〔衛 生 化 学〕
EISEI KAGAKU
31 (2) 87-94 (1985)

Gas Chromatographic Determination of Toluene in Tissue by Using a Head Space Method¹⁾

SHUICHI MIYaura and HIDEO ISONO

*Faculty of Pharmaceutical Sciences, Teikyo University, 1091-1
Sagamiko-machi, Tsukui-gun, Kanagawa 199-01, Japan*

(Received September 12, 1984)

A head space method was studied for the quantitative determination of toluene in tissues by gas chromatography (GC). The method may be summarized as follows. Tissue was minced by extruding it from a plastic syringe into an equilibration vial containing water, sodium hydroxide, olive oil and an internal standard (ethylbenzene). As a standard sample, another equilibration vial having the same components plus authentic toluene but without tissue was used. Both vials were rapidly sealed with Teflon-rubber packing, and then shaken at 35°C for 2.5 h in a water bath. One milliliter of warmed head space gas was subjected to GC. The concentration of toluene in tissue was calculated by comparing T/I (T, peak height of toluene; I, peak height of internal standard) of the tissue sample with that of the standard sample.

This method was found to be useful for determining toluene in the range of 0.002 to 10 mg/g in tissue.

Keywords—head space method; internal standard; GC; toluene tissue; olive oil; solubilization

Introduction

Toluene has been extensively used in chemical and industrial processes as a thinner, and in adhesives, general solvents, *etc.* It is also a subject of abuse by inhalation. If there is the suspicion that an intoxication or a death^{2,3)} might have been caused by inhalation of evaporated substances from thinner or adhesives containing toluene, it is important to determine toluene quantitatively not only in the expired air, but also in blood or urine, in order to clarify the direct cause of the incident. Two quantitative methods^{4,5)} for measuring toluene in blood and urine have been reported previously. Our method was capable of determining toluene in the range of 0.5 to 500 $\mu\text{g/ml}$ in blood and 0.04 to 40 $\mu\text{g/ml}$ in urine.⁴⁾ However, tissues such as liver, kidney and brain were also suitable for determining toluene when blood or urine could

not be obtained because of a long lapse of time after death.

Several methods have been reported for the quantitative analysis of organic solvents in tissue. The methods involve a process of homogenizing tissue to facilitate the vaporization of toluene,⁶⁾ or a process of direct vaporization of organic solvent from a sample of tissue at 90°C in a water bath.⁷⁾ However, these methods are not problem-free; substantial amounts of organic solvents may be lost during tissue homogenation; heating of the equilibration vial (glass vial) at as high a temperature as 90°C produces an extremely high pressure and might cause a glass vial to explode, or might permit leakage of organic solvent gas through the packing when the needle of the gas syringe is inserted for gas-sampling.

Fatty components were found to affect the vaporization of individual organic solvents in

the previous study using blood.⁴⁾ This effect was also expected in tissue samples because they contain large amounts of fatty materials.

In the present paper, the following points were investigated: 1) solubilization of tissue; 2) selecting a suitable internal standard not affected by 1 N NaOH; 3) preparation of an aqueous standard sample without tissue; 4) storage of tissue samples.

Experimental

1. Reagents and Apparatus—1) Toluene, ethylbenzene, chloroform, 1,2-dichloroethane, methanol (special grade), pepsin (activity, 1:100), olive oil and Triton X-100 were from Wako Pure Chem. Ind. Ltd. Pancreatin (practical grade) was from Nakarai Chem. Ltd.

2) Equilibration instrument: A glass vial (vol. 100 ml) with a plastic screw cap having a hole 3.5 mm in diameter (Nichiden-Rika Glass Co. Ltd.) was used with rubber (1.5 mm in thickness, Iruma Rubber Co. Ltd.) and Teflon Tape (0.05 mm in thickness, Nichias Corp.) packings, both cut to the same size as the inner diameter (33 mm) of the screw cap (Fig. 1).

3) Gas syringe: 1 ml (Precision Sampling Corp.).

2. Conditions of Gas Chromatography (GC)
—A Hitachi model 163 gas chromatograph equipped with a hydrogen flame ionization detector was used. Conditions were as follows. 1) Column: 3 mm ϕ $\times$ 1 m, glass tube. 2) Column packing: 20% dioctyl sebacate/Uniport B 60–80 mesh (Gasukuro Kogyo Co. Ltd.). 3) Carrier gas: nitrogen, 30 ml/min. 4) Hydrogen pressure: 0.8 kg/cm². 5) Air pressure: 1.3 kg/cm². 6) Column temperature: 100°C. 7) Injection port: 115°C.

