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# Analysis of *n*-Hexane, 2-Hexanone, 2,5-Hexanedione, and Related Chemicals by Capillary Gas Chromatography and High-Performance Liquid Chromatography

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Analytical methods, using capillary gas chromatography and normal-phase high-performance liquid chromatography, were developed for the analysis of the neurotoxic chemicals *n*-hexane, 2-hexanone, and 2,5-hexanedione and their suspected metabolites. Two gas chromatographic methods, using a 50-m glass capillary OV 101 column and cyclohexane as an internal standard, were employed. In both methods, the injector and detector temperatures were 220 and 280°C, respectively. In method I the following temperature program was used: isothermic at 50°C for 30 min, followed by a temperature increase of 10°C/min to a final temperature of 180°C, which was then maintained for 7 min. This method was used to analyze the following compounds: *n*-hexane, 2,5-dimethylfuran, 2-hexanone, 3-hexanone, hexanal, 1-hexanol, 2-hexanol, 3-hexanol, 5-hydroxy-2-hexanone, γ-valerolactone, 2,5-hexanedione, and 2,5-hexanediol. Method II, which was developed for *n*-hexane and eight of its more common metabolites, used the following temperature program: isothermic at 70°C for 15 min, followed by a temperature increase of 40°C/min to a final temperature of 220°C, which was maintained for 5 min. A linear relationship between peak area and amount injected was observed over a 100-fold range. The minimum detectable amounts ranged from 0.05 to 1 μg, depending on the compound. Normal-phase HPLC, using a 5-μm silica cartridge fitted into an RCM-100 radial-compression separation system, was utilized to analyze 2-hexanone and its metabolites 2,5-dimethylfuran, γ-valerolactone, 5-hydroxy-2-hexanone, and 2,5-hexanedione. The mobile phase was a linear gradient of 3–55% 2-propanol in *n*-hexane in a period of 1 min at a solvent delivery rate of 0.8 ml/min. Compounds were detected by monitoring the ultraviolet absorbance, at 254 nm, of the column eluates and quantitation was achieved by measurement of the peak heights. This method showed linearity over a 100-fold range. The minimum detectable amounts ranged from 2–10 μg depending on the compound. These methods were tested by analysis of chicken plasma to which a mixture of the chemicals was added. The recoveries ranged from 30 to 70% depending on the compound.



Straight-chain hexacarbon solvents are a group of industrial chemicals known to cause a neurotoxic condition described as peripheral neuropathy, central-peripheral distal axonopathy, or polyneuropathy (1). Peripheral neuropathy was first reported in humans as a result of occupational or abusive exposure to *n*-hexane or 2-hexanone (2–6). The toxic condition is manifested as a slowly developing weakness in the hindlimbs which may progress to paralysis (7). Lesions involve giant axonal swelling with focal accumulation of 10-nm neurofilaments, followed by fiber demyelination and degeneration [for reviews see (1,6)]. Subsequent studies have shown that peripheral neuropathy is also induced in many laboratory animals such as chickens, rats, cats, dogs, and monkeys (2,8–16).

A number of metabolism studies of *n*-hexane and related hexacarbons have been carried

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out in animals and humans. The metabolites identified include 2,5-dimethylfuran, γ-valerolactone, and other oxidation products of *n*-hexane (17–24). These metabolism and toxicological studies suggested that 2,5-hexanedione may be the active compound responsible for this toxic condition.

Analysis of *n*-hexane and its metabolites has been carried out primarily by using gas chromatography on steel- (18–20) or glass-packed columns (21,25). The chemicals were detected by either flame ionization detector (FID)[3] (18–21) or mass spectrometry methods (25). However, these methods analyzed only a few chemicals in each column. This report describes analytical methods for the separation and quantification of *n*-hexane and 11 of its related chemicals in one chromatographic system using capillary gas chromatography. Another gas chromatographic method was developed to analyze *n*-hexane and eight of its more common metabolites. Also, an analytical method using normal-phase HPLC for the analysis of 2-hexanone and four of its metabolites is reported. These methods were tested by analysis of chicken plasma to which test chemicals had been added.

