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ROUTINE DIRECT INJECTION GAS—LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE ANALYSIS OF VOLATILE HALOGENATED ANAESTHETICS IN WHOLE BLOOD USING A NEW EXTERNAL INJECTION PORT*

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SUMMARY

This communication describes the design and construction of a new external injection port for the direct gas—liquid chromatographic analysis of volatile compounds in whole blood. Aliquots (4—40 μl) of EDTA anti-coagulated blood containing the volatile compound and a weighed quantity of the internal standard, isobutanol, were injected into the disposable glass wool filter of the heated injection port. The volatiles released were led by means of the carrier gas stream directly onto the chromatographic column. Typical data are presented from chromatography performed with dual 6 ft. × 2 mm I.D. glass columns containing Chromosorb 101 programmed from 110—180° at 6°/min and the external injection port maintained at 180°. The method eliminated the problems usually associated with direct injection methods and permitted the accurate analysis of halothane, methoxyflurane, diethyl ether and ethanol over the approximate range 1—100 mg%. Using this analytical procedure the distribution of halothane between the cells and plasma of human blood at 4° was found to be 2.0 ± 0.2.

INTRODUCTION

Gas—liquid chromatographic procedures for the quantitative analysis of

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volatile anaesthetics in blood or other fluids [1—31], are of two types: either indirect, in which the anaesthetic is separated from the blood prior to injection, or direct, in which the gas chromatography is performed on a sample of whole blood. The indirect techniques (head space methods [1—7], solvent extraction [8—22] and distillation [23,24]) are time consuming [1,2,6,28,30,31], require complicated pre-treatments [29] involving a potential loss of the agent [30] and may be difficult to apply to small samples [30]. Furthermore, solvent extraction procedures may be complicated by the deposition of lipid materials on the columns [2] and by the production of solvent peaks of large area which may interfere with the estimation of the anaesthetic peak and/or result in prolonged elution times [11,14,16]. Direct injection methods [25—31], in contrast, are rapid and apparently simple [16] and can be used with small samples of blood [1]. They may, however, suffer from problems associated with contamination of the columns with non-volatile blood components [2,6,30] and subsequent baseline drift due to the slow elution of such components [2,30], clogging of the syringes used for injection [6,30], distortion and broadening of the peaks [2,6,16,30], ghost peaks [6,30] and poor reproducibility [1,2].

In this paper we describe the design and construction of an external injection port which allows the injection of a small sample of whole blood into the pre-heated carrier gas stream of the gas chromatograph. Flash evaporation removes the non-volatile components and only the volatile components enter the column. This external injection port in combination with the use of an internal standard avoids the problems discussed above and permits the rapid analysis of halothane, diethyl ether, methoxyflurane and ethanol in whole blood over the approximate range of 1—100 mg%.

MATERIALS AND METHODS

Halothane (Hoechst Pharmaceuticals, Montreal, Canada), methoxyflurane (Abbot Laboratories, Vancouver, Canada) and diethyl ether (spectroanalysed; Fisher Scientific, Montreal, Canada) were used without further purification. Isobutanol (reagent grade; Mallinckrodt, St. Louis, Mo., U.S.A.) was dried over anhydrous potassium carbonate and purified by fractional distillation [32]. Ethanol was dried by refluxing with magnesium turnings and purified by distillation [32]. All other chemicals employed were of reagent grade or better. Silicone rubber O-rings 3/16 in I.D. and 5/16 in O.D. HT8 low bleed septa were obtained from Applied Science, State College, Pa., U.S.A. Chromosorb 101, 103, 105, and 107 were purchased from Johns Manville, Denver, Colo., U.S.A. and Reacti-vials and Mininert valves from Pierce, Rockford, Ill., U.S.A.