3. Toluene Standard Solution—Toluene (250 mg) was mixed with 25 ml of methanol in a 25-ml equilibration vial. The vial was closed with a screw cap (having a hole) and a Teflon-rubber packing. A portion of this solution was taken with a microsyringe inserted through the packing. This toluene standard solution was newly prepared when needed.

4. Internal Standard Solution—As an internal standard solution, 5 g of ethylbenzene was dissolved in 25 ml of methanol and then used as described in section 3.

5. 18% (v/v) Olive Oil—Water was added to a mixture of 27 ml of olive oil, 8 ml of methanol and 0.5 ml of Triton X-100 to give a total volume of 150 ml. This mixture was emulsified with vigorous shaking before use.

6. Measurement of Toluene in Liver Tissue
—An equilibration vial, containing 25 ml of 1 N NaOH, 5 ml of 18% (v/v) olive oil, 10 μ l of internal standard solution and 50 μ l of methanol, was closed using a screw cap with Teflon-rubber packing. About 1 g of rabbit liver was put into a plastic syringe (vol. 5 ml) and weighed together with the syringe (a g). The liver tissue was ejected from the syringe into the open vial, which was then capped rapidly. The used syringe was weighed again (b g). Another equilibration vial containing a mixture of the same components as above and 50 μ l of additional toluene standard solution but without the liver tissue and methanol, was prepared as a standard sample. Both vials were shaken at 35°C for 2.5 h in a water bath, then 1 ml of head space gas was taken by using a gas syringe through the packing, and subjected to GC. The procedures and calculation are outlined in Chart 1.

7. Inhalation of Toluene—Toluene (20 ml) was allowed to vaporize in a 12-l desiccator at room temperature for 1 h. A rat was placed in the desiccator, and inhaled the toluene vapor for 30 min.

Results

1. Tissue Solubilization

Approximately 1 g of liver tissue, disrupted with a plastic syringe, was placed in equilibration vials containing 25 ml of various concentrations of NaOH (0.2 through 5 N). They were shaken at 35°C in a water bath (Fig. 2). The NaOH treatment (0.5 through 1.5 N) solubilized the minced liver tissue completely within 1–2 h. Although 0.2 through 2% (w/v) solutions of pepsin or pancreatin

(1) reagents

A toluene standard solution : toluene -----10 mg/ml methanol

B internal standard solution : ethylbenzene -----200 mg/ml methanol

(2) assay & calculation

standard sample

 1_N NaOH

25ml

18% (v/v) olive oil

5ml

A

50 μ l

B

10 μ l

tissue sample

minced tissue

ca. 1g

 1_N NaOH

25ml

18% (v/v) olive oil

5ml

methanol

50 μ l

B

10 μ l

GC peak (1 ml of head space gas)

toluene

ethylbenzene

 T_0 I_0 $K \text{ value} = T_0/I_0$ 35°C
2.5h
shaking T I toluene in tissue
(mg/g) $= T/I \times 1/K \times 1/(a/b) \times 0.5$

Chart 1. Procedures of the Internal Standard Method for Determination of Toluene in Tissue Samples

Fig. 1. Diagram of Equilibration Apparatus

were examined in place of NaOH, they failed to give complete solubilization of the tissue. Therefore, 25 ml of 1_N NaOH was used for tissue solubilization in the experiments below.

Fig. 2. Procedure of Tissue Solubilization

2. Effect of 1_N NaOH on Solvent Vaporization

Sixty microliters of methanol containing 50 μ g of toluene, 400 μ g of chloroform, 125 μ g of ethylbenzene and 125 μ g of 1,2-dichloroethane was added to an equilibration vial previously containing 25 ml of 1_N NaOH. Many similar model samples were prepared

Fig. 3. Effect of 1 N NaOH on the Vaporization of Organic Solvents

○: toluene; ●: ethylbenzene; △: 1,2-dichloroethane; ▲: chloroform.

and shaken at 35°C in a water bath for various times. The head space gas (1 ml) was taken from each sample for analysis by GC.