## MATERIALS AND METHODS

*Chemicals.* *n*-Hexane (99+%), 1-hexanol (98%), 2-hexanol (99%), 3-hexanol (97%), hexanal (99%), 2-hexanone (99+%), 3-hexanone (98%), 2,5-hexanediol (99%), γ-valerolactone (98%), and 2,5-dimethylfuran (99%) were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. 2,5-Hexanedione (98%) was obtained from Eastman–Kodak Company, Rochester, New York. Cyclohexane was purchased from Fisher Scientific, Raleigh, North Carolina. 5-Hydroxy-2-hexanone was a gift from Dr. DiVincenzo, Eastman–Kodak Company, Rochester, New York. 2,5-Dimethylfuran, 2,5-hexanedione, and γ-valerolactone were purified before use. 2,5-Dimethylfuran was distilled using a 15-cm fractionating column and the distillate at 92°C was collected, washed six times with $H_2O$, dried over $MgSO_4$, and stored at −20°C. γ-Valerolactone and 2,5-hexanedione were purified by washing several times with *n*-hexane. Solvents for HPLC were obtained from Fisher. All other chemicals used in this study were obtained in the highest purity available.

*Gas chromatography.* A Varian Model Vista 6000 gas chromatograph equipped with an FID and a 50-m capillary glass OV 101 column was obtained from Varian Associates, Palo Alto, California. Nitrogen was used as a carrier gas at a flow rate of 3 ml/min and the capillary make up flow rate was set at 27 ml/min. Hydrogen and air for the FID were used at flow rates of 33 and 355 ml/min, respectively. Two gas chromatographic methods were developed. In both methods the injector and detector temperatures were set at 220 and 280°C, respectively. Also, cyclohexane was used as an internal standard in both methods. The first method (Method I) was used to analyze *n*-hexane, 2,5-dimethylfuran, γ-valerolactone, 1-hexanol, 2-hexanol, 3-hexanol, hexanal, 2-hexanone, 3-hexanone, 2,5-hexanedione, 5-hydroxy-2-hexanone, and 2,5-hexanediol. In method I the column temperature was programmed as follows: isothermic at 50°C for 30 min, followed by a temperature increase of 10°C/min to a final temperature of 180°C, which was maintained for 7 min. Method II was used to analyze all previous chemicals except hexanal, 3-hexanone, and 3-hexanol. The column temperature in method II was programmed as follows: isothermic at 70°C for 15 min followed by a temperature increase of 40°C/min to a final temperature of 220°C, which was maintained for 5 min. A data processor, Chromatopac EIA (Shimadzu Seisakuisho, Ltd., Kyoto, Japan), was used to determine retention time and peak area.

*HPLC.* The HPLC system consisted of a Model 660 solvent programmer equipped with two Model 6000A pumps, a Model 440 uv detector, a U-6K injector, an RCM-100 radial-compression separation system with a 5-μm silica cartridge and RCSS Guard-PAK silica,

[3] Abbreviation used: FID, flame ionization detector.

(Waters Associates, Inc., Milford, Mass.), and a recorder (Fisher Recordall Series 5000). This HPLC system was used to analyze 2-hexanone and its possible metabolites 5-hydroxy-2-hexanone, 2,5-hexanedione, γ-valerolactone, and 2,5-dimethylfuran. The mobile phase was a linear gradient of 3 to 55% 2-propanol in *n*-hexane in a period of 1 min at a solvent flow rate of 0.8 ml/min. The compounds were detected by monitoring the uv absorbance at 254 nm of the column eluates, and peak heights were measured for quantitation. The baseline absorbance changed suddenly due to the sudden change in the proportion of 1-propanol in the solvent mixture. Since high levels of γ-valerolactone and 2,5-hexanedione interfere with the baseline absorbance, their separation was possible only at small amounts under this condition. γ-Valerolactone has a very low absorbance at this wave length. Also, the cartridge should be equilibrated by the solvent of the initial condition for at least 5 min before injection of the next sample.

*Extraction of plasma.* Plasma from untreated chickens was enriched with either 2-hexanone and its metabolites or *n*-hexane and its suspected metabolites as well as with cyclohexane as an internal standard; 6 N HCl and $Na_2SO_4$ were then added (20 μl acid and 150 mg $Na_2SO_4$/ml plasma) and the chemicals were extracted from the plasma with ether (3 × 2 ml for each ml plasma). The ether extracts were then concentrated under $N_2$ and analyzed by HPLC and gas chromatography.

