A TECHNIQUE FOR THE ANALYSIS OF METHOXYFLURANE IN BLOOD BY GAS CHROMATOGRAPHY

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SUMMARY

A gas chromatographic method for the analysis of methoxyflurane in blood has been developed in which the anaesthetic is extracted from the blood sample by equilibration with silicone fluid. Because of its non-volatility, injection of the silicone extractant into a chromatograph results in a methoxyflurane peak, eluted after 1 minute, that is not accompanied by a solvent peak. The method permits the storage of specimens for several days before chromatography and is well suited to batch analysis. There is a linear response to concentrations of methoxyflurane in the tested range of 1–20 mg/100 ml and the reproducibility is an improvement upon that published for other techniques (±1.8 per cent coefficient of variation for methoxyflurane in blood 10 mg/100 ml).

For an investigation of the analgesic potency of inhalational drugs in obstetrics, a method was required which would enable blood samples containing low concentrations of methoxyflurane to be stored and subsequently accurately analyzed. The published techniques involve either the injection of blood samples directly into the chromatograph or preliminary separation of the anaesthetic agent before analysis.

Although the direct injection technique (Lowe, 1964) offers the advantages of rapidity of analysis and apparent simplicity, the method is not without difficulties which include injection hazards, baseline disturbances and the distortion of peak shape. All arise from the need to evolve rapidly the volatile fraction from a protein residue and to separate subsequently the non-polar anaesthetic from a relatively enormous quantity of water vapour. In addition, special techniques are required for the storage of samples before analysis.

A number of techniques have been described for the extraction of the anaesthetic from blood prior to analysis. These include gas equilibration (Yamamura et al., 1966; Butler, Kelly and Zapp, 1967; Fink and Morikawa, 1970), distillation (Gadsen et al., 1962) and chemical extraction (Wolfson, Ciccarelli and Siker, 1966). This last method offers the advantage that the extraction process can be commenced immediately after sampling and thereafter there is little risk of leakage of the sample from the extractant. However, the commonly employed agents which can successfully extract methoxyflurane from blood interfere with the subsequent chromatographic analysis. Preliminary attempts to produce satisfactory reproducibility using carbon disulphide as the extractant, as described by Wolfson, Ciccarelli and Siker (1966), were disappointing. Subsequently, after considering the desirable features of an extraction solvent (see Appendix I), a number of liquids with promising characteristics were tested. These included, among others, carbon tetrachloride, n-heptane, toluene, dichloromethane, trichloroethylene, benzene, ether and halothane. None was entirely satisfactory. All were volatile and produced large chromatographic peaks which either interfered with the sample peak or were so slowly eluted as to reduce seriously the frequency with which successive analyses could be made.

As an alternative, a solvent was sought which would be sufficiently non-volatile to accumulate at the proximal end of the column and not to produce a demonstrable chromatographic peak. However, this build-up might seriously interfere with the characteristics of the column. Silicone fluids are noted for their low volatility and are also structurally similar to the silicone polymers used as stationary liquid phases on chromatographic columns. Their accumulation would, therefore, be less likely to interfere with column performance. The dimethyl silicone polymers (Midland Silicones Ltd) are marketed as fractions of specified viscosity (the second part of the serial number indicates the viscosity in centistokes) and not as pure substances of specific chain length. Silicone fluid MS.200/20c.S combines a...
ANALYSIS OF METHOXYFLURANE IN BLOOD

viscosity low enough for extraction purposes with a very low vapour pressure (<0.5 mm Hg at 200°C). The specific gravity (0.96) is sufficiently different from that of blood to enable their separation by centrifugation. It is miscible in all proportions with all the volatile anaesthetics whereas a saturated solution in water contains less than 0.2 per cent silicone fluid. Preliminary experiments demonstrated that this silicone fluid has an oil/gas partition coefficient for methoxyflurane of 906 at 21°C. The oil/blood partition coefficient was calculated to be 71.7 and from this an extraction ratio of 98.6 per cent was predicted.

METHOD

Pretreatment of silicone fluid MS.200/20c.S.