As shown in Fig. 3, the concentrations of ethylbenzene, toluene and 1,2-dichloroethane in the head space were each found to be constant up to at least 4 h of shaking. Toluene and ethylbenzene were stable during shaking for 8 h in spite of the presence of 1 N NaOH (data not shown). However, the concentration of chloroform in the head space gradually decreased during shaking and, after 4 h, was only 70% of the initial value (at 10 or 20 min); this decrease of chloroform may reflect decomposition by 1 N NaOH, and means that chloroform is unsuitable for use as an internal standard.

3 Effect of Liver Content on Solvent Vaporization

Liver tissue was homogeneously solubilized with 1 N NaOH. This original liver solution (1 g of liver in 25 ml of 1 N NaOH) was diluted to liver concentrations of 0 (corresponding to only 1 N NaOH solution), 20, 40, 60, 80 and 100% (corresponding to the original liver solution) with 1 N NaOH. To 25 ml of each diluted liver tissue solution in an equi-

libration vial was added 60 μ l of methanol containing 50 μ g of toluene, 125 μ g of ethylbenzene and 125 μ g of 1,2-dichloroethane. The mixture was shaken at 35°C for 2.5 h, then the head space gas was analyzed by GC. The peak height of each organic solvent decreased with increase of liver concentration. The percentages of the peak height in the original liver solution (100% sample) with respect to the height of only 1 N NaOH solution (0% sample) were as follows: 67% for toluene, 50% for ethylbenzene and 96% for 1,2-dichloroethane. The peak height ratios (K values) of toluene to each of the other organic solvents obtained at different liver concentrations were compared with those obtained at 0% liver concentration (only 1 N NaOH). The latter K values were taken as 100 (Table I).

If the toluene concentration of the original liver solution is measured by the internal standard method employing liver-free 1 N NaOH solution as a standard sample, +35% error was obtained with ethylbenzene as an internal standard and -43% error with 1,2-dichloroethane. These results suggest that the above errors were caused by the presence or absence of fat in samples. These errors could be decreased to +12% for ethylbenzene and -9% for 1,2-dichloroethane by diluting the original liver solution (100% sample) to 20% with 1 N NaOH. However, when an extremely small amount of liver tissue was utilized, it was difficult to obtain an accurate value, because the liver weight was markedly affected by the adhesion of body fluid and fatty tissue.

Therefore, olive oil, as a substitute for fat, was deliberately added to the 0% and 100% samples to minimize the difference in fat content between them.

4 Effect of Addition of Olive Oil on Solvent Vaporization

Various amounts of olive oil were added

TABLE I. Effect of Liver Content on *K* Value

Internal standard	Liver concentration (%)					
	0	20	40	60	80	100
1,2-Dichloroethane	100	91	83	76	72	70
Ethylbenzene	100	112	117	120	127	135

Liver concentration 100%: original liver concentration (1 g of liver per 25 ml of 1 N NaOH).

K value: peak height ratio of toluene to internal standard.

The *K* value shown by liver content 0% was taken as 100. The other *K* values were compared with the above *K* value.

TABLE II. Effect of Olive Oil on *K* Value

	Olive oil content (%)					
	0	1	2	3	4	5
Standard sample ^{a)}	100	100	100	100	100	100
Tissue sample ^{b)}	130	105	100	99	101	99

^{a)} 25 ml of 1 N NaOH.

^{b)} 25 ml of 1 N NaOH containing 1 g of liver.

K value: peak height ratio of toluene to ethylbenzene. The *K* values of the standard samples containing different amounts of olive oil were taken as 100.

The *K* value shown by each of the tissue samples was compared with that of the corresponding standard sample.

to the original liver solution (25 ml) or 1 N NaOH solution (25 ml), both of which had been supplemented with 60 μ l of methanol containing 0.5 mg of toluene and 1 mg of ethylbenzene. The concentration of both organic solvents in the head space significantly decreased with increasing amount of olive oil in the samples. Table II lists the relative values (*K* values) of peak height ratio as described in Table I. The differences of *K* values between the standard sample (0% sample) and tissue sample (100% sample) were found to be decreased to only a few percent by addition of olive oil. Thus, olive oil was hereafter used at a concentration of 3% (v/v) in sample solutions.