## RESULTS AND DISCUSSION

*n*-Hexane and other chemicals analyzed in this study (Fig. 1) constitute compounds with a wide range of physical and chemical properties. Their boiling points range from 69°C for *n*-hexane to 217°C for 2,5-hexanediol. These chemicals include paraffins, alcohols, an aldehyde, ketones, an hydroxyketone, a lactone, and an aromatic compound. Due to the diversity of these chemicals it is difficult to develop an analytical method that can separate all of them in one chromatographic system,

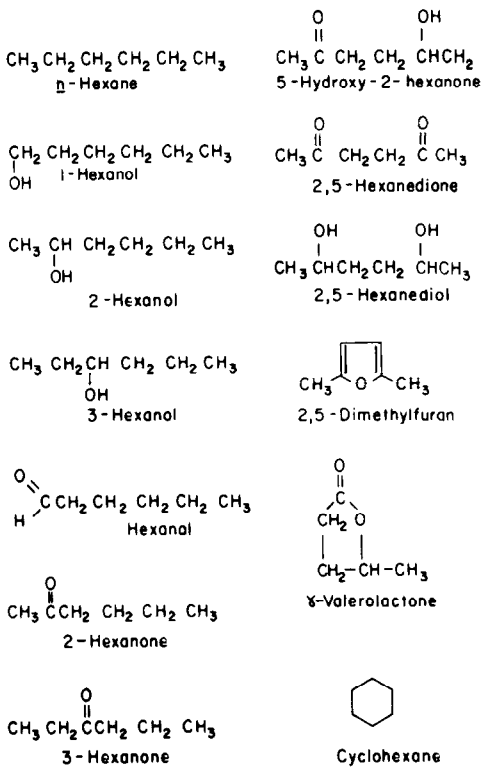


FIG. 1. Structure of cyclohexane and *n*-hexane and possible metabolites of *n*-hexane.

especially for compounds with close chemical structures. However, by using the powerful separation technique of capillary gas chromatography, this separation was achieved.

Figure 2 presents a typical gas chromatogram of cyclohexane and *n*-hexane and 11 of its possible metabolites using method I. The retention times of these chemicals are presented in Table 1. A linear relationship between peak area and the amount injected was observed over a range of 0.2–10 μg. The minimum detectable amounts were 0.05 μg for *n*-hexane, cyclohexane, and 2,5-dimethylfuran, and 0.2 μg for the rest of the compounds.

Figure 3 presents a typical gas chromatogram of *n*-hexane and eight of its most common metabolites with cyclohexane as an internal standard using method II. These compounds have been identified as metabolites of *n*-hexane in many animal species (17–24). This method was faster than the previous one,

