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A RAPID ANALYTICAL METHOD FOR MEASURING TOLUENE IN BIOLOGICAL SPECIMENS

R. G. PETERSON* and J. V. BRUCKNER

Department of Neurobiology and Anatomy and Department of Pharmacology, The University of Texas Medical School at Houston, P.O. Box 20708, Houston, Texas 77025 (U.S.A.)

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SUMMARY

A rapid and accurate method for the direct extraction and quantitation of toluene in blood and tissues has been developed. The technique involved extraction with methanol, selective adsorption onto Tenax, and desorption from the Tenax with heat and injection into a gas chromatograph. Measurement of standards indicated values were linear over a range of tissue concentrations of 1–1,734 μg per gram of sample. The procedure described in this paper is a rapid, accurate method for measuring volatile hydrocarbon solvent in both blood and solid tissues of experimental animals. This technique should be applicable to basic research including animal studies, as well as to clinical cases involving abuse of or industrial exposure to solvents.

INTRODUCTION

Techniques currently available for measuring levels of volatile solvents in blood and tissues have a number of inherent problems. Solvent levels have previously been measured either by injecting blood or tissue homogenates directly into a gas chromatograph[1–5], or by allowing an equilibrium of solvent to develop between these specimens and a known volume of air in a closed container before sampling the vapor concentration[1–3,6–8]. Although these methods may be suitable for blood, we have found in our studies with toluene that substantial amounts of solvent were lost during tissue homogenization. In addition, contamination resulting from direct injection of biological materials into the gas chromatograph column will markedly shorten the column's lifetime. In an effort to circumvent these problems, we have developed a technique based upon principles that other investigators have used to measure very low solvent concentrations in urine[9,10] and in water[11].

* Present address for reprint requests: R. G. Peterson, Ph.D., Department of Anatomy, Indiana University School of Medicine, 1100 W. Michigan Street, Indianapolis, Ind. 46202, U.S.A.

EXPERIMENTAL

Evaporation containers

Methanol, as will subsequently be described, was used to extract toluene from blood and tissue samples. The methanol extract was placed onto small pieces of filter paper in a cylindrical PTFE container. The container consisted of a 3/8-in. PTFE tube plugged in both ends with 3/8-in. PTFE rod (Fig. 1). One end of the container was connected to a tube leading to a nitrogen manifold, while the other end had a hole into which a Tenax column (sample column) could tightly fit. Nitrogen was blown through the container over the methanol-impregnated paper into a glass sample column packed with Tenax. Toluene and xylene were selectively delayed in the Tenax sample column at room temperature, whereas little methanol was retained.

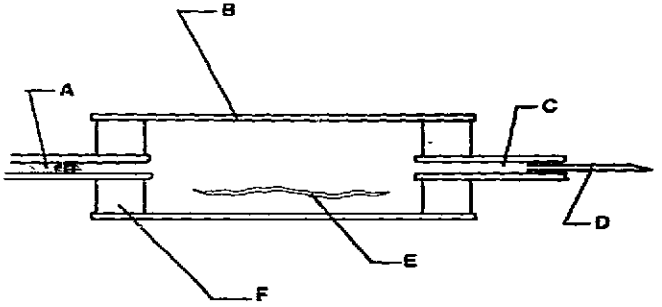

Fig. 1. Diagram of evaporation container utilized in toluene analysis. Nitrogen enters through the needle and PTFE tubing and flows over the filter paper containing the sample and into the Tenax column. A, Glass column (3 mm O.D.) containing Tenax; B, PTFE tubing (3/8 in. I.D.); C, PTFE tubing (1/16 in. O.D.); D, needle (18 gauge); E, filter paper; F, PTFE plug (3/8 in. O.D.).

Tenax sample columns

The sample columns consisted of Tenax (60–80 mesh; Applied Science Labs., State College, Pa., U.S.A.) in Pyrex glass tubes (3 mm O.D., 1.5 mm I.D., and 16 cm in length). Silanized glass wool wads were used on either end of a 3-cm packing of Tenax. Columns were conditioned at the maximum operating temperature (350–370°) in the system before being used.

