Research Methods

Gas Chromatographic Determination of Methoxyflurane in Blood

R. H. Gadsden, Ph.D., W. M. McCord, M.D., E. F. Woods, Ph.D., E. E. Bagwell, M.S.

Gas chromatography is developing into an excellent tool for the measurement of gases in biological media.[1, 2, 3] However, certain difficulties arise when using this method in the preparation of blood samples for the determination of anesthetic agents of low volatility. It is necessary, for technical reasons, to transfer the agent to be measured from the aqueous (polar) solution in blood to an organic solvent (nonpolar) solution for injection onto the special chromatographic column. We propose a method for isolating methoxyflurane • from small quantities of blood by a distillation technique, its dehydration, and subsequent measurement by gas chromatography. The isolation, detection, and quantitative measurement may be accomplished within 30 minutes. Accuracy, based on experiments in reproducibility and recoveries, is good. The volume of sample (blood) required is small: 4 ml. for human beings and 2 ml. for dogs.

Apparatus

The gas chromatograph utilized in this investigation was a Beckman Model GC-2 with a thermal conductivity detector and Bristol Dynamaster recorder. All chromatograms were carried out at the following instrument settings: temperature, 70° C.; attenuation, 1; detector current, 250 ma. The column was the Beckman No. 17296 of which we used a 4-foot length for purposes of chromatography. This is a ¼-inch copper tubing which has hexadecane as the fixed phase on a supporting column of 30–50 mesh firebrick. Helium was used as the carrier gas and maintained at 40 p.s.i. throughout. The Beckman micro liquid sampler (Beckman No. 22400) was used for injection of samples into the gas chromatograph apparatus.

An all-glass distilling apparatus (fig. 1) was used for distillation of the samples. The method of distillation is described under the methods section.

Method and Materials

Separation of methoxyflurane from blood was accomplished by a distillation technique. (Attempts at an extraction method resulted in removal of blood lipids and chromogens. This led to eventual gross, permanent contamination of the chromatograph column.) Distillation, or transfer, of volatile organic materials (alcohols) from blood by aeration and distillation has been accomplished by a number of workers.[4, 5, 6] However, with the column and the selected thermal conductivity detector, an anhydrous sample is essential for chromatography. We accomplished the separation of the small amount of methoxyflurane present in the sample by addition of iso-amyl alcohol and by distilling the mixture through a Vigreaux column set up for reflux fractionation and through a water cooled condenser arranged for condensation and collection (fig. 1). The organic fraction of distillate was dehydrated before injection on the chromatograph column. With this technique, considerable fractionation occurred, all the iso-amyl alcohol and methoxyflurane distilled

• 2,2-dichloro-1,1-difluoroethyl methyl ether.

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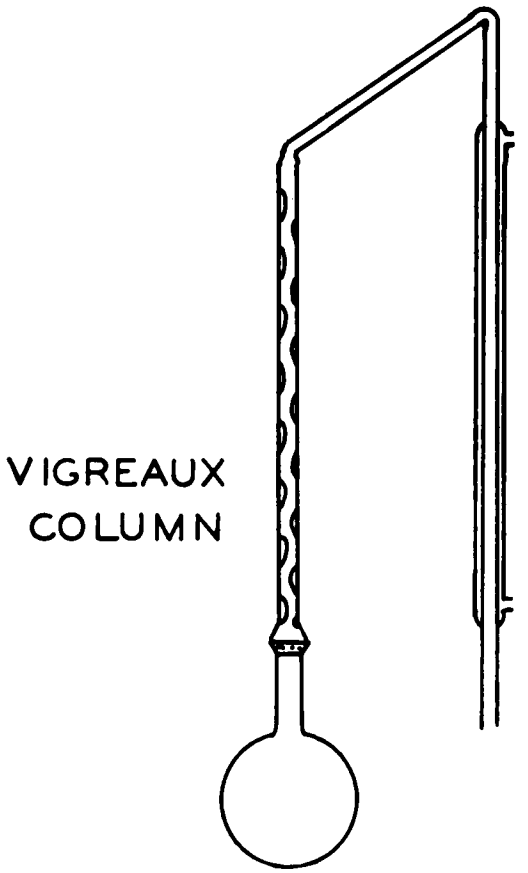

FIG. 1. Vigreaux distilling column: distilling flask 50 ml. capacity, S. T. 14/20; Vigreaux column 30 cm.; water jacket of condenser 20 cm. Ball and socket joint employed between Vigreaux column and condenser for greater mobility.

over, and a minimal amount of water collected with the distillate.