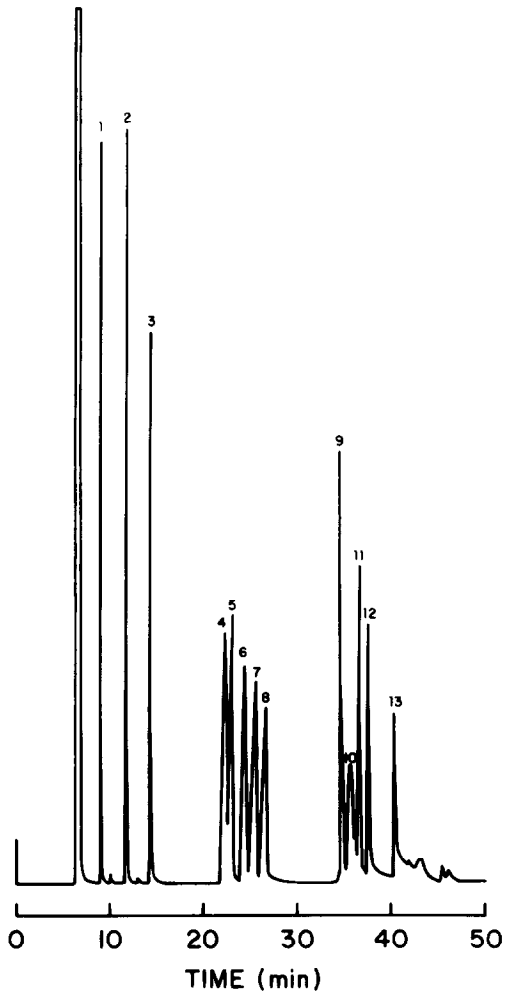


FIG. 2. Separation of cyclohexane and *n*-hexane and 11 of the possible metabolites of *n*-hexane by OV 101 capillary gas chromatography (method I). System used: isothermic at 50°C for 30 min followed by a temperature increase of 10°C/min to a final temperature of 180°C, which was maintained for 7 min. See text for more details. Peaks: 1, *n*-hexane; 2, cyclohexane; 3, 2,5-dimethylfuran; 4, 3-hexanone; 5, 2-hexanone; 6, hexanal; 7, 2-hexanol; 8, 3-hexanol; 9, 1-hexanol; 10, 5-hydroxy-2-hexanone; 11, 2,5-hexanedione; 12, γ-valerolactone; 13, 2,5-hexanediol.

since the 10 compounds were separated in less than 20 min. The retention times of these chemicals are listed in Table 1. The relationship between peak area and the amount injected was also linear.

An advantage of these gas chromatographic methods is that in the analysis of [$^{14}$C]*n*-hexane, [$^{14}$C]2,5-hexanedione, or [2-$^{14}$C]-hexanone and their metabolites from biological samples, the radioactive products which emanate from the FID can be collected and analyzed by liquid scintillation counting. This is especially important when a metabolite is present in a very small quantity. A preliminary study showed that 80% of the radioactivity present in a [$^{14}$C]2,5-hexanedione sample, as quantitated by liquid scintillation counting, was recovered from the gas chromatograph (Nomeir and Abou-Donia, unpublished results). Such findings were consistent and reproducible.

Since *n*-hexane and its mono and dihydroxylated metabolites do not absorb the uv light at 254 nm, HPLC was only used for the analysis of 2-hexanone and its metabolites 5-hydroxy-2-hexanone, 2,5-hexanedione, γ-valerolactone, and 2,5-dimethylfuran. These chemicals contain a carbonyl or an aromatic chromophore that can be measured at 254 nm. The method resulted in a baseline separation of these compounds within 12 min (Fig. 4). The retention times of these chemicals are presented in Table 1. γ-Valerolactone had a very low uv absorbance at 254 nm relative to all other chemicals and its separation from 2,5-hexanedione was only possible using small amounts (Fig. 5).

The drastic change of solvent composition (from 3 to 55% in 1 min) was necessary since 2-hexanone and 2,5-dimethylfuran were separated in this column at a solvent composition of very low polarity, while 5-hydroxy-2-hexanone, γ-valerolactone and 2,5-hexanedione were only eluted from the column at a solvent composition of high polarity. This drastic change in solvent composition resulted in a sudden drift in the baseline (Fig. 4) and this drift was magnified by increasing the sensitivity (Fig. 5). Due to this baseline drift it was more consistent to use peak height, instead of peak area, when correlating the amount injected with the detector response. Such correlation showed linearity over at least two orders of magnitude (2–1,000 μg). The minimum detectable amounts with this method were 10 μg for 2,5-dimethylfuran, 4 μg for 2,5-hexanone and 5-hydroxy-2-hexanone, 2 μg for

TABLE 1

RETENTION TIME OF CYCLOHEXANE, *n*-HEXANE, AND ITS POSSIBLE METABOLITES USING CAPILLARY GAS CHROMATOGRAPHY[a] AND HPLC

| | Gas Chromatography | | | | |
|---|---|---|---|---|---|
| | Method I[b] | | Method II[c] | | HPLC[d] |
| Compound | Retention time (min) | Relative RT | Retention time (min) | Relative RT | Retention time |
| *n*-Hexane | 9.08 | 0.75 | 6.15 | 0.84 | |
| Cyclohexane | 12.12 | 1.00 | 7.30 | 1.00 | |
| 2,5-Dimethylfuran | 14.72 | 1.21 | 8.20 | 1.12 | 4.10 |
| 3-Hexanone | 23.05 | 1.90 | | | |
| 2-Hexanone | 24.45 | 2.02 | 11.44 | 1.57 | 6.83 |
| Hexanal | 25.62 | 2.11 | | | |
| 2-Hexanol | 27.05 | 2.23 | 12.45 | 1.71 | |
| 3-Hexanol | 28.26 | 2.33 | | | |
| 1-Hexanol | 35.50 | 2.93 | 16.49 | 2.26 | |
| 5-Hydroxy-2-hexanone | 36.50 | 3.01 | 17.28 | 2.37 | 11.37 |
| 2,5-hexanedione | 37.60 | 3.10 | 17.62 | 2.41 | 12.50 |
| γ-Valerolactone | 38.60 | 3.18 | 18.29 | 2.51 | 11.95 |
| 2,5-Hexanediol | 41.40 | 3.42 | 19.75 | 2.71 | |

[a] A 50-μm glass capillary OV 101 column and injector and detector temperatures of 220 and 280°C, respectively, were used in both methods.