When 1 µl of MS.200/20c.S is analyzed in a chromatograph under the same conditions and attenuation as those used for analysis of analgesic levels of methoxyflurane in blood, a number of peaks are produced which are due to traces of short chain polymers of relatively high volatility. By heating the fluid to 150–200°C in a glass vessel while drawing air through it with a vacuum pump for 2 hours or so, these traces can be eliminated so that subsequent injections of the treated fluid produce no deviation of the baseline (fig. 1).

Chromatographic technique.

Column conditions. Our gas chromatograph is a Pye Series 104 Model 4 isothermal instrument fitted with a flame ionization detector. A 5-foot-long, ¼-inch o.d. glass column, packed with 10 per cent S.E.30 on 60/80 mesh Universal B (Phase Separations Ltd), has been found satisfactory for the analysis of methoxyflurane since the drug is eluted as a symmetrical peak with only slight tailing. The carrier gas is nitrogen (50 ml/min). A column oven temperature of 80°C produces a consistent baseline free of artefacts and the retention time for methoxyflurane at that temperature is 60 sec. Despite good peak shape for the vapour samples, there was pronounced tailing of peaks when methoxyflurane was injected dissolved in 1-µl aliquots of silicone fluid because of slow evolution of vapour from the non-volatile vehicle. An injection port temperature of 160°C restored peak shape without introducing baseline instability.

The effect of repeated injections of silicone fluid. Silicone fluid, being a non-volatile substance, accumulates in the proximal end of the column and might modify its characteristics. In order to establish the extent of this effect, 1-µl aliquots of a 10 mg/100 ml solution of methoxyflurane in silicone fluid were injected successively at intervals of 70 sec. The effect of 50 such injections on the peak areas so produced was insignificant though the peak heights fell by 20 per cent after 30 injections (fig. 2).
Coefficients of variation of peak heights and areas of groups of 5 consecutive injections were calculated. For areas, the value remained below 1.5 per cent for the first 35 injections, the maximum by the end of 50 injections being 2.5 per cent. The peak heights for the first 25 injections, however, showed coefficients of variation of 3–6 per cent. After heating the column to 200°C for 30 min, the peak heights returned to their original level and it was only after approximately 100 injections that there was visible "wetness" of the packing material at the injection port end of the column. The original conditions could be restored by repacking the proximal 2 inches of the column or by heating it to approximately 200°C for 30 min. Our routine when such reconditioning becomes necessary is to exchange the column for one that has already been repacked at its injection port end. More than 3,000 analyses have now been made using the same two columns in this way without any progressive deterioration in either linearity, response time or reproducibility.

**Extraction technique.**

The equilibration of silicone fluid with blood must be undertaken with only gentle agitation since excessive vigour produces an emulsion that is difficult to separate. When a 2-ml sample of blood is equilibrated with 2 ml of silicone fluid in a slowly rotating (30 r.p.m.) mixing jig at room temperature, a fine dispersion is achieved which can be easily separated by centrifuging. The period necessary for complete equilibrium to be achieved has been investigated (fig. 3). A series of "bijou" bottles containing blood and silicone fluid was equilibrated for periods from 1 min to 24 hr. After 30 min, the percentage extraction remains virtually constant.

The technique that we now adopt is that a "bijou" bottle is fitted with an aluminium foil seal and weighed on an analytical balance. Two ml of silicone fluid is introduced and the whole is weighed again. Approximately 2 ml of heparinized blood is then injected beneath the silicone layer, thus preventing loss of methoxyflurane to the atmosphere, and the bottle is reweighed. With the screw cap firmly in place, it is possible to leave the mixture for several days before analysis. The bottle is clipped to a rotating mixer and gently agitated for 45 min. The mixture is separated by centrifuging for about 10 min at 1250 g and supernatant silicone fluid is sampled using a 1-μl SGE syringe (Scientific Glass Engineering Ltd). After introducing the needle into the fluid, the plunger is pumped slowly several times and finally withdrawn beyond the 1-μl mark. The needle is withdrawn from the fluid and, using the reproducibility adaptor, the plunger is depressed to the 1-μl mark. The needle is wiped clean of any fluid. With a smooth motion, it is then passed through the injection port septum to a constant depth directly into the column packing and the plunger depressed to empty the syringe. The needle is immediately withdrawn. The peak area is recorded using a Vitatron Model UR.400M integrating potentiometric recorder. The chromatogram so produced presents the methoxyflurane peak on an otherwise flat baseline (fig. 4). The blood level of methoxyflurane may be calculated by comparing the area with that of a silicone/methoxyflurane standard peak.