Oxalated blood was collected for all analyses. Care was taken to avoid excess suction during withdrawal of the sample. Specimens obtained by cannulation were collected by hydrostatic pressure and gravity flow. If distillation of the samples was not carried out immediately, they were stored under refrigeration. Daily analysis of the same blood specimen stored at 4° C. revealed that methoxyflurane was stable in blood for at least five days.

Reagents:

Sodium tungstate: 100 Gm. anhydrous Na_2WO_4 and 100 Gm. anhydrous Na_2SO_4 were dissolved and diluted to 1 liter with distilled water.

Sulfuric acid: 200 Gm. anhydrous Na_2SO_4 were dissolved and diluted to 1 liter with N H_2SO_4.

Iso-amyl alcohol: Practical or reagent grade was redistilled and checked for purity by gas chromatographic analysis until only the single peak of iso-amyl alcohol was obtained at minimal attenuation. Five kilograms of the original alcohol yielded 1–1.5 kg. of the purified material for distillation and chromatography.

K_2CO_3: anhydrous, reagent grade.

Methoxyflurane (Penthrane, compound No. DA-759, Abbott Laboratories): see section on preparation of standards.

Procedure

Distillation and Chromatography

Blood Samples. 4 ml. each of sodium tungstate and sulfuric acid reagents were pipetted into the round-bottom distilling flask. Several small glass boiling beads were added, and mixing was accomplished by swirling. Next in order were pipetted 2 ml. of blood and 0.25 ml. of iso-amyl alcohol reagents. (The volume of sample employed depends strictly upon the blood level of anesthetic. The aforementioned aliquot was most convenient for maintenance anesthetic levels in the dog experiments cited; in experiments in human beings, 4-ml. samples were used.) The flask was stoppered, swirled for thorough mixing of the contents and allowed to stand five minutes to effect complete precipitation of the blood proteins. This, subsequently, prevented foaming of the mixture when heat was applied for distillation. The flask was attached to a Vigreaux column by a water seal. A small tube, 5–6 mm. inside diameter and 7.5–8 cm. long, was set directly under the delivery tip of the condenser as a distillate receiver. A low flame from a microburner was applied to the flask until the dense white vapor, consisting mainly of iso-amyl alcohol and methoxyflurane, collected in the upper portion of the Vigreaux column. At this point the rate of heating was increased to accomplish the distillation. The distillate was collected until the receiving tube was filled to approximately 95 per cent of its length. The upper layer

of distillate (consisting of iso-amyl alcohol, methoxyflurane, and a trace of water) was transferred with the aid of a rubber bulb pipet to a small (1 ml. capacity) glass-stoppered flacon. Dehydration of the sample obtained was accomplished by the addition of a small amount (10–15 mg.) of anhydrous potassium carbonate. The flask was stoppered and allowed to stand for 15 minutes before applying to the chromatograph column. A 20-μl. aliquot of the mixture was injected on the chromatograph column under the conditions stated in the section on apparatus. The relative elution peaks for methoxyflurane and iso-amyl alcohol are shown in figure 2. In order to obtain the iso-amyl alcohol peak on this recording, it was necessary to increase the attenuation to 200. (With "ageing" of the column, the peak distance for iso-amyl alcohol becomes shortened, but the peak distance for methoxyflurane is not reduced. Life span of the *n*-hexadecane column under these conditions was approximately 150 analyses.) Routinely, the recorder was removed from the system subsequent to the elution of methoxyflurane. Under these conditions a period of 19–20 minutes was allowed for complete elution of the iso-amyl alcohol and return to stable base-line at maximal sensitivity before injection of the next sample.

