derivatization procedure and maintained at −15°C as quantitative standards.

RESULTS AND DISCUSSION

Gas chromatography utilizing temperature programming provided separation of the acetylated chlorothiophenols while maintaining an analysis time of less than 30 min. A representative chromatogram is shown in Fig. 3. When the column was consistently equilibrated between programming runs, less than one percent difference was observed in the acetate retention times. However, there were contaminating substances which coextracted with the chlorothiophenols. These coextracted substances caused a deterioration in peak height reproducibility over a period of time. This problem was corrected by daily replacement of the injection port glass insert with its silanized glass wool plug. It was also necessary to preextract the distilled water used in the hydrolysis procedure with methylene chloride–hexane (1:10), to remove some impurities that appeared as extraneous peaks. For quantification purposes, the height of the chlorothiophenol acetate peaks obtained from sample ex-



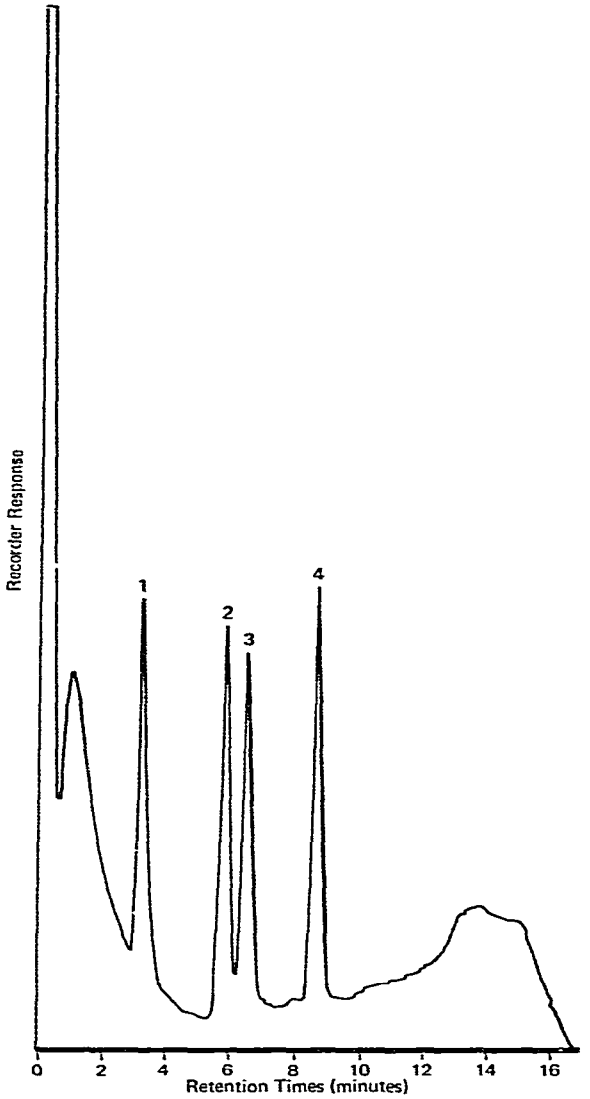


Fig. 2. Hydrolysis, derivatization, and extraction of chlorophenylmercapturic acids.

Fig. 3. Gas chromatographic separation of chlorothiophenol acetate standards. Chromatographic conditions are given in text. Each acetate represents 120 pg. Peaks: 1 = 4-chlorothiophenol acetate; 2 = 2,5-dichlorothiophenol acetate; 3 = 3,4-dichlorothiophenol acetate; 4 = 2,4,5-trichlorothiophenol acetate.

tracts was compared to peak heights obtained with known amounts of chlorothiophenols following acylation.

There were significant differences between recoveries in blanks and in control urines when they were fortified prior to hydrolysis. The recovery of four chlorothiophenols ranged from 4 to 15% lower in blanks than in control urines, indicating that urine deterred loss. In addition, a comparison was made between control urine samples fortified prior to hydrolysis and control urine samples fortified following hydrol-

ysis. We observed a 0–7% loss of chlorothiophenols during the hydrolytic process.