The external injection port

Design

The design of the external injection port is shown diagrammatically in Fig. 1; Fig. 2 shows detailed engineering drawings. The external injection port consists essentially of a heat reservoir and a rotary valve both of which

contain channels for directing the flow of carrier gas. Both the reservoir and rotary valve are constructed of brass, their respective plane face plates being separated by a 1/32-in. Teflon gasket against which the valve rotates. Mounted on the front of the rotary valve are: (a) a spring which can be used to tighten the valve against the Teflon gasket to achieve a gas tight seal, (b) a gas chromatographic injection port of conventional design sealed with a septum and (c) a pair of handles which allow rotation of the valve. The reservoir is maintained at 180° with a heater set into a well drilled into the body of the reservoir; a similar well contains a thermometer. The base of the port is attached, by a gas tight seal, to the injection port system of the gas chromatograph and can be readily modified for use with a variety of instruments. The carrier gas enters the port through a Swagelok fitting sealed into the side of the reservoir; this requires a diversion of the carrier gas stream prior to the point where it enters the original injection port of the gas chromatograph.

Fig. 3 illustrates the flow paths of the carrier gas within the external injection port when the rotary valve is in the "on" operational and the "off" non-operational positions. In the "on" position the carrier gas passes successively through the fixed channels a and b located, respectively, in the body of the heat reservoir and the rotary valve. It then proceeds via the fixed channel c located in the reservoir, into a glass U-tube (3/16 in. O.D.) connecting c with the fixed channel d located in the body of the reservoir. The carrier gas then passes through fixed channels e and f located, respectively, in the rotary valve and the reservoir and enters the gas chromatography column through the base of the injection port. The U-tube is located on the left side (see Fig. 2) and is sealed into position with silicone rubber O-rings lightly lubricated with a high-temperature vacuum grease. Before insertion the ends of the U-tube were also lubricated with the same vacuum grease. A lever attached to the reservoir (see Fig. 2) prevents the ejection of the U-tube when the valve

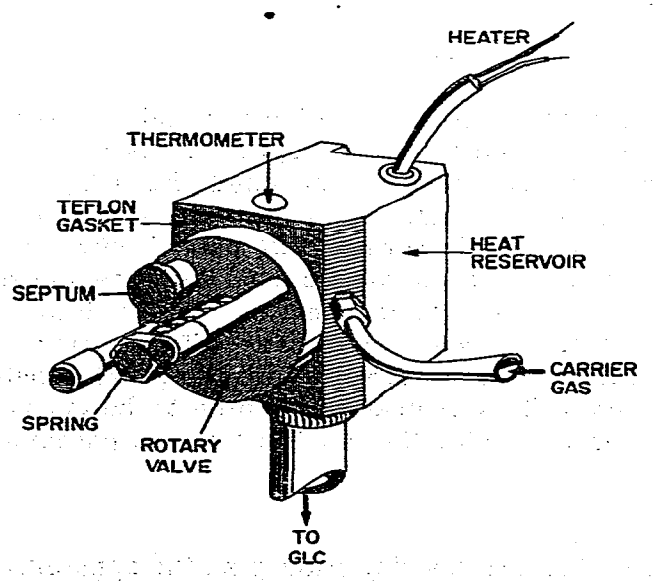

Fig. 1. Diagrammatic representation of the front view of the external injection port.