Equipment

A Varian 940 gas chromatograph, equipped with an Altex automatic sampler (Tefzel slider injection valve with pneumatic actuator), was utilized (Fig. 2). A copper column (150 cm × 1/8 in. O.D.) packed with Tenax 60–80 mesh was used. Flow-rates were 300, 30 and 300 ml/min for helium, hydrogen and air, respectively, while the operating temperatures were: column, 190°; injection port, 210°; flame detector, 230°. The output of the electrometer was monitored with a strip-chart recorder. When analyzing the toluene and xylene content of Tenax sample columns, the sample loop on the automatic sampler was replaced with a holder for the columns (Figs. 2, 3). This mechanism consisted of PTFE tubes which were attached to the automatic sampler via 2 spring-loaded uprights with special seals for the ends of the Tenax sample

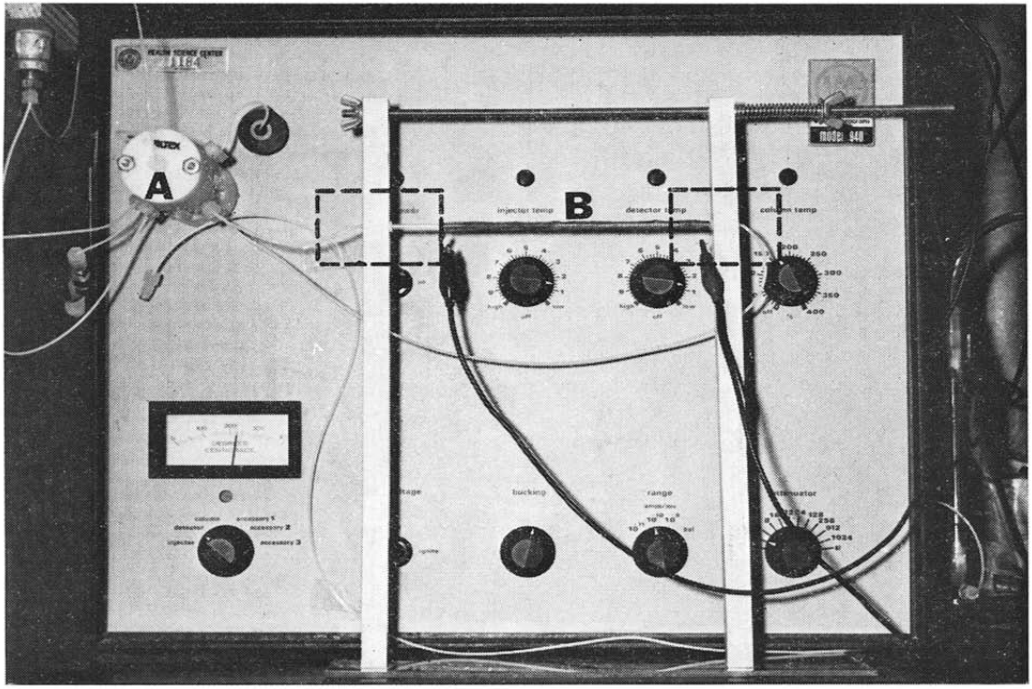

Fig. 2. Frontal view of the gas chromatograph, automatic sampler (A), and Tenax column (B) with heating device used in toluene analysis. The areas outlined in dashed lines are detailed in Fig. 4 to show the seal structure.

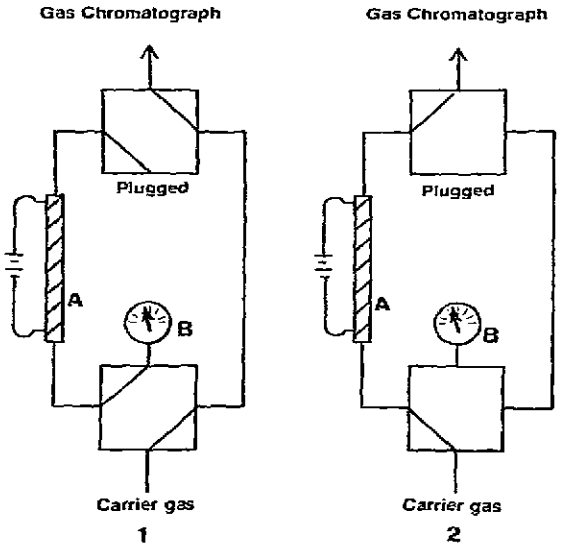

Fig. 3. Diagrammatic representation of the injection valve with the Tenax column in place surrounded by the heating coil (A). In position 1 the column is sealed. When the valve is moved from position 2 to position 1, some pressure is maintained and can be read on the gauge (B) if there are no leaks in the system. The column is heated in position 1. When the temperature is high enough the valve is moved to position 2 and the solvents travel into the gas chromatograph.