**Evaluation of the technique**

**Accuracy and reproducibility.**

Several workers have recommended the use of an internal standard to eliminate inaccuracies arising from poor reproducibility of delivered volume by microsyringes (Cervenko, 1968; Wolfson, Ciccarelli and Siker, 1966). As a test of the value of this technique, a quantity of halothane was added to a solution of methoxyflurane (5 mg/100 ml) in silicone fluid so that the heights of the two peaks were similar on chromatography. Analysis of the coefficients of variation of six successive 1-μl injections of the resultant solution with respect to peak heights and peak areas of each agent and of the ratios of both (table I) shows that no advantage is derived from the use of an internal standard. This suggests that the reproducibility of 1-μl SGE syringes used...
throughout this investigation was sufficiently good to be disregarded. The reason for the poorer reproducibility of the halothane peaks is probably that elution was too rapid under the existing column conditions for optimum peak shape to be achieved.

Table I. The effect of an internal standard (halothane) on the reproducibility of the chromatographic technique.

<table>
<thead>
<tr>
<th>Chromatogram</th>
<th>Methoxyflurane</th>
<th>Halothane</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak area</td>
<td>1.63</td>
<td>2.70</td>
<td>1.85</td>
</tr>
<tr>
<td>Peak height</td>
<td>3.08</td>
<td>4.31</td>
<td>2.28</td>
</tr>
</tbody>
</table>

To test the accuracy and reproducibility of the technique, five standards of known concentrations of methoxyflurane in blood in the range of 1–20 mg/100 ml were prepared. From each standard, five 2-ml samples of blood were extracted with 2 ml of MS.200/20c.S (i.e. 25 extractions in all). Five chromatographic analyses of 1-μl aliquots of each of the extracts were subsequently performed, the peak areas being recorded. A standard solution of methoxyflurane in silicone fluid was also prepared in order to calculate the blood methoxyflurane concentration.

Preparation of standards.

Both blood and silicone standards were prepared in an identical manner. One hundred ml weighed glass bottles were filled with citrated whole human blood or silicone fluid and reweighed. Using a 10-μl syringe, each was then inoculated with a quantity of methoxyflurane appropriate to produce blood standards of approximately 1, 2, 5, 10 and 20 mg/100 ml and a silicone fluid standard of 10 mg/100 ml. The bottles were sealed with aluminium-foil-lined screw caps and agitated gently for 12 hr to ensure complete solution of the anaesthetic. The densities of blood (1.045) and silicone fluid (0.949) were determined and used in the calculation of the actual concentrations.

Calculation of the results.

The expression for the concentration of methoxyflurane in blood, together with its derivation, is given in Appendix II.

Linearity and reproducibility.

All the analyses were recorded at the same amplifier attenuation. The results of the evaluation are summarized in Table II.

Linear and log/log plots of the mean measured (column 2) against the standard (column 1) concentrations indicated very close agreement with linearity. The data for each concentration were submitted to a hierarchical analysis of variance in order to give an estimate of the variance of individual analyses about the mean value for all analyses and also the variance of individual extractions about the mean value for all extractions. These are expressed in Table II (columns 3 and 4) as coefficients of variation (standard deviation as a percentage of the mean concentration). The individual variances were also used to calculate the total variance of our routine analytical technique which consists of two analyses of one extraction of a sample. Thus:

Total variance of routine technique = \( \frac{\text{Variance of single extraction}}{\text{Number of extractions}} + \frac{\text{Variance of single analysis}}{\text{Number of analyses}} \)
TABLE II. The reproducibility and linearity of the method.