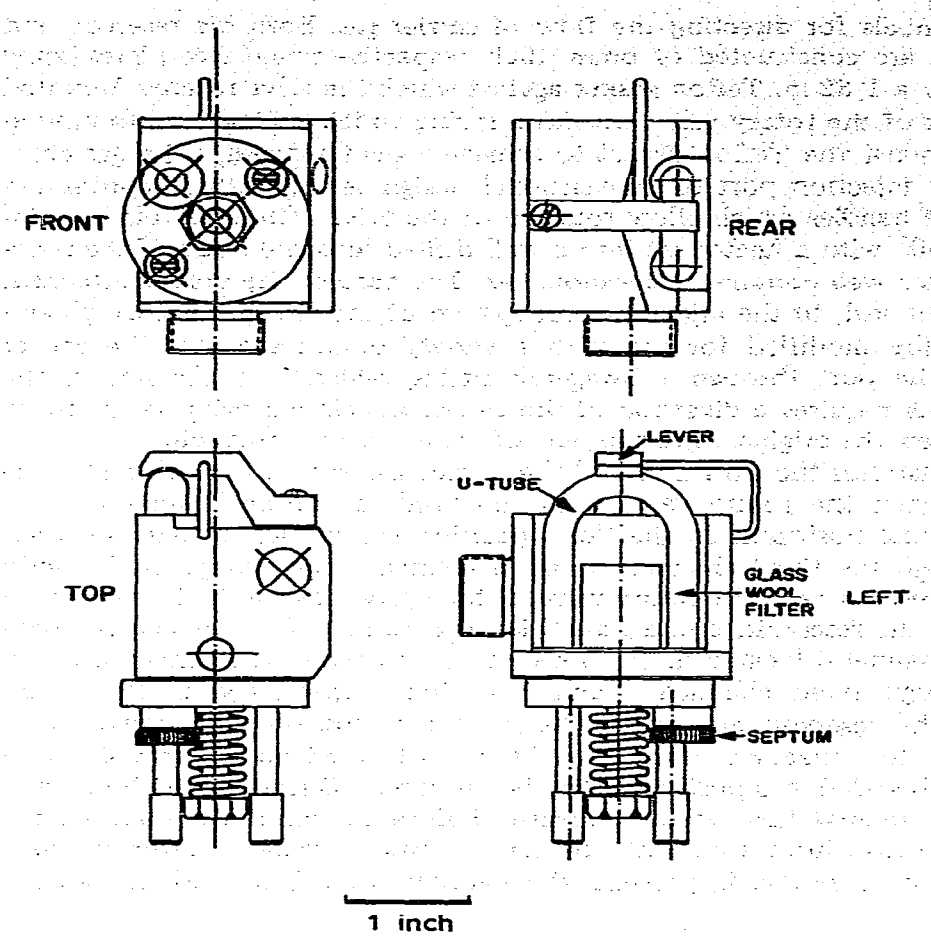

Fig. 2. Engineering drawings of the external injection port.

is in the "on" position (approximate pressure 20—25 p.s.i.) and also serves to maintain a gas tight seal. Holes situated in the appropriate positions in the Teflon gasket allow free passage of gases between the fixed channels in the reservoir and the rotary valve. When the valve is in the "on" position the needle of a Hamilton gas chromatographic syringe will pass through both the septum of the injection port mounted on the front of the valve and the Teflon gasket and enter the U-tube permitting injection directly into the pre-heated carrier gas stream. When the valve is in the "off" position the U-tube is by-passed and the carrier gas passes directly into the gas chromatograph through channels a, b and f. This enables the U-tube to be changed simply and rapidly without interfering with the gas flows in the chromatograph.

Operation

Prior to the first operation of the external injection port the apparatus is maintained at 180° with a pressure on the Teflon gasket of 15—20 pounds for

24—36 h. Under these conditions the Teflon gasket softens and is moulded into the shape of the inside surfaces of the rotary valve and heat reservoir. This ensures a good seal and provided the reservoir is maintained at 180° remoulding is only necessary when a new gasket is fitted.

With the rotary valve in the "on" position a sample of blood (4—40 μl) is injected directly into a loose glass wool filter plug inserted into the U-tube. To avoid clogging of the needle of the syringe by coagulated blood components injection was accomplished with a chaser technique in which the blood sample in the syringe barrel is separated from a plug of water by a bubble of air. When the analysis is completed the rotary valve is switched to the "off" position and the U-tube is replaced. On switching to the "on" position the equipment is ready for another injection. Changing the U-tube occupies between 15 and 60 sec.