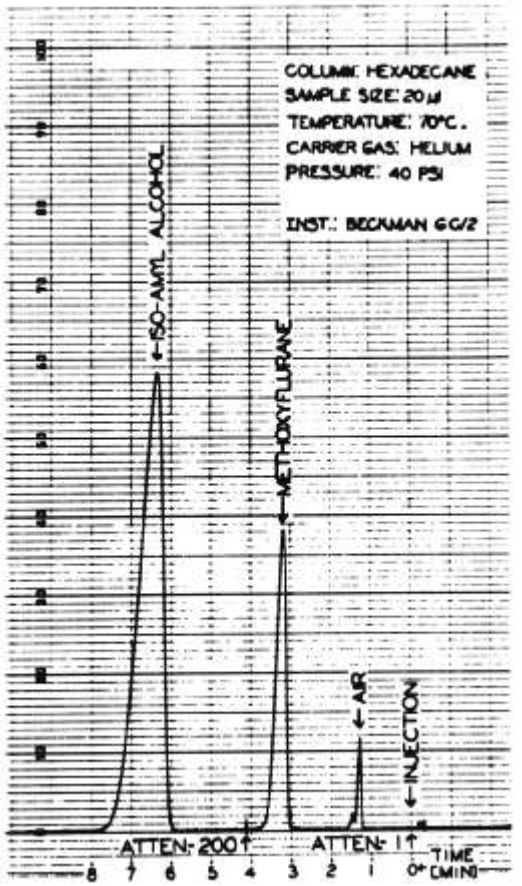

FIG. 2. Recorder tracing to illustrate relative elution peaks for air, methoxyflurane and iso-amyl alcohol.

PREPARATION OF METHOXYFLURANE STANDARDS

Purification of Methoxyflurane. The anesthetic grade of methoxyflurane was distilled from an all-glass Vigreaux (3-foot column) distilling apparatus, collecting the fraction boiling at a constant 105° C. The purity of the distillate was checked by gas chromatographic analysis until the single peak of methoxyflurane was obtained at minimum attenuation. The specific gravity (w/v) of the distillate was determined after purity was assured by this method.

PREPARATION OF STANDARDS

A stock standard (560 mg./100 ml.) was prepared by addition of 0.4 ml. of the methoxyflurane to approximately 50 ml. distilled water in a 100-ml. flask. Dissolving of the methoxyflurane was aided by the addition of 1 ml. of Span 80 (Atlas Powder Co., Wilmington, Delaware). Span 80 is a surface active agent of high molecular weight that permits preparation of aqueous solution of the relatively water insoluble methoxyflurane. It did not interfere with the distillation or chromatography of the methoxyflurane and iso-amyl alcohol. The mixture was diluted to a final volume of 100 ml. with distilled water. Working standards were prepared by diluting 10, 7.5, 5, and 2.5 ml. of the stock standard to 100 ml. with distilled water yielding standards of 56, 42, 28, and 14 mg./100 ml., respectively. (The concentration of the stock standard would depend upon the specific gravity of the anesthetic. The standards here were based upon a specific gravity of 1.4046 at 29° C. for the purified methoxyflurane.)

