Journal of Chromatography, 164 (1979) 63—72
Biomedical Applications

CHROMBIO. 369

QUANTITATIVE ANALYSIS OF VOLATILE HALOTHANE METABOLITES IN BIOLOGICAL TISSUES BY GAS CHROMATOGRAPHY

R.M. MAIORINO, I.G. SIPES, A.J. GANDOLFI and B.R. BROWN, Jr.

Department of Anesthesiology, University of Arizona, Health Sciences Center, Tucson, Ariz. 85724 (U.S.A.)

(Received March 26th, 1979)

SUMMARY

A simple and sensitive gas chromatographic method for the determination of 2-chloro-1, 1-difluoroethylene (CDE) and 2-chloro-1,1,1-trifluoroethane (CTE), two highly volatile metabolites of halothane, in blood, liver and isolated hepatic microsomes is described. The entire head-space in equilibrium with a known volume or weight of the sample is injected into the gas chromatograph equipped with a flame ionization detector. Quantification is accomplished with standards prepared by fortifying blank samples with known concentrations of CDE and CTE which are treated under the same conditions as the samples. Detection limits for CDE and CTE were 2 pmole/ml in blood and 10 pmole/g in liver and the mean relative standard deviations are no greater than ± 6% except for CTE in hepatic microsomes (± 9%). A preliminary study of blood CDE and CTE levels in humans anesthetized with halothane is reported.

INTRODUCTION

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is a volatile anesthetic widely used for clinical applications. Traditionally halothane was considered to be an inert anesthetic, but since 1964 it has been shown to undergo biotransformation to trifluoroacetic acid, N-trifluoracetyl-2-ethanolamide, N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine, inorganic chloride, fluoride and bromide [1—4]. Recently, two volatile metabolites of halothane, 2-chloro-1,1,1-trifluoroethane (CTE) and 2-chloro-1,1-difluoroethylene (CDE), have been identified in the expired air of halothane anesthetized rabbits [5] and man [6]. These two metabolites could be related to the observed hepatotoxicity of halothane which occurs under hypoxic conditions [7].

Presently, methods for the isolation and quantification of these two highly volatile low molecular weight metabolites in biological tissues are non-existent. The boiling points for both compounds are extremely low (CTE + 6.9°, CDE —17.7°). This property offers the possibility of quantification by head-space gas chromatographic analysis. This principle has been applied to the analysis of

ethanol [8] and inhalation anesthetics [9] in blood. This paper presents a simple and sensitive method for the determination of CTE and CDE in hepatic microsomes, blood and liver in which quantification is accomplished by a head-space technique utilizing gas chromatography with flame ionization detection. The head-space technique does not require extraction, separation or other isolation steps which would cause extensive losses of these volatile substances.

EXPERIMENTAL

Materials

Halothane (Ayerst Laboratories, New York, N.Y., U.S.A.), CTE and CDE (PCR Research Chemicals, Gainesville, Fla., U.S.A.) were determined by gas chromatography to be 99+% pure. Pesticide quality N,N-dimethylformamide (DMF) and *n*-heptane (Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.) were used without further purification. Sampling vessels were 1- and 5-ml screw cap microreaction vials with PTFE lined rubber septums (Supelco, Bellefonte, Pa., U.S.A.). Gas tight, push-button valve Precision Sampling syringes (5 ml) were obtained from Alltech, Arlington Heights, Ill., U.S.A.

Biological samples

Immediately after exposure of male rats to halothane (1% for 2 h in air) blood samples were removed from the tail vein with a syringe coated with heparin solution and 200-μl aliquots were transferred to 1-ml septum vials kept on ice. In certain cases, animals were sacrificed and blood was obtained in a similar manner from the inferior vena cava. From these same animals the liver was removed and a 2.0-g portion was transferred to a Dounce homogenizing tube containing 6.0 ml of cold 50 m*M* Tris—1.15% potassium chloride buffer (pH 7.4). After homogenization, 200 μl of the homogenate were transferred to a 1-ml septum vial kept on ice.