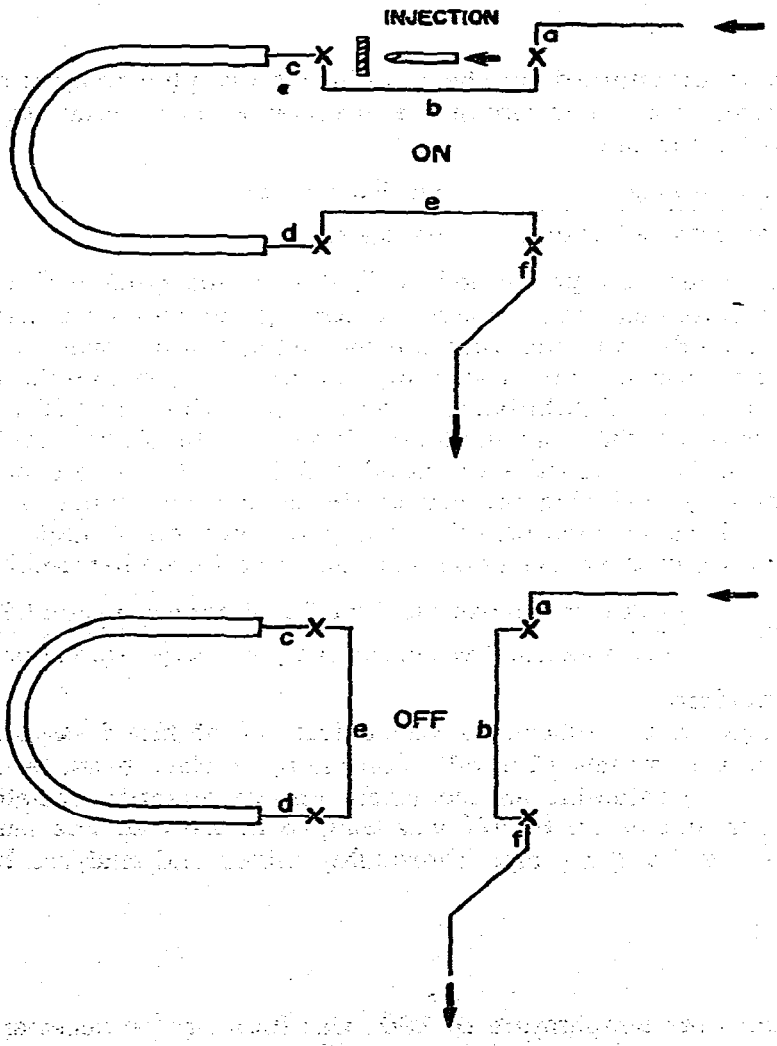

Fig. 3. Schematic carrier gas flow diagram of the external injection port.

Gas chromatography

Gas chromatography was performed on a Hewlett-Packard 7610A gas chromatograph fitted with dual flame ionisation detectors using dual 6 ft. × 2 mm I.D. glass columns containing Chromosorb 101 (80—100 mesh) programmed from 110—180° at 6°/min. The injection port of the chromatograph was maintained at 200°, the external injection port at 180° and the flame ionisation detector at 250°. Gas flow-rates employed were as follows: carrier 25 ml/min; auxillary 35 ml/min, hydrogen 50 ml/min and air 470 ml/min. Both helium and nitrogen could be used as carrier gas. Range resistance of the electrometer was set at $10^8\ \Omega$.

Peak areas were measured with a Hewlett-Packard 3370B electronic integrator operated at Up and Down slope sensitivities of 0.1 mV/min. Injection volumes were chosen to yield a minimum anaesthetic peak area of $5 \times 10^4\ \mu V$ sec.

Analytical methods

Response factors were determined by the gas chromatographic analysis of solutions of known composition containing the agent and the internal standard isobutanol with the equation:

$$\text{Response factor} = \frac{\text{peak area agent}}{\text{peak area isobutanol}} \times \frac{\text{wt. isobutanol}}{\text{wt. agent}}$$

Analyses of blood samples were performed on EDTA anti-coagulated blood using Reacti-vials each equipped with a magnetic stirring bar and a Mininert valve. Water was added to the vial such that the remaining volume, when the vial was filled to the rim, was 0.5 ml. A standard solution (50 μl) containing an accurately known quantity of isobutanol in water (approximately 260 mg per 100 g) was added and the vial was completely filled with the blood sample to be analysed (450 μl). The quantities of blood and internal standard employed were established by weighing the vial at the appropriate times. The samples were mixed and gas chromatography was performed on aliquots of 4—40 μl. The concentration of the anaesthetic was calculated from the formula:

$$\text{Anaesthetic concentration} = \frac{\text{peak area anaesthetic} \times \text{wt. I.S.} \times \text{concentration I.S.}}{\text{peak area I.S.} \times \text{wt. blood sample} \times \text{response factor}}$$

where I.S. = internal standard.