<table>
<thead>
<tr>
<th>(1) Concentration of methoxyflurane in standards (mg/100 ml)</th>
<th>(2) Mean measured concentration in standards (mg/100 ml)</th>
<th>(3) Coefficient of variation of a single chromatographic analysis (%)</th>
<th>(4) Coefficient of variation of a single extraction procedure (%)</th>
<th>(5) Coefficient of variation of the technique* (%)</th>
<th>(6) 95% confidence limits of the technique* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.98</td>
<td>0.91</td>
<td>5.4</td>
<td>3.8</td>
<td>5.4</td>
<td>± 11.1</td>
</tr>
<tr>
<td>2.01</td>
<td>2.00</td>
<td>3.9</td>
<td>2.7</td>
<td>3.8</td>
<td>7.9</td>
</tr>
<tr>
<td>5.22</td>
<td>5.34</td>
<td>1.5</td>
<td>3.1</td>
<td>3.3</td>
<td>6.7</td>
</tr>
<tr>
<td>10.17</td>
<td>10.08</td>
<td>2.4</td>
<td>2.0</td>
<td>2.6</td>
<td>5.3</td>
</tr>
<tr>
<td>20.51</td>
<td>20.38</td>
<td>0.8</td>
<td>0.9</td>
<td>1.1</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* These figures correspond to a technique consisting of two successive analyses of one extraction of the sample.

The variability of the routine technique is indicated in column 5 also as the coefficient of variation. Clearly, this value may be reduced, if desired, by increasing the number of extractions and analyses per sample. The 95 per cent confidence limits for the technique are shown in column 6. Each figure indicates the range (expressed as a percentage of the mean) about the mean measured concentration within which 95 per cent of the measurements by the routine technique would be expected to fall.

In this study, the coefficients of variation fell with increasing concentration, suggesting that it was a function of random error in the chromatographic technique. An important element in this is that, as a fixed amplifier attenuation was maintained, low concentrations resulted in small peaks which approached the limit of resolution of the digital presentation of the integrator (equivalent to a variation of ±3 per cent at a concentration of methoxyflurane of 1 mg/100 ml). It is unlikely that weighing inaccuracies contributed significantly to the variance of the method since this would introduce a variation that would have been independent of the concentration.

The effect of storage.

Capped “bijou” bottles containing 2 ml of a 10 mg/100 ml solution of methoxyflurane in blood plus 2 ml of silicone fluid were sampled daily for 12 days. There was a steady decline in methoxyflurane concentration which amounted to 0.3 per cent of the sample concentration per day. This rate of loss, which is partially accounted for by the necessity to open the bottles daily for sampling, is small in comparison with the coefficient of variation of the analytical method and is neglected in practice when storage for up to 4 days before analysis is undertaken.

DISCUSSION AND CONCLUSION

This gas chromatographic technique offers advantages over other methods for the measurements of anaesthetics in blood. A chemical extraction technique avoids the difficulties associated with the injection of blood directly into the chromatograph and does not call for the use of sophisticated injection assemblies (Yokota et al., 1967) subtractive precolumns (Jacobs, 1964) or special precautions when handling microlitre syringes (Lowe, 1964). Since the blood is put into the extractant immediately after sampling, there is a reduced risk of loss of anaesthetic by evaporation. The high extractant/blood partition coefficient eliminates inaccuracies arising during gas equilibration techniques which are affected by discrepancies in the blood/gas partition coefficient (Yamamura et al., 1966; Butler, Kelly and Zapp, 1967).

The non-volatile nature of the silicone fluid extractant allows for a considerable saving in chromatographic analysis time (70 sec between repeat injections) and permits convenient use of frequent calibration checks and repeat analyses of individual samples. The technique is particularly suitable for batch analyses. For example, although one solitary complete analysis takes 56 min, a batch of 20 samples can be analyzed with an individual analysis time of 4–5 min. This includes duplicate injections of each specimen.

The viscosity of MS.200/20c.S, far from complicating handling, appears to improve the consistency of delivered volume of microlitre syringes as compared with less viscous liquids. The reproducibility of the syringes has not necessitated the use of an internal standard.