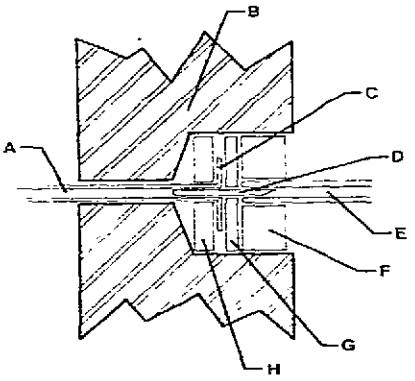

Fig. 4. Detailed drawing of the seal for the ends of the Tenax column. The locations of these areas are shown in Fig. 2 by dashed lines. A, PTFE tubing (1/16 in. O.D.); B, metal frame; C, flange of PTFE tubing; D, needle; E, glass column (3 mm O.D.) containing Tenax; F, PTFE washer; G, fluorosilicon washer; H, metal washer.

columns (Fig. 4). Before the Tenax column was placed onto the apparatus, an electrical coil was placed around the sample column so that it could be heated to release the toluene.

Methanol extraction of toluene

An initial experiment was performed in order to determine the time necessary for extraction of toluene from blood and tissues by methanol. Six mice were injected intraperitoneally with 0.2 ml ($\approx$ 5 μC) of ^{14}C-labeled toluene. Upon induction of anesthesia by the toluene within 5–8 min after injection, blood was drawn by cardiac puncture and the liver and brain of each animal removed. Both blood and tissue specimens were placed into methanol [blood–methanol (1:4, v/v); tissue–methanol (1:4, w/v)]. Methanol aliquots (100 μl) were removed 1/2 h after beginning the extractions. The solid tissues were then crushed with a PTFE rod while within the extraction fluid. Additional 100 μl methanol aliquots were taken from both tissues and blood specimens at 1, 2, 4, 24 and 72 h after the initial extractions were begun. All samples were immediately placed into scintillation fluid for counting by a Mark III (Searle) scintillation counter with a DPM accessory.

Standards, ranging in concentration from 0.2–346.8 μg of toluene per ml methanol, were utilized to determine the recovery of toluene from biological specimens. Each standard also contained a constant quantity of xylene (87 μg per ml of methanol). These concentrations of toluene in methanol are equivalent to tissue toluene concentrations ranging from 1–1,734 μg/g.

One standard, containing 87 μg of toluene per ml of methanol, was added to brain, liver and blood specimens *in vitro* in order to study toluene and xylene recovery. Additional standards containing lower concentrations of toluene were also studied. Portions of brain and liver were crushed while within a vial containing the methanol-based standard [tissue–methanol (1:4, w/v)]. Blood was diluted with methanol-based standard [blood–methanol (1:4, v/v)]. An additional methanol-based standard was diluted with water [water–methanol, (1:4, v/v)] so that peak heights could be directly compared. The biological specimens were permitted to remain in the methanol-based standard for 1 h, before aliquots of methanol were withdrawn for toluene and xylene

gas chromatographic analysis. Absolute peak heights and ratios of peak heights were measured for determination of toluene concentrations.

In another experiment designed to assess toluene recovery, 6 mice were injected intraperitoneally with 0.2 ml ($\approx$ 5 μC) of ^{14}C-labeled toluene. After the animals became anesthetized, they were killed by cervical dislocation. A small portion of the liver from each mouse was excised and divided into two portions. The brain was also removed and cut into two hemispheres. One portion of each organ was weighed and immediately oxidized in a IN 4101 L.S. sample oxidizer (Intertechnique) recovering the original toluene as radioactive CO_2. The other portion was immediately placed into methanol, weighed and diluted (1:4, w/v) with methanol. After each tissue had been permitted to remain in methanol for 1/2 h, the tissue was crushed with a PTFE rod. After allowing an additional 3–4 h for toluene extraction, 100-μl samples of the methanol extract were placed into scintillation fluid for counting. This experiment differed from the preceding *in vitro* study, in that the toluene measured in the tissues were not spiked *in vitro*, but was absorbed by the animal *in vivo*.

Animal treatment

Mice were exposed for 3 h to a vapor concentration of 15 mg of toluene per liter of air (4,000 ppm). Air (5–10 ml) was injected subcutaneously into the backs of the mice 15 min before each sacrifice period. Air samples were removed from the air blebs just prior to sacrifice of the animals after 3 h of toluene exposure and at 2 h post exposure. The mice were sacrified by cervical dislocation, blood obtained by right ventricular cardiac puncture, and tissue specimens rapidly excised.