[b] Method I: isothermic at 50°C for 30 min followed by an increase in temperature at a rate of 10°C/min to a final temperature of 180°C, which was maintained for 7 min. See text for details.

[c] Method II: Isothermic at 70°C for 15 min followed by an increase in temperature at a rate of 40°C/min to a final temperature of 220°C/min, which was maintained for 5 min. See text for details.

[d] HPLC solvent system was a linear gradient of 3–55% 2-propanol in *n*-hexane in a period of 1 min at a solvent flow rate of 0.8 ml/min on a 5-μm silica cartridge.

2,5-hexanedione, and 100 μg for γ-valerolactone. A useful application of this method would be in metabolism studies using radioactive chemicals since fractions could be collected for quantitative analysis by liquid scintillation counting.

These analytical methods were developed to analyze *n*-hexane and its metabolites in various tissues and excreta of treated animals. In order to test these methods for this purpose, an extraction procedure was developed to recover these chemicals from chicken plasma. Figure 6 presents a typical gas chromatogram (Method I) of chicken plasma extract to which *n*-hexane and related chemicals had been added. It is clear that *n*-hexane and related chemicals are recovered from the plasma by this method of extraction. However, a portion of 5-hydroxy-2-hexanone may be converted to 2,5-dimethylfuran in the gas chromatographic column. This possibility was confirmed by injecting a dry solution of 5-hydroxy-2-hexanone into the gas chromatographic column. Thus, this conversion may account for the low recovery of 5-hydroxy-2-hexanone which was observed. (Fig. 6). Also, the conversion of 5-hydroxy-2-hexanone was found to occur in dry ether if left at room temperature for a week or longer before the analysis by HPLC. Table 2 shows the percentage of recovery of *n*-hexane and other chemicals from chicken plasma analyzed in this study. The recoveries of *n*-hexane and cyclohexane were relatively low. This was found to be mainly due to loss during evaporation rather than to extraction. 2,5-Hexanediol could not be determined with this method since its retention time coincided with some endogenous

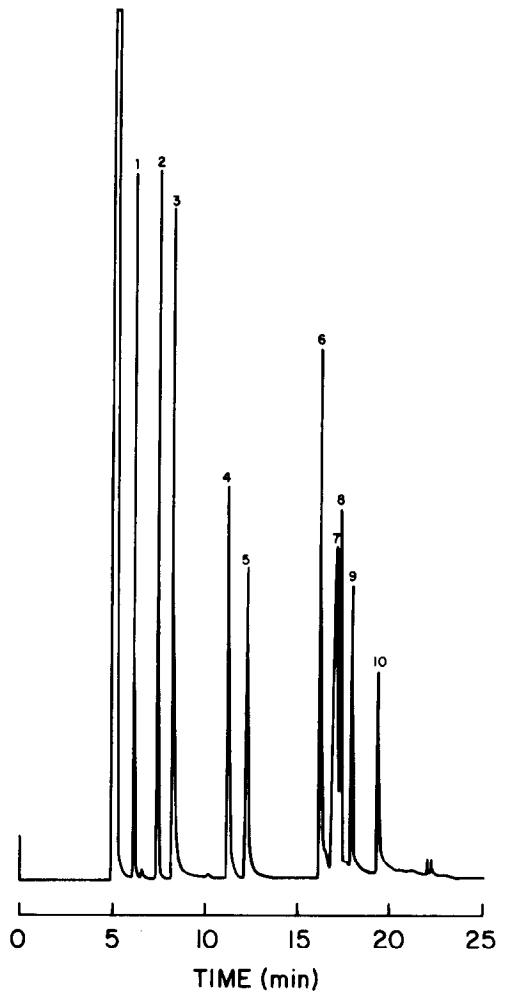


FIG. 3. Separation of cyclohexane, *n*-hexane and eight common metabolites of *n*-hexane by OV 101 capillary gas chromatography (method II). System used: isothermic at 70°C for 15 min followed by a temperature increase of 40°C/min to a final temperature of 220°C, which was maintained for 5 min. Peaks: 1, *n*-hexane; 2, cyclohexane; 3, 2,5-dimethylfuran; 4, 2-hexanone; 5, 2-hexanol; 6, 1-hexanol; 7, 5-hydroxy-2-hexanone; 8, 2,5-hexanedione; 9, γ-valerolactone; 10, 2,5-hexanediol.