Finally, the reproducibility of the whole method is better than other published figures. Lowe (1964)
gives a coefficient of variation of \( \pm 5.2 \) per cent for methoxyflurane in blood (10 mg/100 ml) with a direct injection method and Butler, Kelly and Zapp, using a gas equilibration method give a coefficient of variation of \( \pm 4-6 \) per cent. The technique presented here has a coefficient of variation of \( \pm 2.6 \) per cent for methoxyflurane 10 mg/100 ml. Thus, the technique described combines reliable storage with an analytical method that is rapid and accurate in the subanaesthetic range for methoxyflurane. Trials now in progress indicate that the technique may also be applied to the analysis of other volatile organic anaesthetics.

**APPENDIX I**

**Characteristics of an ideal solvent.**

1. It should be insoluble in blood.
2. The sample substance should be highly soluble in it, in order to ensure a high extraction ratio (i.e. a high extractant/blood partition coefficient).
3. Once equilibration is complete, the solvent should be easily separable from blood.
4. It should not interfere with the sample peak during chromatography and should have no deleterious effect upon either column or detector.
5. It should allow repeat injections to be carried out rapidly.
6. It should be inert with respect to blood and substance to be analyzed.
7. Its viscosity should not complicate handling with microlitre syringes.

**APPENDIX II**

**Proof of the equation for the concentration of methoxyflurane in blood.**

Let \( M_s \) be the weight of methoxyflurane in the sample and let \( M_{sl} \) and \( M_{si} \) be the weights of methoxyflurane in blood and silicone at equilibrium. The silicone/blood partition coefficient is represented by \( \lambda_{sl/b} \) at 21°C. At equilibrium, the mass of methoxyflurane remaining in the blood will be

\[
M_{BE} = (M_{sl} \times V_{olb}) / (\lambda_{sl/b} \times V_{ols}) \quad \ldots (1)
\]

where \( V_{olb} \) and \( V_{ols} \) are the volumes of blood and silicone respectively. From the chromatograms, the peak areas are related to the concentrations of methoxyflurane in the sample and the standard by

\[
A_{si} / A_{std} = [M_{si}] / [M_{std}] \quad \ldots (2)
\]

where \( A_{si} \) and \( A_{std} \) are the peak areas for sample silicone and standard silicone and \( [M_{si}] \) and \( [M_{std}] \) are the concentrations of methoxyflurane in sample and standard silicone fluids respectively. The concentration of methoxyflurane \( [M_{si}] \) in silicone fluid is given by

\[
[M_{si}] = M_{si} / V_{ols} = ([M_{std}] \times A_{si}) / A_{std} ;
\]

thus, at equilibrium, the mass of methoxyflurane extracted will be

\[
M_{si} = ([M_{std}] \times A_{si} \times V_{ols}) / A_{std} \quad \ldots (2)
\]

If one substitutes for \( M_{si} \) in \( (1) \)

\[
M_{BE} = ([M_{std}] \times A_{si} \times V_{ols}) / (A_{std} \times \lambda_{sl/b}) \quad \ldots (3)
\]

By combining expressions \( (1), (2) \) and \( (3) \) the concentration of methoxyflurane in blood may be derived:

\[
[M_B] = ([M_{std}] \times (A_{si} / A_{std})) \times (V_{ols} / V_{ols}) + (1 / \lambda_{sl/b})
\]

This equation is used in the calculation of the partition coefficient and the methoxyflurane concentrations of unknown blood samples.

**ACKNOWLEDGEMENTS**

We would like to record our thanks to Dr W. W. Mapleson, Ph.D., for his valuable assistance during the statistical analysis of this work, to Mr P. Allott, B.Sc., for the use of his computer program, and to Professor W. W. Mushin, C.B.E., for his help and encouragement.

The packed chromatographic columns used in this investigation were obtained from Jones Chromatography & Co., 71 Ridgeway, Machen, Newport NP1 8RD.

Silicone Fluid MS.200/20c.S is obtainable from Midland Silicones Ltd, Barry, Glamorgan.