Microsomes were isolated from livers of rats not exposed to halothane by differential centrifugation according to the method described by Sipes et al. [10]. Aliquots of microsomal suspensions (2 ml) were placed into 5-ml vials and mixed with 0.5 ml of a NADPH generating system [10] to give a protein concentration of 5 mg/ml. The vials were then purged with nitrogen, tightly capped and allowed to equilibrate to 37° for 10 min. The reaction was then initiated by the addition of 18.8 μmoles of liquid halothane (2 μl). After a 30-min incubation at 37° with constant shaking, the reaction was terminated by addition of 20 μl of 6 *N* hydrochloric acid. Blood samples were stored at 3° and liver and microsomal samples were frozen at —76° until analyzed.

Gas chromatography

Gas chromatographic analysis was performed on a Varian 1440 gas chromatograph equipped with a flame ionization detector (FID) and a Varian CDS 101 integrator. The integrator was programmed to measure separated peak areas by the tangent baseline method. Separation was attained with a 1.8 m × 2 mm I.D. stainless-steel column packed with Porapak Q, 100—120 mesh. The injection port was maintained at 180°, the column at 150°, and the detector at 240°. Flow-rates were 30 ml/min for the nitrogen carrier gas, 300 ml/min for

air, and 30 ml/min for hydrogen. The electrometer was set at range 10^{-12} and attenuation 2. The identity of volatile metabolites which were detected by this gas chromatographic system were confirmed on a 3300 Finnigan gas chromatograph—mass spectrometer equipped with a Finnigan 6110 data system.

Analytical procedure

Prior to analysis the samples were equilibrated at room temperature for 1 h, vortexed for 20 sec and placed on a block heater at 37° for a minimum of 20 min. The needle of a gas-tight syringe was inserted through the septum of the vial into the vapor phase, without touching the liquid. The valve was opened, and the plunger was drawn slowly to the 5.0-ml mark of the syringe over a time period of 15—20 sec. Before removing the syringe from the vial, the push-button syringe valve was closed. The head-space sample was then compressed by depressing the plunger until the syringe pressure approximately equaled the chromatograph inlet pressure (35 p.s.i.). Injection of the compressed head-space sample into the column was performed by inserting the syringe needle into the injection port, opening the syringe valve, and then depressing the syringe plunger in a rapid, smooth motion. Each chromatographic determination, including the elution of halothane, required 20—25 min. The concentrations of CTE and CDE in microsomal, blood, and liver samples were determined with the aid of standard calibration curves.

CTE and CDE standards

Separate stock solutions of CTE and CDE were prepared by slowly bubbling the respective gas into 5.0 ml of dimethylformamide (DMF), which is an excellent low-volatility carrier for these compounds, in screw capped glass vials and their concentrations were determined by weight difference. Serial dilutions of the stock solutions with additional DMF were made (0.0048 to 6.8 μmoles/ml) and 2.0—5.0 μl of these solutions were added to septum vials containing 200 μl of blood, 200 μl of liver homogenate, or 2.5 ml of microsomal incubation media.

These standards were freshly prepared for each phase of the study, and were treated in the same manner as the biological samples. The integrator peak areas per syringe of head-space vapor were plotted against the corresponding standard concentrations of these metabolites. At least three standards in duplicate were prepared that encompassed the integrator peak areas of the particular biological samples that were assayed. Blank samples containing only DMF did not interfere with the gas chromatographic analysis of CTE and CDE. In addition, CTE and CDE standards were prepared in *n*-heptane in a similar manner as the DMF standards for recovery studies.

Statistics

Linear regression analysis, sample concentration and relative standard deviation computations were performed on a programmable Wang 500 calculator (Wang Instruments).

RESULTS AND DISSCUSSION

The high volatilities of CTE and CDE in aqueous solutions allow for the success of head-space analysis, although an optimum equilibration temperature (37°) and equilibration time (20 min) are necessary for reproducible chromatographic peaks. The FID was chosen for quantitative analysis since the sensitivity of the tritium electron capture detector was much lower. In addition, the excellent linear range (10^7) of the FID allowed for determination of the parent halothane as well as its metabolites per injection of head-space gas.