Measurement procedure

(1) The concentration of toluene in air removed from air blebs was determined by injecting samples directly into the gas chromatograph.

(2) Following cervical dislocation, blood was collected by right ventricular cardiac puncture into a heparinized PTFE syringe. This syringe was subsequently sealed with a PTFE cap until other tissues could be removed and placed into methanol for toluene extraction.

(3) Tissues (0.5–2.0 g) were removed immediately after blood collection. Approximately 2 min were required to remove the liver and brain from each animal. Liver and brain samples were each placed into a glass scintillation vial containing methanol (1:4, w/v). In order to assure adequate toluene extraction into the methanol, each tissue was crushed with a 1/2-in. PTFE rod while within the vial and permitted to remain there for at least 1 h at room temperature.

(4) Blood samples were similarly placed into methanol (1:4, v/v) for extraction.

(5) After the 1-h extraction period, 20-μl portions of each methanol extract were placed into PTFE evaporation containers (Fig. 1). Each container was immediately fitted with a Tenax sample column and placed onto a nitrogen manifold, which was maintained at approximately 1/2 p.s.i. for about 10 min.

(6) Tenax sample columns containing the toluene samples and xylene internal standards were sequentially placed onto the column holder (Fig. 2). After checking the seals of each sample column with pressure (by valving from position 2 to position 1 as illustrated in Fig. 3, pressure is maintained in the line), the sample column was heated for a 30-sec period to about 200° (position 1, Fig. 3). The sample column was

then valved into the input line of the gas chromatograph (position 2, Fig. 3) and heated for about 2 min to a maximum temperature of 370°. The chromatograph output signal was recorded on a strip-chart recorder and the concentrations of toluene calculated by measuring ratios of heights of toluene and xylene peaks and comparing these with a standard curve.

RESULTS

Analyses of a series of toluene standards, in concentrations corresponding to tissue levels commonly encountered, demonstrated that results were well within the linear range of the measurement technique (Fig. 5). A representative series of chromatograms from a standard containing toluene and xylene are shown in Fig. 6. Accurate duplication of standards and samples can be obtained using this technique. It has, however, been found that some preselection of sample columns is necessary to assure uniform peak heights, in that each sample column has individual characteristics. These variations in sample columns are not a problem if some preselection is exercised and if standards are run on the same sample columns as are the samples.

Results of the experiment designed to ascertain the time required to extract toluene from tissue and blood samples with methanol are shown in Table I. Maximal

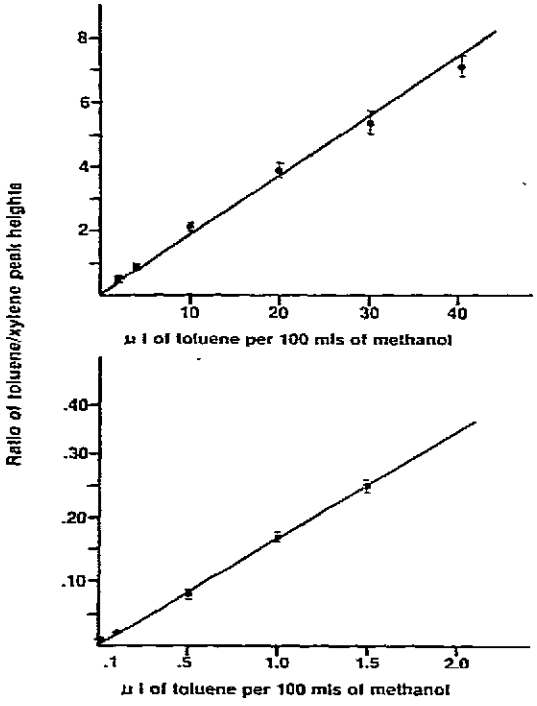

Fig. 5. Standard curve obtained from measurement of a series of standards equivalent to 1–1,734 μg of toluene per gram of tissue. The top graph represents the lower concentration range, while the bottom graph shows the upper range. Brackets encase mean ± the standard error for each concentration of toluene (6 determinations per concentration).