**REFERENCES**


**UNE TECHNIQUE EN VUE DE L'ANALYSE DU METHOXYFLURANE PRESENT DANS LE SANG**

**A L'AIDE D'UNE CHROMATOGRAPHIE GAZEUSE**

**SOMMAIRE**

Une méthode de chromatographie gazeuse destinée à analyser le méthoxyflurane présent dans le sang a été mise au point. Dans cette méthode, l’anesthésique est extrait de l’échantillon de sang par équilibration avec un silicone fluide. Du fait de la non-volatilité de celui-ci, l’injection du silicone d’extraction dans un chromatographe entraîne un pic de méthoxyflurane, élue après une minute et qui ne s’accompagne pas d’un pic du solvant. Ce procédé permet le stockage d’échantillons pendant plusieurs jours avant d’effectuer la chromatographie. Il convient bien à une analyse par fournées. Il existe une réponse linéaire aux concentrations de méthoxyflurane dans la gamme testée de 1 à 20 20 mg/100 ml et la faculté de reproduction constitue un progrès par rapport à ce qui a été publié pour d’autres techniques (Coefficient de variation de \( \pm 1.8 \) pour cent pour une concentration de méthoxyflurane dans le sang de 10 mg/100 ml).
GASCHROMATOGRAPHISCHE BESTIMMUNG VON METHOXÝFLURANE IM BLUT
ZUSAMMENFASSUNG
Zur Bestimmung von Methoxyflurane im Blut wurde eine gaschromatographische Methode entwickelt, wobei das Anaesthetikum der Blutprobe durch Equilibrierung mit Silikon-Flüssigkeit entzogen wird. Wegen seiner Nicht-Flüchtigkeit führt die Injektion des Silikon-Extraktes in einen Chromatographen zu einem Methoxyflurane-Peak, der nach einer Minute eluiert wird und nicht von einer Lösungsmittelzacke begleitet ist. Die Methode erlaubt die Lagerung der Proben für einige Tage bis zur Chromatographie und ist für die Analyse größerer Mengen gut geeignet. Die Reaktion auf verschiedene Konzentrationen von Methoxyflurane von 1 bis 20 mg/100 ml ist linear; die Reproduzierbarkeit stellt eine deutliche Verbesserung gegenüber anderen bisher veröffentlichten Methoden dar (±1,8 Prozent Variationskoeffizient für Methoxyflurane von 10 mg/100 ml).

UNA TECNICA PARA EL ANALISIS DE METOXIFLURANO EN LA SANGRE MEDIANTE CROMATOGRAFIA GASEOSA
RESUMEN
Ha sido desarrollado un método cromatográfico gaseoso para el análisis de metoxiflurano en la sangre en el cual el anestésico es extraído de la muestra de sangre mediante equilibración con líquido de silicona. A causa de su falta de volatilidad, la inyección del extractante de silicona en el cromatógrafo produce un pico de metoxiflurano, eluido después de un minuto, que no es acompañado por un pico del solvente. Este método permite el almacenamiento de muestras durante varios días antes de la cromatografía y es muy adecuado para el análisis de lotes. Hay una respuesta lineal a concentraciones de metoxiflurano entre los límites ensayados de 1–20 mg/100 ml y su reproducibilidad es una mejora en comparación con otras técnicas publicadas (±1,8 por ciento el coeficiente de variación de metoxiflurano en sangre 10 mg/100 ml).

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XXIInd FRENCH CONGRESS ON ANAESTHESIOLOGY AND RESUSCITATION
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Faculté de Médecine, Rue des Saints-Pères, Paris 5e

“PROBLEMS OF ANAESTHESIA, ANALGESIA AND RESUSCITATION IN OBSTETRICS”
Organized by: La Société Française d’Anesthésie, d’Analgesie et de Réanimation; l’Association des Anesthésiologistes Français; La Société Nationale de Gynécologie- Obstétrique

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1. Physiology of the pregnant woman—foeto-placental circulation
2. Physio-pathology of the foetus and the newborn
3. Placental transfer of drugs
4. Teratogenesis — uterine physiopharmacology
5. Obstetric analgesia
6. Problems of resuscitation in pregnancy
7. General anaesthesia for the pregnant woman
8. Biological and physical monitoring of the foetus
9. Regional analgesia for the pregnant woman
10. Foetal distress
11. Problems of resuscitation during labour
12. Psycho-prophylactic analgesia
13. Influence of anaesthesia on foetus and newborn
14. Resuscitation of the newborn