Sample chromatogram

Typical gas chromatograms of the direct injection of the head-space from rat blood containing CDE, CTE and halothane and from blood from a non-halothane anesthetized rat show adequate resolution on Porapak Q from normal blood constituents (Fig. 1). Chromatograms of liver homogenates and microsomal suspensions yielded very similar separation and resolution of CDE and CTE. In Fig. 1, the peaks with retention times of 2.2 and 3.4 min corresponded to those of authentic CDE and CTE, respectively, and represented blood concentrations of 2.1 and 8.4 nmole/ml. In addition, mass spectrometry confirmed these peaks to be CDE and CTE. The first two peaks eluting before CDE were air and water. At low concentrations of CDE (< 0.1 nmole/ml) the peak was reduced to a shoulder of the water peak, thus the integrator was programmed to measure peak areas by the tangent baseline method to avoid integration of part of the water. Mass spectrometric analysis of the peak that elutes after CTE was identified as acetone. From mass spectral analysis, the unknown peak that eluted prior to halothane appears to be an alcohol. This chromatographic system is also capable of resolving trifluoroethanol and 2-bromo-2-chloro-1,1-di-

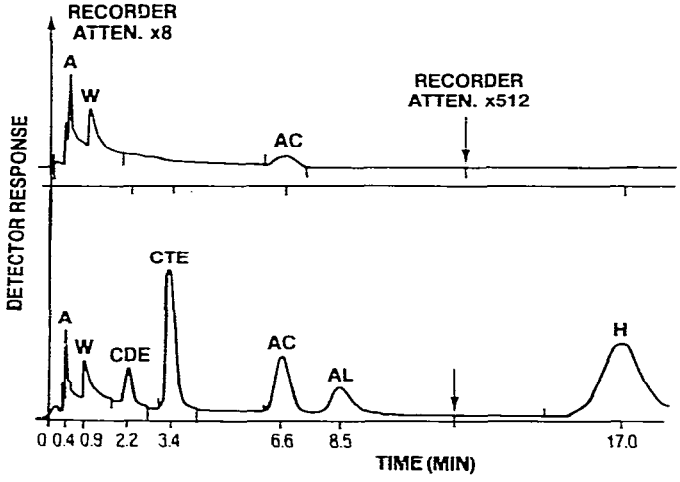

Fig. 1. Lower: typical gas chromatogram of vial head-space gas of 200 μl of inferior vena cava blood from a rat anesthetized with 1% halothane for 2 h. Upper: blank; identical conditions as in lower chromatogram, except with blood from an untreated rat. Peaks: A = air, W = water; CDE = 2-chloro-1,1-difluoroethylene (2.1 nmole/ml); CTE = 2-chloro-1,1,1-trifluoroethane (8.4 nmole/ml); AC = acetone; AL = alcohol; H = halothane.

fluoroethylene, other possible halothane metabolites, which were not found in any of the biological tissues investigated.

Linearity

Typical calibration curves of CDE and CTE described in the procedure are shown in Figs. 2 and 3. A linear relationship between detector response of the head-space gases and concentration of the gases in the liquid phase exists within the concentration range studied. Correlation coefficients were greater than 0.999. The applicability of the excellent wide linear range obtained is illustrated in Fig. 3. The standard concentrations of CDE and CTE (Fig. 3) reflect the experimental range of peripheral venous blood concentrations in rats and humans exposed to halothane, while the concentrations given in Fig. 2 represent

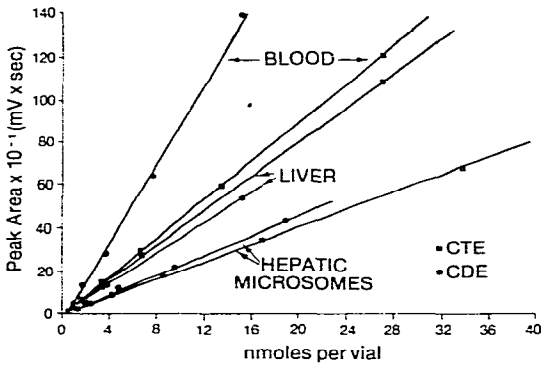

Fig. 2. Gas chromatographic calibration curves for the determination of CDE and CTE in central venous blood, liver homogenate and hepatic microsomal suspension. Detector response is measured as the integrator peak area per syringe of head-space gas plotted against the total vial content of CDE or CTE. Each point represents the mean of 3 replicate standards.