The effect of storage on the halothane concentrations of blood samples was tested as follows. Reacti-vials (1.0 ml), containing a glass bead, were filled with blood samples, containing an accurately known quantity of halothane, in such a manner that no air bubble was trapped in the vial. The samples were stored at 4° for 7 and 14 days, thoroughly mixed and analysed by gas chromatography.

RESULTS

An external injection port temperature of 180° was found to be necessary to maintain a gas tight seal between the Teflon gasket and the face plates of

the rotary valve and the heat reservoir. Under these conditions the glass wool plug served the dual function of providing a large, heated surface area for evaporation of the volatiles and of a trap for the non-volatile components. The rate of evaporation was presumably controlled by both the pre-heating of the carrier gas stream in the body of the heat reservoir and the dispersion of the injected blood sample. When liquid was deposited on a portion of the U-tube free of glass wool the fluid beaded and evaporated slowly, a phenomenon accompanied by peak broadening on gas chromatography. Successive injections of volumes of blood up to a total of 10 μl could be made before it was necessary to insert a fresh U-tube but use of samples of between 10 and 40 μl necessitated the insertion of a fresh U-tube after each injection. The O-rings used to seal the U-tube into the port gave some bleeding problems when new. These could be eliminated, however, by heating the rings for 16 h at 160° before insertion into the port. The effective life of the rings was improved by light lubrication with a high-temperature vacuum grease. Routinely the septum of the injection assembly, mounted to the front of the rotary valve, was changed once a week; it could, in any case, be used for at least 35—40 injections.

Preliminary experiments were conducted to evaluate the use of methanol, ethanol, *n*- and isopropanol, *n*-, *sec*-, *tert*- and isobutanol and isoamyl alcohol as internal standards for the estimation of halothane and methoxyflurane on one or more of the column packings Chromosorb 101, 103, 105, or 107 under either isothermal or temperature-programmed conditions. Isobutanol was found to be a cheap and convenient internal standard since it could be readily purified, was soluble in blood at the concentrations employed and had a retention time intermediate between that of halothane and methoxyflurane; it also proved to be a good internal standard for both ethanol and diethyl ether. The use of a temperature programme resulted in the splitting out of the water peak from the halothane peak permitting accurate measurements of peak area. Chromosorb 101 was the preferred packing material since it gave little or no bleed and was very stable; the column has been in routine use for more than 12 months. Furthermore Chromosorb 101 can be employed, under the same operating conditions, for the rapid analysis of halothane, methoxyflurane, ethanol and diethyl ether. Using the conditions specified above the system exhibited little or no baseline drift. Such drift when it occurred could be corrected by a single blank program run or by a short period of conditioning at 180°. Table I lists the sensitivity of the flame ionisation detector and the characteristics of the electrometer and integrator when halothane, methoxyflurane, ethanol, diethyl ether and isobutanol were analyzed as described above. Fig. 4 shows sample chromatograms obtained from these analyses.

Table II shows the results obtained by analysis of blood samples containing accurately known weights of halothane, methoxyflurane, diethyl ether and ethanol over the approximate concentration range 1—100 mg%. As can be seen excellent recoveries were obtained over the entire concentration range for all the compounds investigated. Reacti-vials proved to be efficient for storing blood samples containing halothane for periods as long as two weeks at 4°. Such storage permits efficient staging of analyses when the experimental design requires multiple samplings.

TABLE I

PERFORMANCE CHARACTERISTICS OF THE FLAME IONISATION DETECTOR, ELECTROMETER AND ELECTRONIC INTEGRATOR

These performance characteristic data correspond to peaks A–E of the sample chromatograms in Fig. 4.