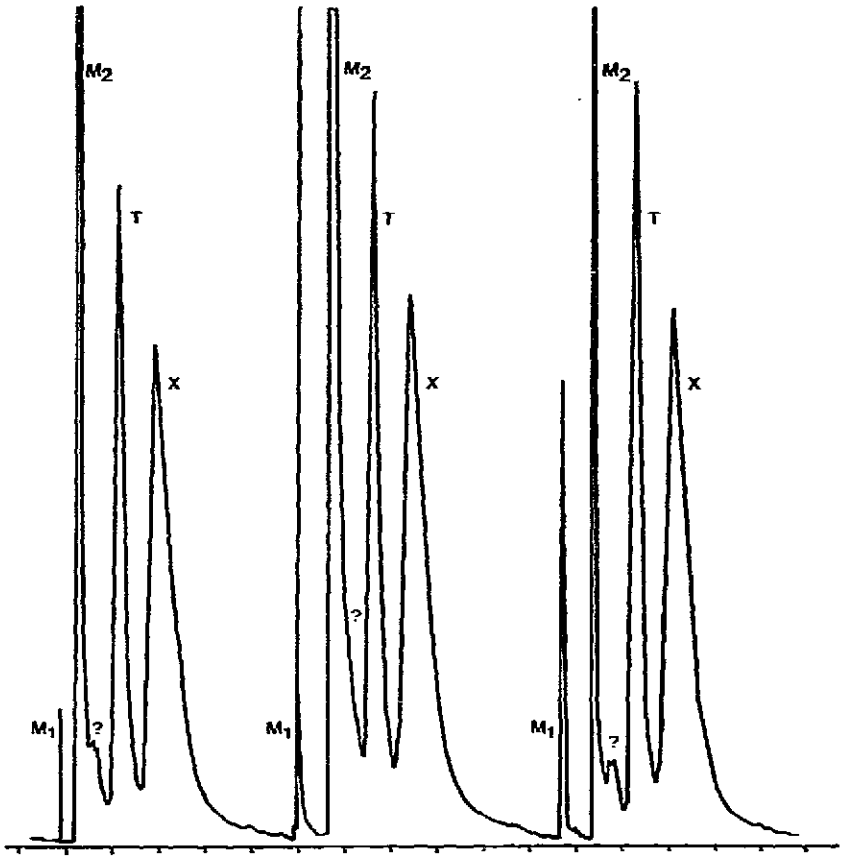

Fig. 6. Representative gas chromatographic tracing in triplicate of a toluene–xylene standard. The M_1 peak is a methanol peak which appears while the sample column is being pressurized for the leak check. M_2 is a methanol peak which appears after the column is heated and valved into the gas chromatograph. The heights of M_1 and M_2 vary, depending upon the exact amount of methanol remaining in the sample column. The small peaks and shoulders following M_2(?) are apparently contaminants of methanol in that they are also evident when assaying methanol alone. Toluene (T) and xylene (X) peaks follow the apparent contaminant peaks. The toluene peak appears approximately 1 min after injection, while the xylene peak appears in approximately 2 min.

extraction occurs within 1/2 h from blood and liver and within 1 h from brain. Additional time in methanol does not enhance toluene recovery from any of the three biological specimens, nor result in toluene loss from the methanol extract.

Results from a study of recovery of toluene and xylene in an *in vitro* system, in which biological specimens were diluted with the methanol-based standard containing both xylene and toluene, are shown in Table II. Toluene and xylene peak heights for blood, liver and brain are not significantly different from standard peak heights, indicating good retention of toluene and xylene by the methanol. The ratios of the respective peak heights also remain quite constant in each case. Good retention is also observed when concentrations as low as 0.87 μg toluene per ml methanol are similarly added to the biological specimens. Analyses performed after 1 week on

TABLE I

TIME SEQUENCE OF TOLUENE EXTRACTION FROM BIOLOGICAL SPECIMENS

Extraction time (h)	*Toluene recovery (dpm per 100 µl of methanol extract) ±S.E. (6 mice)*		
	Blood	*Liver*	*Brain*
½	543.9 ± 16.2	5,638 ± 1,072	578.1 ± 28.9
1	536.1 ± 23.9	5,616 ± 970	754.7 ± 31.1
2	520.9 ± 28.8	5,579 ± 960	741.0 ± 27.1
4	525.2 ± 24.3	5,580 ± 948	724.8 ± 26.4
24	510.9 ± 28.1	5,614 ± 919	706.5 ± 29.5
72	476.2 ± 37.9	5,519 ± 911	661.5 ± 37.7

TABLE II

RECOVERY OF TOLUENE AND XYLENE* FROM BLOOD, LIVER AND BRAIN IN AN *IN VITRO* SYSTEM

Diluent for standard	*Gas chromatographic peak heights*		*Recovery (%)*	*Toluene–xylene peak height ratio***
Water	Toluene	40.76 ± 4.28	100	1.84 ± 0.18
	Xylene	22.38 ± 3.59	100	
Blood	Toluene	45.50 ± 4.64	112	1.95 ± 0.21
	Xylene	23.63 ± 3.28	106	
Liver	Toluene	45.04 ± 4.82	111	1.91 ± 0.21
	Xylene	23.79 ± 3.58	106	
Brain	Toluene	38.71 ± 4.59	95	1.77 ± 0.25
	Xylene	22.29 ± 3.91	100	

* 87 µg of toluene and xylene were added to each ml of methanol.
** Mean ±S.D. of 12 determinations per sample.

methanol aliquots from these samples yield values identical to the original measurements.