5. Correlation between Equilibration and Solubilization Times

The liver from a rat which had inhaled toluene (section 7) was divided into portions

Fig. 4. Effect of Shaking Time on Determination of Toluene

of about 1 g. Toluene concentration in each tissue sample was measured by the internal standard method after shaking at 35°C in a water bath for various times. As shown in Fig. 4, the observed concentration of toluene gradually increased until 1.5 h, and thereafter reached a constant value of about 0.83 mg/g. At this time, the minced liver tissue was visibly solubilized. These results suggested that shaking time for solubilization of liver tissue coincided with the time required for equilibration of toluene into the head space. Therefore, a shaking time of 2.5 h seemed to be sufficient both for solubilization and solvent vaporization.

6. Measurement of Toluene in Liver Tissue

Toluene (0.2, 0.4, 0.6, 0.8, or 1.0 mg) was added to 1 g of minced liver tissue, and the concentration of each was measured (Fig. 5). The variations of experimental values against theoretical ones were only about $\pm 5\%$.

In the case of the addition of 0.2 mg of toluene to 1 g of kidney or brain tissue disrupted with the plastic syringe, recoveries of toluene were approximately 100% (kidney, $n=6$, mean $\pm$ S.D. = 0.2 ± 0.007 mg; brain, $n=7$, mean $\pm$ S.D. = 0.2 ± 0.004 mg). This

Fig. 5. Correlation between Added and Recovered Values

●: Values recovered.

result suggests that this method is also available for the measurement of toluene in the kidney and brain.

7. Gas Chromatograms of Liver Tissue Samples

Figure 6 shows gas chromatograms obtained from liver tissue samples containing toluene or without toluene. One unknown peak was detected from liver. Although new olive oil did not show any peak, old olive oil stored for 4 years gave four peaks. They were particularly noticeable when the sensitivity of GC was increased for measuring relatively low concentrations of toluene. The five peaks from the oil and liver tissue did not overlap either the toluene or the ethylbenzene peak. The peaks from the old oil were thought to be due to the degradation of the oil during long storage.

Kidney and brain often gave several unknown peaks on increasing gas chromatographic sensitivity to a maximum. However, they had early retention times, and so did not affect the quantitative analysis of toluene.

8. Storage of Samples

Two rats (A and B) were allowed to inhale toluene under the same conditions as described in section 7. The removed liver was divided into portions of about 1 g and each was stored in a glass vial (vol. 3 ml) with a polyethylene cap at -20°C . They were individually analyzed after a storage period of 0, 1, 2, 3 or 7 d (Table III). Rat A showed a toluene concentration of 0.42 mg/g in the liver at 0 time of storage and rat B gave 0.71 mg/g. When the liver samples of rat A were stored for 1 and 2 d, the toluene concentrations were 0.40 and 0.43 mg/g, respectively. The liver samples of rat B showed values of 0.70 and 0.72 mg/g after storage for 3 and 7 d, respectively. The storage of tissue samples in this way was possible without loss of toluene in samples for at least 7 d.

Discussion

Since conventional tissue homogenation using a homogenizer may result in the loss of a substantial amount of toluene, we developed a convenient method to minimize loss of toluene by employing a plastic syringe.

For the quantitative analysis of toluene in blood or urine, a 25-ml equilibration vial was used in the previous paper.⁴⁾ This vial could be used for the present method in place of a 100-ml vial if the volume (25 ml) of 1 N NaOH was decreased to 5ml. However, the 25-ml vial required 1–2 h more for tissue solubilization than did the 100-ml vial. Therefore, the present method employed a 100-ml vial.

When the present method was conducted at a shaking temperature of 55°C , which had been adopted for the quantitative analysis of ethanol in blood,⁸⁾ the time for tissue-solubilization was shortened from 1–2 h at 35°C to 30–50 min at 55°C . However, a shaking tem-

Fig. 6. Gas Chromatograms of Head Space Gas of Liver Tissue Samples Containing Toluene

(A): Range 10^2 , attenuation 8.

a: Solubilized liver tissue (liver 1 g per 1 N NaOH 25 ml) 25 ml; b: a+18% (v/v) olive oil 5 ml; c: b+toluene standard solution (8 mg/ml methanol) 50 μ l+internal standard solution (ethylbenzene, 200 mg/ml methanol) 10 μ l.

a): component from liver; M: methanol; T: toluene; E: ethylbenzene.