Performance characteristic	Isobutanol	Halothane	Methoxy-flurane	Diethyl ether	Ethanol
Concentration (mg%)	11	98	52	60	56
Volume of injection (μl)	15	15	40	10	10
Detector output at maximum (A)	2.56×10^{-10}	3.44×10^{-10}	4.18×10^{-10}	6.5×10^{-10}	9.61×10^{-10}
Integrator input at maximum (mV) (=electrometer output)	2.56	3.44	4.18	6.50	9.61
Peak area (μV·sec)	7.422×10^{4}	1.363×10^{5}	9.543×10^{4}	1.222×10^{5}	2.354×10^{5}

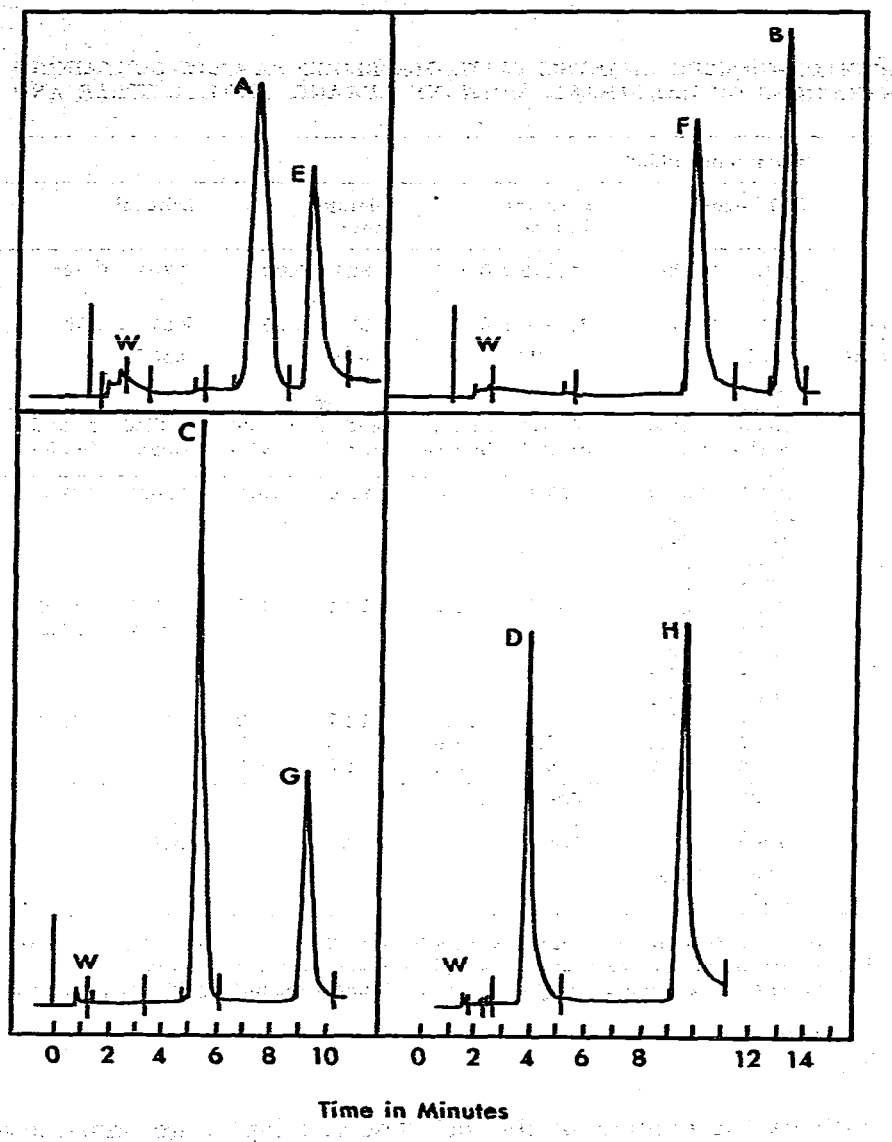

Fig. 4. Sample chromatograms from the analysis of halothane (A), methoxyflurane (B), diethyl ether (C) and ethanol (D) using, in each case, isobutanol (E, F, G, H) as internal standard. The water peak in each chromatogram is labelled W. The vertical lines crossing the baselines are event markers arising from the integrator.