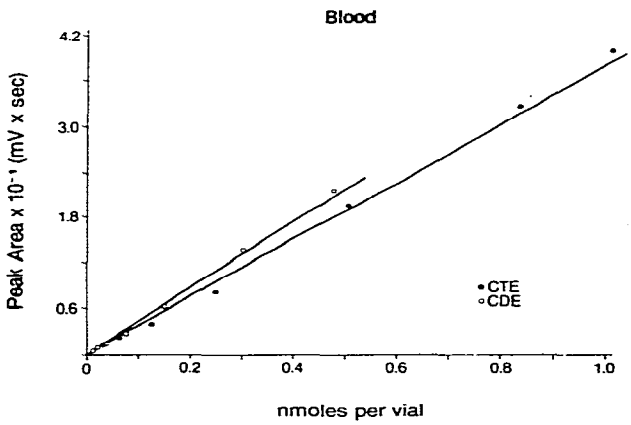

Fig. 3. Gas chromatographic calibration curves for the determination of CDE and CTE in peripheral venous blood. Conditions same as Fig. 2. Note that concentration ranges of CDE and CTE in central venous blood are approximately 50 and 30 times greater respectively than the corresponding concentration ranges in peripheral venous blood. Each point represents the mean of 4 replicate standards.

the range for central venous blood and liver of rats exposed to halothane and in vitro hepatic microsomal incubations with halothane. This represents an overall linear range of approximately 10^3, well within the acceptable linear range of the FID (10^7).

Precision

The precision of the method was determined by comparing the integrator peak areas of replicate control samples of blood, liver homogenate, or microsomal suspension fortified with known concentrations of CDE and CTE. The results presented in Tables I and II show that the mean relative standard deviations did not exceed 6% except for CTE in microsomal suspension (9%).

Recovery

The amounts of CDE and CTE recovered from the various biological samples are presented in Tables I and II. Depending on the sample up to 84% of CDE and 60% of CTE could be recovered by single sampling of the head-space. The percent recovered was calculated from standards prepared in heptane which were injected directly onto the gas chromatographic column. The higher recovery of CDE over CTE obtained from all biological samples probably reflects the greater volatility of CDE.

In order to determine the total recovery of the method, repeated injections were made of the head-space of microsome standards into the gas chromatograph until the detector gave no further response to CDE and CTE. Between each injection, the vials were allowed to re-equilibrate before additional sampling of the head-space. The total amount in each vial was computed by comparing the sum of the integrator peak areas with the peak areas obtained from

TABLE I

RECOVERY AND PRECISION OF THE GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF CDE IN BIOLOGICAL TISSUES

Replicate samples	CDE added (nmoles)	CDE recovered (nmoles)	Recovery (%)	Mean peak area (mV × sec)	Rel. S.D. (%)
Blood	0.0095	0.0079	83	0.40	7.5
(4)	0.15	0.12	80	6.2	6.0
	0.95	0.84	88	43	1.7
			Mean 84±4 S.D.		Mean 5.1
Liver	1.9	1.2	63	62	1.4
(3)	3.8	2.7	71	136	2.8
	15	10	67	533	1.2
			Mean 67±4 S.D.		Mean 1.8
Microsome	1.2	0.46	38	24	3.1
(3)	4.8	2.3	48	119	5.2
	19	8.3	44	426	10.3
			Mean 43±5 S.D.		Mean 6.2