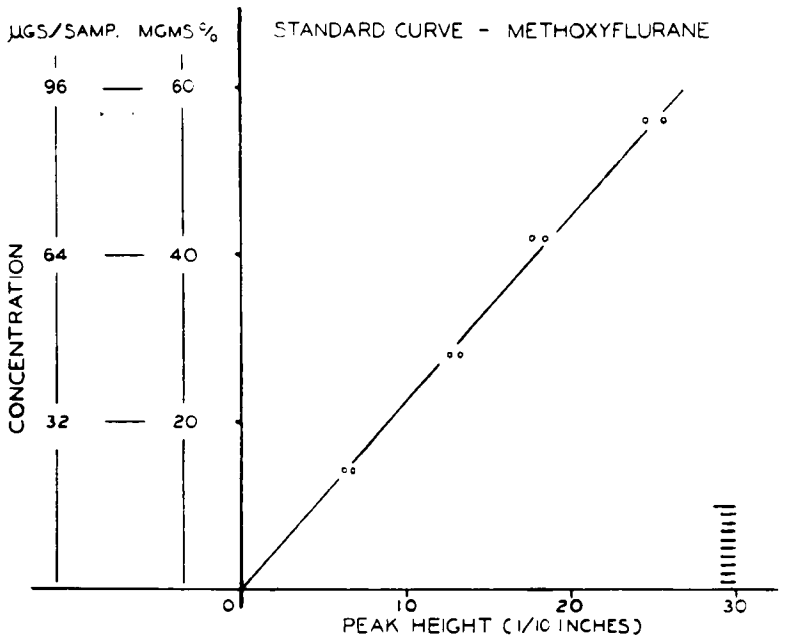

FIG. 3. Standard gas chromatogram curve for methoxyflurane concentration: mg./100 ml. represents concentration of original sample; micrograms per sample represents absolute amount per 20 µl. injected on chromatograph column. Peak height is in one-tenth inch units.

STANDARDIZATION

Two milliliters of the working standards were treated in the same manner as described in the distillation technique for blood samples. After allowing an adequate period for dehydration, 20-µl. aliquots were injected on the chromatograph column. Great care was taken to develop the injection technique: rapid insertion of the needle into the injection port; immediate, forceful, rapid delivery of the sample into the apparatus; and immediate withdrawal of the needle. A standard curve based upon peak height analysis is shown in figure 3. Each of the points represents a separate distillation of the respective working standards. Curve area analysis yielded no greater consistency of duplication; thus we utilized the peak height method of calculation. Before each run of blood analyses two working standards (56 and 28 mg./100 ml.) were distilled and chromatographed. These peak heights were used for subsequent calculation of blood concentrations. Any blood sample distillate attaining a recorder peak height greater than 35 was redistilled, using a 1 ml. or less aliquot of the original blood sample. With a recorder peak height obtained less than 5, the distillation was repeated on 4 ml. or more of the original sample. With these manipulations, peak heights were kept within the limits of the standard curve.

Results

REPRODUCIBILITY AND RECOVERY EXPERIMENTS

Experiments were carried out to determine the reproducibility of the method. Results of these experiments are shown in table 1. Blood samples of "low" (less than 15 mg./100 ml.) and "high" (15–75 mg./100 ml.) concentration were distilled in triplicate. Each peak height represents a single injection of one of the triplicate distillates on the chromatograph column. Concentrations were calculated with respect to peak heights obtained for standards in the aforementioned manner. Compared to the average blood concentration for each set of experiments, the variation in reproducibility was within ± 5 per cent.

Recovery experiments were performed to test the adequacy of distillation and subsequent chromatography of the distillate. Results of these experiments are shown in table 2. Blood was pooled from several animal experiments. The pooled samples were chromatographed and methoxyflurane concentra-

tions determined. The concentrations (total) noted in the table are stated in absolute terms and refer to the micrograms of anesthetic per 20-μl. aliquot of distillate injected on the column. Two milliters of blood (15, 22 and 54.5 mg./100 ml., respectively) were mixed with 2 ml. of working standard (14 and 28 mg./100 ml.), distilled and chromatographed. Each of these experiments was carried out in triplicate. Variations in the method of distillation and chromatography based upon recovery experiments were within ± 5 per cent.