(B): Range 10, attenuation 2.

a: Solubilized liver tissue 25 ml; b: a+18% v/v olive oil 5 ml; c: b+toluene standard solution (0.08 mg/ml methanol) 50 μ l+internal standard solution (ethylbenzene, 2 mg/ml methanol) 10 μ l; d: a+18% (v/v) olive oil stored for 4 years 5 ml; e: d+toluene standard solution (0.08 mg/ml methanol) 50 μ l+internal standard solution (ethylbenzene, 2 mg/ml methanol) 10 μ l.

arrows: components from old olive oil.

TABLE III. Effect of Storage of Liver Tissue Samples on Toluene Detection

	Storage (d) at -20°C				
	0	1	2	3	7
Rat-A	0.42 ± 0.016	0.40 ± 0.026	0.43 ± 0.017	-	-
Rat-B	0.71 ± 0.037	-	-	0.70 ± 0.035	0.72 ± 0.025

These values are toluene mg/g liver, expressed as the mean $\pm$ S.D. for six samples.

—: not measured.

perature as high as 55°C raises the following problems. 1) Organic solvent gas leaks from the vial through the packing at the time of sampling the head space gas. 2) The large difference of temperature between the head space and the gas syringe gives rise to vari-

ations in the measured value. Therefore, after completely solubilizing the liver tissue at 55°C , this sample was shaken again at 35°C for more than 30 min, and then analyzed. From 1 g of liver tissue containing 0.5 mg of toluene, 99% of toluene was recovered by shaking

at 55°C. The shaking at 35°C gave a recovery of more than 95%. The toluene concentrations found in the liver of a rat that had inhaled toluene were 0.50 mg/g at a shaking temperature of 35°C and 0.47 mg/g at 55°C. Thus, the difference between the above two solubilization temperatures had no significant effect on the measured values.

Although toluene, ethylbenzene and 1,2-dichloroethane were stable during treatment with 1 N NaOH at 35°C for 4 h (Fig. 3), 1,2-dichloroethane in the head space decreased to 62% on changing the temperature from 35°C to 55°C. The concentrations of toluene and ethylbenzene were not changed by this treatment at 55°C. Consequently, by using 55°C for solubilization and ethylbenzene as an internal standard, the time required for analysis was shortened to approximately half of the time in the case of 35°C.

A variation of K values was caused by differences in fat content between the standard and tissue samples (Table I). Therefore, an excess amount of olive oil, as a substitute for fat, was added to both samples in order to reduce this variation. As a result, the peak height of toluene decreased to approximately

one-eighth of the intact value observed in the absence of olive oil. Although the addition of olive oil reduced the detection limit, the added olive oil reduced the difference between the peak height ratios (K value) of toluene to internal standard presented by the two samples. As some unknown peaks from olive oil stored for a long time were detected close to those of toluene and ethylbenzene, it is necessary for the oil to be previously examined for volatile components.

We again examined the utility of chloroform, 1,2-dichloroethane and ethylbenzene for use as internal standards, although they were all suitable for the quantitative determination of toluene in blood or urine.⁴⁾ Only ethylbenzene was suitable for the present method. Chloroform and 1,2-dichloroethane were unsuitable, because their peaks were often hidden in a large peak of methanol and they were unstable in the solution of 1 N NaOH.

When the concentrations of toluene and internal standard (Chart 1) were both raised ten times or reduced to one-hundredth, the present method could cover the wide range of 0.002 to 10 mg toluene per g of tissue.

References and Notes

- 1) The 3rd Annual Meeting of Forensic Toxicology, Tokyo, June 1984.
- 2) S. Tadokoro, "Toxicology," ed. by K. Uruguchi, Y. Ueno, Y. Kasuya, H. Kitagawa and H. Sakai, Chijinshokan, Tokyo, 1979, pp. 296–305.
- 3) S. Tadokoro, *Igaku No Ayumi*, **88**, 300 (1974).
- 4) S. Miyaura, R. Mikawa and H. Isono, *Eisei Kagaku*, **29**, 83 (1983).
- 5) K. Okamoto, *Hanzaigaku Zasshi*, **47**, 104 (1981).
- 6) T. Kojima and H. Kobayashi, *Nippon Hoigaku Zasshi*, **27**, 255 (1973).
- 7) E. Zuccato, F. Marcucci and E. Mussini, *Anal. Lett.*, **13**, 363 (1980).
- 8) "Standard Methods of Chemical Analysis in Poisoning—With Commentary—," ed. by the Pharmaceutical Society of Japan, Nanzando, Tokyo, 1982, p. 64.