Recovery of the toluene, from biological specimens taken from animals dosed *in vivo* with ^{14}C-labeled toluene, was also good. Toluene content was measured by two techniques in this particular experiment: (1) direct oxidation with recovery and measurement of toluene as radioactive CO_2; (2) methanol extraction and measurement of radioactive toluene. Experimental results were as follows: oxidized liver 341.2 ± 60.8; extracted liver 249.6 ± 25.8; oxidized brain 41.7 ± 3.6; extracted brain 38.3 ± 2.9. Values (dpm per mg of tissue) are expressed here as mean ± standard error for groups of 6 animals. For neither tissue was there a significant difference in the quantity of toluene recovered by either technique.

Concentrations of toluene in biological specimens following a 3-h exposure of mice to toluene are shown in Table III. The liver and brain contain substantially higher concentrations of toluene than does the blood, with levels in the liver exceeding those present in the brain. Measurement of toluene levels in animals sacrificed 2 h post exposure reveals that quantities of the solvent have decreased markedly during this time. The concentration of toluene within the air bleb has also decreased during the post-exposure period, although not at so rapid a rate as blood and tissue levels.

TABLE III

TOLUENE CONCENTRATIONS IN MICE EXPOSED TO 4,000 ppm OF TOLUENE VAPOR

Values are expressed as mean ±S.E. for groups of 7 mice at each exposure time.

Exposure time	*Air bleb (mg/l)*	*Blood (μg/g)*	*Liver (μg/g)*	*Brain (μg/g)*
3 h	5.9 ± 0.4	193.9 ± 13.8	639.1 ± 145.9	428.1 ± 104.1
2 h *post*	2.1 ± 0.2	37.4 ± 4.4	115.8 ± 16.4	67.1 ± 6.8

DISCUSSION

The presently described method is a direct quantitative procedure which allows analysis of toluene levels in both blood and solid tissues. The most direct methods which have been previously used are those in which whole blood or tissue homogenates are injected directly into the gas chromatographic column[1–5]. In such cases the column often collects solid debris and must be replaced frequently. The present method involves limited tissue disruption within a closed container, utilizes a tissue extract containing volatile tissue components and avoids toluene evaporation loss which occurs with other procedures. Recovery of toluene from blood, liver and brain is quite good, as demonstrated here in both *in vitro* and *in vivo* systems.

Zlatkis *et al.*[9] have also reported a technique which is capable of demonstrating trace amounts of organics in biological material using Tenax as an adsorbent. Their experimental procedure is quite different from the one described here, principally in that their method is more complex, requires more equipment, and is more time consuming. Our technique requires relatively inexpensive accessories for gas chromatography. The present, simplified procedure can be performed in less than 2 h (including extraction time) with multiple duplicate samples. The number of samples which can be analyzed simultaneously is limited only by the number of Tenax sample columns and evaporation containers available, and by the time required for gas chromatographic analysis.

Our technique is sensitive over a wide range of solvent concentrations. Although the method has not been used for analysis of organic solvents other than toluene, with some modification it should be applicable for measurement of any solvent which is selectively adsorbed onto Tenax. The range of linearity of this technique is apparently quite broad. If the internal standard concentration is adjusted, an even wider range of toluene concentrations in tissue may be measured.

Measurement of toluene levels in air blebs is a useful index of levels of the solvent in blood and tissues. After ratios between toluene concentrations in the air bleb and each biological specimen are established by actual analysis, determination of the air bleb concentration and application of the predetermined ratio provides a non-invasive method for monitoring body solvent burdens. Such a technique is relatively simple and rapid. In more extensive studies which will be reported elsewhere, the air bleb to tissue ratio remains relatively constant throughout solvent exposure, but increases during recovery from exposure. Thus, this technique appears useful in providing estimates of tissue solvent levels, but has limitations which restrict its accuracy.

ACKNOWLEDGEMENT

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