As an illustrative example of the general usefulness of this gas–liquid chromatographic procedure, in Table III are included the results of a preliminary study of the equilibrium distribution of halothane between the cells and plasma of human blood at 4°. This experiment was performed as follows. To Reacti-vials completely filled with EDTA anti-coagulated human blood was added, with a microlitre pipette whose tip was located near the base of the vial, a known volume of a solution of halothane in 0.85% saline equal to

TABLE II

GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF WHOLE BLOOD SAMPLES CONTAINING KNOWN CONCENTRATIONS OF HALOTHANE, METHOXYFLURANE, DIETHYL ETHER AND ETHANOL

	Compound added							
	Halothane		Methoxy-flurane		Diethyl ether		Ethanol	
Response factor (isobutanol = 1)	0.207 ± 0.008		0.242 ± 0.009		0.924 ± 0.001		0.797 ± 0.024	
Retention time (min)*	6.21 ± 0.20		12.31 ± 0.13		5.36 ± 0.15		3.21 ± 0.08	
Relative retention time (Isobutanol = 1)	0.697		1.397		0.608		0.382	
	Added (mg%)	Found (mg%)	Added (mg%)	Found (mg%)	Added (mg%)	Found (mg%)	Added (mg%)	Found (mg%)
	102.7	105.2	110.4	111.3	111.6	115.3	119.7	121.7
	102.3	103.6						
	94.1	90.9						
	93.5	97.3						
	92.8	90.4						
	54.7	53.1	54.7	54.2	67.4	66.6	62.2	63.6
	15.3	15.0					24.4	23.5
	14.9	13.8						
	14.2	14.3						
	13.2	12.2						
	11.4	12.1	10.3	10.7	12.7	11.9	11.5	11.7
	4.7	4.3	5.0	4.9	5.2	5.5		
	1.2	1.0	0.7	0.8	1.0	0.7		
t**	0.26		0.69		0.5		0.9	
d.f.***	12		4		4		3	
	N.S. §		N.S.		N.S.		N.S.	
After 1 week storage	18.8	17.8						
After 2 weeks storage	18.4	18.6						

*In these studies the retention times for isobutanol were 8.91 ± 0.56, 8.81 ± 0.56, 8.81 ± 0.56 and 8.41 ± 0.48 for analysis of halothane, methoxyflurane, diethyl ether and ethanol respectively.
**Paired *t* test [39]. Differences between quantities added and found were not significantly different from zero.
***d.f. = degrees of freedom.
§N.S. = not significant.

exactly one tenth of the volume of the vial. The vial caps were immediately replaced thus displacing any excess blood and insuring a bubble free sample (i.e., no head space). The vials were rotated slowly at 4° for various time periods and were then centrifuged at 2800 *g* for 15 min. The plasma was analysed for halothane and the concentration of the anaesthetic in the cellular fraction calculated from the equation:

$$[\text{blood}_{halo}] = 0.9\ Hc\ [\text{cell}_{halo}] + (1—0.9\ Hc)\ [\text{plasma}_{halo}]$$

where

$[blood_{halo}]$ = added concentration of halothane in blood (29.7 mg%)

$[plasma_{halo}]$ = concentration of halothane in plasma as determined by gas chromatography

Hc = volume fraction of undiluted blood occupied by cells

0.9 = dilution factor due to addition of the saline solution of halothane to blood

$[cell_{halo}]$ = concentration of halothane in the cellular component of blood

From this equation the distribution of halothane between cells and plasma can be calculated.

The halothane solution was prepared by saturating 0.85% saline at 37° with the anaesthetic. Under these conditions the saturation concentration of halothane as determined by gas chromatography was 297±8 mg% (n=5); at 4° the value obtained was 481±12 mg% (n=4). Possible losses of halothane were estimated in separate control experiments by omitting the centrifugation step and determining the concentration of halothane in the whole blood after the period of equilibration. Statistical analysis of these data (n=9) indicated that there was no difference between the quantity of halothane added and that found by gas chromatographic analysis. Significant haemolysis did not occur during the period of time used in these equilibration experiments.