Coutts *et al.* used aqueous sodium bicarbonate as the reaction medium for the acetylation of phenols. However, in modifying this method for derivatization of thiophenols, it was determined that the use of aqueous sodium hydroxide as the reaction medium produced higher acetate yields. Another advantage in using sodium hydroxide was the elimination of the frequent venting necessary with sodium bicarbonate. To determine the optimum derivatizing conditions, normalities of sodium

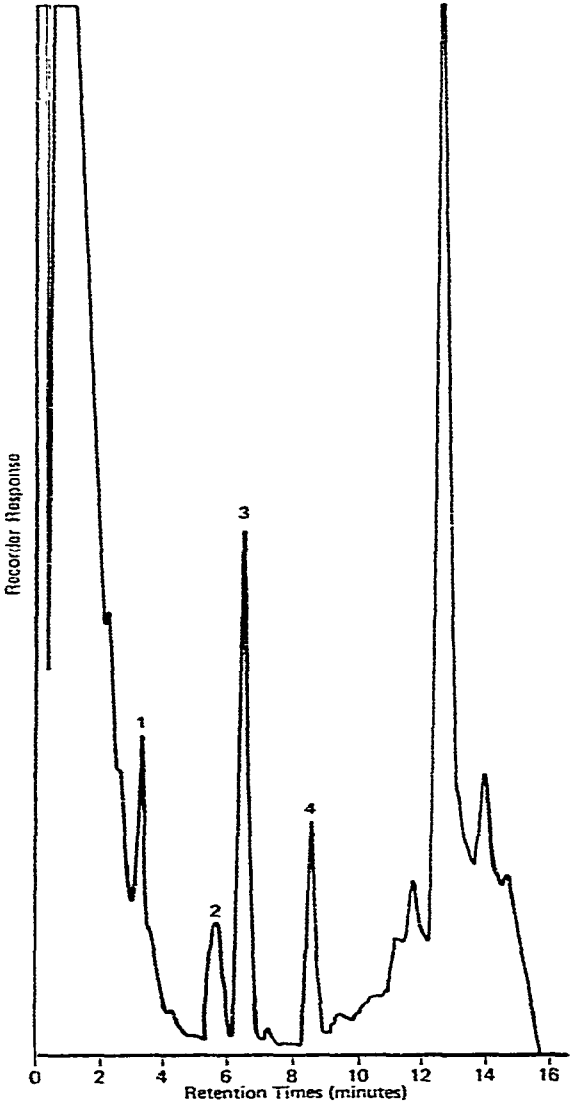


Fig. 4. Representative chromatogram of chlorothiophenols found in rat urine following treatment with lindane. Chromatographic conditions are given in text. Peaks: 1 = 4-chlorothiophenol acetate (84 ppb, 10^9); 2 = 2,5-dichlorothiophenol acetate (49 ppb); 3 = 3,4-dichlorothiophenol acetate (215 ppb); 4 = 2,4,5-trichlorothiophenol acetate (91 ppb).

hydroxide, ranging from 0.1 to 1 *N*, and volumes of acetic anhydride, ranging from 1 to 10 ml, were examined. The optimum reaction medium providing the most consistent and efficient derivatization proved to be 50 ml of 0.4 *N* sodium hydroxide and 5 ml of acetic anhydride. A reaction temperature of 65°C had the highest yield of acetate derivatives. Because this method required no sample transfer steps, sample loss was minimized and analysis time reduced.

Extraction of the acetates from the reaction medium was a simple and efficient procedure. However, the acetates were adsorbed by the anhydrous sodium sulfate when insufficient hexane wax used in the extraction or in the rinse. An extraction volume of 5 ml followed by a 3-ml rinse proved adequate to prevent adsorption. Further extraction of the reaction medium with a more polar solvent showed no residual acetates.

The derivatized standards were examined for determination of stability at room temperature and at −15°C with no detectable degradation after three days. After the stability of the standards was ascertained, the four chlorothiophenols were derivatized on four consecutive days and then analyzed with good reproducibility (S.D. 4.3%). Once the reproducibility of the standards was established, fortified control urine samples were analyzed for recovery values. Recoveries of 4-chlorothiophenol, 2,5-dichlorothiophenol, 3,4-dichlorothiophenol, and 2,4,5-trichlorothiophenol from the fortified control urine samples were 84%, 93%, 82%, and 92%, respectively. Due to the high levels of chlorothiophenols found in the hydrolyzed urine, no effort was made to determine the method sensitivity. A representative chromatogram of the chlorothiophenol acetates from a treated rat is shown in Fig. 4.

In summary, mercapturic acid biosynthesis is a vital detoxification mechanism, the full importance of which is still being investigated. Therefore, an improvement in the analysis of mercapturic acids would be advantageous. We have described a simple and rapid procedure for the analysis of chlorophenylmercapturic acids as chlorothiophenol acetates. With this method, we feel that important toxicological information can be obtained.

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