TABLE 1. Reproducibility of Method*

Sample	Peak Height (1/10 inches)	Concentration (mg./100 ml.)	Average Concentration (mg./100 ml.)	Percentage of Average Concentration
A—Samples of "low" methoxyflurane concentration (4 cc. distilled):				
I	5.1	5.8	5.97	97.2
	5.4	6.2		103.9
	5.2	5.9		98.8
II	8.1	9.3	9.00	103.3
	7.7	8.8		97.8
	7.8	8.9		98.9
III	9.7	11.1	11.50	96.5
	10.3	11.8		102.6
	10.2	11.6		100.9
B—Samples of "high" methoxyflurane concentration (2 cc. distilled):				
I	7.9	18.0	18.17	99.1
	8.2	18.7		102.9
	7.8	17.8		98.0
II	14.3	32.6	31.63	103.1
	13.7	31.3		99.0
	13.6	31.0		98.0
III	17.6	40.2	41.26	97.4
	18.4	42.0		101.8
	18.2	41.6		100.8
IV	22.7	51.8	53.90	96.1
	23.9	54.6		101.3
	24.2	55.3		102.6

* Each peak height cited represents a separate blood sample distillation and chromatographic elution of that distillate.

TABLE 2. Recovery Experiments

Sample	Concentration (μg.)	Added (μg.)	Total* (μg.)	Found (μg.)	Recovery (per cent)
Ia	24.0†	22.4‡	46.4	44.4	95.7
			46.4	47.4	102.2
			46.4	48.1	103.7
Ib	24.0	44.8‡	68.8	67.2	97.7
			68.8	69.8	101.5
			68.8	66.2	96.2
IIa	35.2†	22.4	57.6	57.9	100.5
			57.6	59.9	104.0
			57.6	55.7	96.7
IIb	35.2	44.8	80.0	76.2	95.3
			80.0	83.7	104.6
			80.0	81.8	102.3
IIIa	55.2†	22.4	77.6	81.3	104.8
			77.6	74.6	96.1
			77.6	77.8	100.3
IIIb	55.2	44.8	100.0	95.8	95.8
			100.0	96.5	96.5
			100.0	104.2	104.2

* Micrograms methoxyflurane per 20-μl. aliquot of distillate (from blood + working standard) chromatographed.

† This value represents micrograms methoxyflurane per 20-μl. aliquot of distillate chromatographed. (Original blood concentrations 15.0, 22.0 and 34.5 mg./100 ml., respectively. Two milliliters distilled.)

‡ This value represents added micrograms methoxyflurane per 20-μl. aliquot of distillate chromatographed. (Original working standard concentrations 14.0 and 28.0 mg./100 ml., respectively. Two milliliters added to blood and distilled.)

ANIMAL EXPERIMENTS

Eight mongrel dogs were subjected to inhalation of increasing concentrations of methoxyflurane. Arterial and venous blood samples were withdrawn 30 minutes after the animals were placed on the next higher inspired concentration of the anesthetic. Utilizing the analytical technique described, these samples were distilled and chromatographed. Another series of experiments was carried out in which 12 dogs were subjected to inhalation of a constant level (0.5 per cent) of methoxyflurane. Arterial and venous samples were taken at various time intervals and analyzed for methoxyflurane content. Ana-

lytical data obtained for dogs correlated well with values reported by Chenoweth and associates.[7] The method of analysis employed by these workers was that of infrared spectroscopy. Correlation of measured blood concentrations of methoxyflurane with the blood vascular parameters of blood pressure and ventricular contractile force appear elsewhere.[8]

A limited number of human blood analyses have been performed to date. Blood samples were obtained by venipuncture of the left external jugular. Utilizing a 0.5 per cent inspired concentration of methoxyflurane, the venous blood levels attained ranged from 2.7 to 12.5 mg./100 ml. (12 samples). These values were in agreement with data reported by Walts *et al.*[9]

Summary

A method of distillation of blood for the isolation of methoxyflurane and its subsequent gas chromatographic detection and measurement was described. The described distillation method is recommended for isolation of agents of low volatility from biological media as a preparative method for gas chromatography.

Accuracy, based upon experiments in method reproducibility and recoveries, was within ± 5 per cent. These data were not selected and represent a testing of the method from the standpoint of chromatographic conditions (type column, column temperature, injection technique, carrier gas pressure, attenuation) as well as adequacy of sample distillation, distillate dehydration and subsequent elution and detection.

Limited data on human subjects are mentioned. These agreed with results reported by other investigators.

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