TABLE III

DISTRIBUTION OF HALOTHANE BETWEEN THE CELLS AND PLASMA OF EDTA ANTI-COAGULATED HUMAN BLOOD AT 4°

Samples A and B were obtained from the same individual on two separate occasions; they had haematocrits of 0.489 and 0.486 respectively. Sample C, obtained from a different individual, had a haematocrit of 0.507. Assuming that equilibrium is achieved after 16 h then the mean ratio:

$$\frac{[cell_{halo}]}{[plasma_{halo}]} = 2.0 \pm 0.2 \ (n = 11).$$

Sample A		Sample B		Sample C	
Incubation time (h)	$\frac{[cell_{halo}]}{[plasma_{halo}]}$	Incubation time (h)	$\frac{[cell_{halo}]}{[plasma_{halo}]}$	Incubation time (h)	$\frac{[cell_{halo}]}{[plasma_{halo}]}$
3.5	0.6	19.5	1.8	18.0	1.8
13.0	1.1	20.5	2.1	18.5	2.4
16.5	1.9	22.0	2.3	20.5	2.0
17.5	2.0	22.0	1.8	21.5	2.4
		23.0	1.9		

DISCUSSION

Direct injection methods for the gas chromatographic analysis of volatile anaesthetics in blood can be divided into three general classes: (a) those in which the sample is injected into the injection port of the chromatograph [25,26,29], (b) those in which injection is made into a removable glass liner inserted into the injection port [27,30], and (c) those in which injection is made into a heated pre-column device isolated from the columns and then, after a period of time, the volatiles are swept onto the column by a stream of carrier gas via a switching valve [28,31]. The analytical system described here appears to combine all the advantages of these systems without any of their disadvantages. Thus it permitted the rapid, direct, quantitative analysis of the blood concentrations of four volatile compounds on a stable, readily available column packing. The difficulties associated with baseline drift [2,30], ghost peaks [6,30], interference from water [6,30], poor reproducibility [1,2], contamination of the columns with non-volatile components [2,6,30], and non-uniform evaporation which necessitated a preheating period of the sample within the pre-column device [28,31] were not encountered. The use of an internal standard coupled with the measurement of peak areas, rather than peak heights, obviated the need for calibration curves and eliminated problems associated with measurements of broadened or distorted peaks. Reference to Table II demonstrates that, with our procedure, accurate analyses can be performed over a wide range of concentrations.

The external injection port can be easily constructed from inexpensive materials and has proved to be reliable and simple to operate for routine purposes. Installation requires a minimum of modification of existing chromatographs and the port can be readily adapted for use with other chromatographs. Furthermore the U-tube assembly appears to offer distinct advantages over previous systems [28,31] since it is easily accessible and can be rapidly changed without interrupting the carrier gas flow.

The equilibrium distribution of halothane between the cells and plasma of human blood at 4° determined in this study, 2.0±0.2 (see Table III), differs from that reported by Han and Helrich [33] who found that 70% of the halothane was in the plasma. The latter value was, however, estimated indirectly from calculations based upon the Ostwald solubility coefficients of halothane in plasma and cells at 38°. Moreover the Ostwald solubility coefficients were determined after shaking the samples with liquid halothane and mercury for 1 h.

Our data would indicate that the solubility of halothane in blood should increase with haematocrit. Experimental observations on this relation are, however, in conflict. Mapleson et al. [34] and Steward et al. [35] found that in rabbits and dogs, respectively, halothane solubility decreased as the haematocrit decreased. Furthermore, Steward et al. attribute solubility variations to the greater solubility in the cells. Cowles et al. [36], however, found that haematocrit did not affect the solubility of halothane in dog blood. Similar discrepancies have been noted with studies of human blood. Several investigators [29,33,36] have reported a decrease in solubility with an increase in haematocrit while other studies have shown that the solubility of halothane

is independent of the haematocrit [37,38], that halothane is less soluble in blood of low haematocrit [19,37] or more soluble in blood of high haematocrit [38]. On the other hand, other factors affect solubility, including lipid concentration [4,38] and the albumin-to-globulin ratio [37]. In view of the results discussed above it is impossible to assess fully the significance of published data in this area. Our preliminary experiments do however demonstrate that the gas chromatographic procedure described here provides a facile method of examining this interesting controversy. Experiments designed to study this problem in more detail are currently in progress.

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