chemicals extracted from the plasma. This problem could be overcome by using the radioactive compound and collecting this peak for liquid scintillation counting.

In a comparable experiment 2-hexanone and its metabolites were added to the plasma, and then extracted and analyzed by gas chromatography and HPLC. The results showed that the recovery of extraction for these chemicals was similar to that obtained with the gas chromatograph (Table 2).

The minimum detectable amounts of *n*-hexane and metabolites, (0.05 to 1 μg) with the capillary gas chromatographic methods and those of 2-hexanone and metabolites using the HPLC method (2–10 μg) are within the reported levels in humans and test animals which have been exposed to these chemicals. For example, the following metabolites (expressed as μg/ml) were detected in urine samples of men exposed to an *n*-hexane concentration of 411 ± 366 mg/m$^3$ in the environment of a shoe factory: 2-hexanol, 0.1–0.4; 2,5-hexanedione, 2.9–30.0; γ-valerolactone, 0.7–5.5; and 2,5-dimethylfuran, 1.7–17.5 (18). Also, when rats were exposed to 1000 ppm of *n*-hexane for 8 h, the concentration in the blood was 0.5 μg/ml, while the concentration

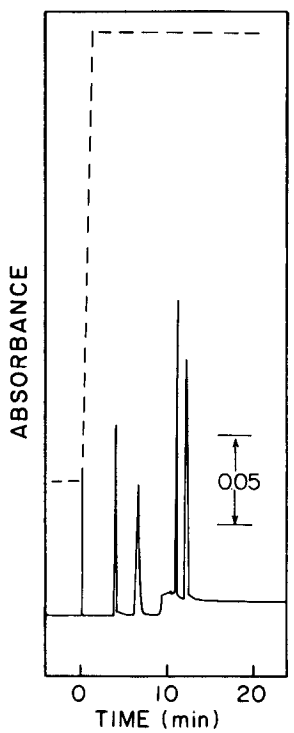


FIG. 4. Separation of 2-hexanone and its metabolites by normal phase HPLC on 5-μm silica. The solvent system used was a linear gradient of 3–55% of 2-propanol in *n*-hexane in a period of 1 min at a solvent flow rate of 0.8 ml/min. Peaks from left to right: 2,5-dimethylfuran, 2-hexanone, 5-hydroxy-2-hexanone, and 2,5-hexanedione. The broken line indicates solvent gradient.

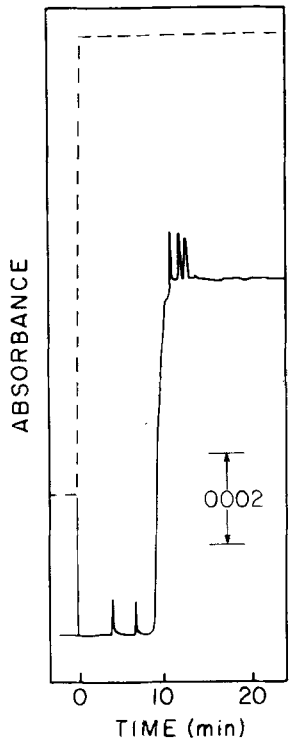


FIG. 5. Separation of 2-hexanone and its metabolites by normal-phase HPLC on 5-μm silica. Peaks from left to right: 2,5-dimethylfuran, 2-hexanone, 5-hydroxy-2-hexanone, γ-valerolactone, and 2,5-hexanedione. Same HPLC system as in Fig. 6. The broken line indicates solvent gradient.