TABLE II

RECOVERY AND PRECISION OF THE GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF CTE IN BIOLOGICAL TISSUES

Replicate samples	CTE added (nmoles)	CTE recovered (nmoles)	Recovery (%)	Mean peak area (mV × sec)	Rel. S.D. (%)
Blood	0.032	0.018	56	1.2	6.8
(4)	0.51	0.31	61	19	7.2
	1.7	1.05	62	65	5.5
			Mean 60±3 S.D.		Mean 6.5
Liver	0.84	0.49	58	30	1.8
(3)	6.8	4.5	66	276	7.2
	27	18	67	1083	6.6
			Mean 64±5 S.D.		Mean 5.2
Microsome	2.1	0.74	35	45	13.0
(3)	8.4	2.9	35	179	6.1
	34	11	32	665	8.0
			Mean 34±2 S.D.		Mean 9.0

CDE and CTE heptane standards. The data presented in Table III for microsomes show high recovery of CDE and CTE except at very low concentrations. The low recoveries at lower concentrations may be partly due to the measurement of smaller peak areas, adsorption of the compounds within the system, or partitioning of the compounds in the PTFE parts of the sampling syringe. Similar results were obtained with blood and liver homogenate standards. In any event, the single sampling of the head-space gas appears to be reasonably linear and precise for the accurate determination of the CDE and CTE levels in biological tissues.

TABLE III

TOTAL RECOVERY OF CDE AND CTE FROM HEPATIC MICROSOMAL SUSPENSION BY REPEATED GAS CHROMATOGRAPHIC HEAD-SPACE ANALYSIS

Added (nmoles)		Recovered (nmoles)		Recovery* (%)	
CDE	CTE	CDE	CTE	CDE	CTE
0.24	0.42	0.12	0.35	50	83
1.4	2.5	1.1	2.2	79	88
5.7	10	5.0	9.8	88	98
23	40	23	40	100	100

*Each recovery is the mean value of 2 replicate standards.

Sample reproducibility

Five replicate blood samples taken from a rat exposed to halothane were analyzed and found to have a relative standard deviation (S.D.) of the peak areas of 4% for CDE and 8% for CTE, indicating good reproducibility. The relative S.D. of the weights of the five aliquots of blood was 4%, suggesting that the variability in the peak areas is partly due to the variability in the blood weights.

Effect of protein-lipid content of sample

The volatility and detection of CTE and CDE is dependent on the composition of the aqueous phase, that is the concentration of protein and lipid in the sample. Calibration curves of CTE with two different dilutions of liver homogenate are shown in Fig. 4. The decreased recovery with increased concentration of liver indicates that the liquid to gas partition coefficient is highly dependent on the protein-lipid content in the sample. The solubility dependence of anesthetic agents on the composition of blood [11—13] and tissue homogenates [11] has been well documented.

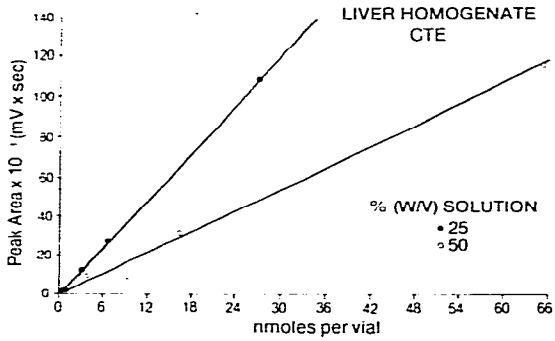

Fig. 4. Gas chromatographic calibration curves of CTE in liver homogenate as a function of homogenate composition. Conditions same as Fig. 2. Each point represents the mean of 3 replicate standards.

Detection limits

Detection limits were approximately 2 pmole/ml for CDE and 2.5 pmole/ml for CTE in blood, and 15 pmole/g for CDE and 10 pmole/g for CTE in liver under the conditions used. Incubation of hepatic microsomal suspension for 30 min containing 2 μl of halothane (18.8 μmole) and 5.0 mg/ml of microsomal protein, yielded detection limits of approximately 0.1 pmole/mg protein for CDE and CTE.

Applications

The assay procedure described above has been applied to the study of the metabolism of halothane in the hypoxic rat model [14]. It has also been used to study the reductive metabolism of halothane by isolated microsomal systems under anaerobic conditions [14].

The applicability of the head-space method for the determination of the vol-

atile metabolites of halothane in man, was tested by drawing venous blood samples from a patient during and after halothane anesthesia. Blood concentration—time curves of CDE and CTE on a semi-logarithmic scale are shown in Fig. 5. There was a rapid increase in the production of CDE and CTE during anesthesia and the levels peaked at the end of anesthesia. The half-lives of elimination of CDE and CTE were estimated to be 1¼ h and 3¼ h respectively. Since CDE and CTE are reductive metabolites of halothane and reductive metabolism of halothane preceeds its toxicity this assay method could be used as a clinical monitor for possible development of halothane hepatotoxicity in patients undergoing surgery in conjunction with drug pretreatment.

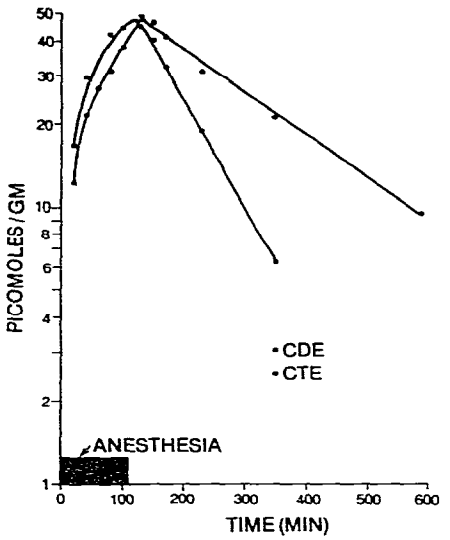

Fig. 5. Peripheral venous blood concentration vs. time curves for CDE (▪) and CTE (•) during and after anesthesia with 1.5% halothane for 110 min in a human patient undergoing surgery. Note that metabolite levels rose rapidly during anesthesia and peaked at the end of anesthesia. Half-lives of elimination were $t_{½}$ CDE = 1¼ h and $t_{½}$ CTE = 3¼ h.

ACKNOWLEDGEMENTS

This investigation was supported by grant 1-R01-CA21820-01 from the National Institutes of Health, Bethesda, Md. The mass spectrometric assistance of Sai Chang, Ph.D. and the excellent technical assistance of Dale S. Horne and Matthew Miller are gratefully acknowledged.

REFERENCES

1 K. Rehder, J. Forbes, H. Alter, O. Hessler and A. Stier, Anesthesiology, 28 (1967) 711.
2 E.N. Cohen, J.R. Trudell, H.N. Edmunds and E. Watson, Anesthesiology, 43 (1975) 392.
3 R.A. van Dyke, M.B. Chenoweth and A. van Poznak, Biochem. Pharmacol., 13 (1964) 1239.

4 A. Stier, H. Alter, O. Hessler and K. Rehder, Anesth. Analg. (Cleveland), 43 (1964) 723.
5 S. Mukai, M. Morio, K. Fujii and C. Hanaki, Anesthesiology, 47 (1977) 248.
6 J.H. Sharp, J.R. Trudell and E.N. Cohen, Anesthesiology, 50 (1979) 2.
7 G.E. McLain, I.G. Sipes and B.R. Brown, Jr., Anesthesiology, (1979) in press.
8 P.K. Wilkinson, J.G. Wagner and A.J. Sedman, Anal. Chem., 47 (1975) 1506.
9 H. Yamamura, B. Wakasugi, S. Sato and Y. Takebe, Anesthesiology, 27 (1966) 311.
10 I.G. Sipes, T.L. Podolsky and B.R. Brown, Jr., Environ. Health Perspect., 21 (1977) 171.
11 H.J. Lowe and K. Hagler, in R. Porter (Editor), Gas Chromatography in Biology and Medicine, Ciba Foundation Symposium, J. and A. Churchill, London, 1969, p. 86.
12 A.L. Cowles, H.H. Borgstedt and A.J. Gillies, Anesthesiology, 35 (1971) 203.
13 P.E. Reid, D.E. Brooks, Y.C. Pang, R. Muelchen and K.M. Leighton, J. Chromatogr., 146 (1978) 297.
14 R.M. Maiorino, I.G. Sipes, A.J. Gandolfi and B.R. Brown, Jr., Drug Metab. Dispos., submitted for publication.