(μg/g wet tissue wt) in some tissues was liver, 1.23; kidney, 5.8; brain, 3.0; and sciatic nerve, 46 (26). In these rats, 2-hexanone and 2,5-hexanedione were also detected in the blood at concentrations (μg/ml) of 0.7 and 1.4, respectively, while their concentrations (μg/g) in the tissues ranged from 0.31 to 26.5 and from 0.4 to 8.58, respectively (26). In another study, the compound 2,5-hexanedione was found in serum at peak concentrations ranging from approximately 50 to 600 μg/ml in rats treated with a single oral dose of 6.6 mmol/kg of *n*-hexane, 2-hexanol, 2-hexanone, 2,5-hexanediol, 5-hydroxy-2-hexanone, or 2,5-hexanedione (27). A dermal 50 mg/kg dose of [$^{14}$C]2,5-hexanedione, applied on a protected area on the back of hens, was rapidly absorbed and distributed throughout the body (28). Most of the radioactivity in plasma, which reached a peak concentration at 4 h, was identified as 5-hydroxy-2-hexanone, with lower levels of 2,5-hexanedione and 2,5-dimethylfuran. The concentrations of these chemicals in various tissues ranged from 0.1 to 4.35 μg/g tissue.

In summary, we have developed gas chromatographic and HPLC methods for the separation and analysis of *n*-hexane and its suspected metabolites. While these methods can be used to analyze *n*-hexane and metabolites in biological samples, the use of radioactive compounds will greatly enhance the capability of these methods for detection at both the qualitative and quantitative level.

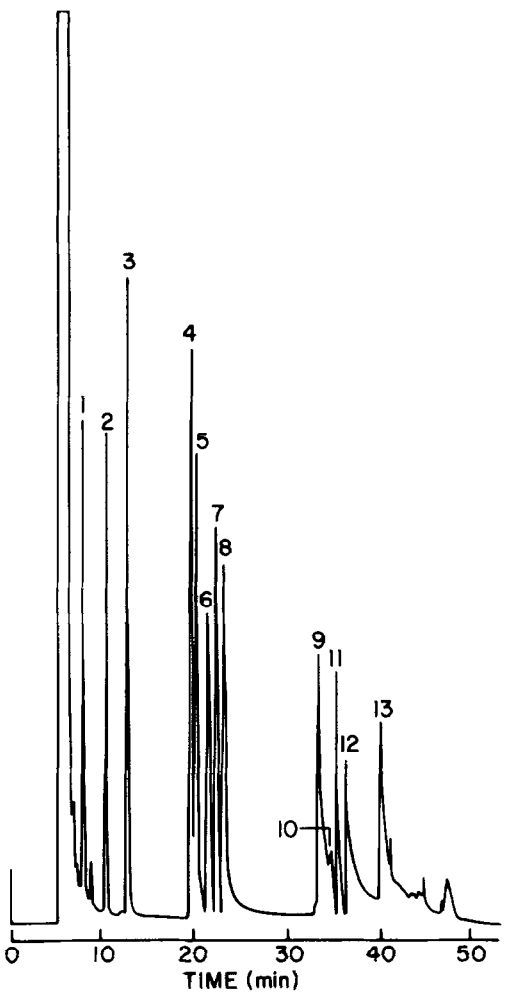


FIG. 6. Gas chromatogram of the extract of chicken plasma fortified with *n*-hexane and related chemicals. The gas chromatographic system and peak numbers are as described for method I in Fig. 2.

TABLE 2

RECOVERY OF EXTRACTION OF *n*-HEXANE AND SUSPECTED METABOLITES FROM CHICKEN PLASMA[a]

| Compound | Percentage of recovery |
|---|---|
| *n*-Hexane | 35 ± 5 |
| Cyclohexane | 37 ± 4 |
| 2,5-Dimethylfuran | 61 ± 6 |
| 3-Hexanone | 75 ± 3 |
| 2-Hexanone | 78 ± 4 |
| Hexanal | 70 ± 6 |
| 2-Hexanol | 79 ± 3 |
| 3-Hexanol | 75 ± 4 |
| 1-Hexanol | 66 ± 8 |
| 5-Hydroxy-3-Hexanone | 30 ± 8 |
| 2,5-Hexanedione | 62 ± 3 |
| γ-Valerolactone | 55 ± 5 |

[a] Plasma samples were enriched with a mixture of the chemicals, HCl and $Na_2SO_4$ were then added, and compounds were extracted with ether. The extract was concentrated under $N_2$ and analyzed by capillary gas chromatography (method I). The data are the average of three replicates ± SD.

## ACKNOWLEDGMENTS

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