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Determination by Ion-exchange Chromatography of Trifluoroacetic Acid as a Biliary Metabolite of Isoflurane in the Rabbit

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ABSTRACT

The optimal composition of eluant for trifluoroacetic acid (TFAA) analysis by ion-exchange chromatography was determined and a preliminary study to detect TFAA, a metabolite of isoflurane, in bile of rabbits during and after inhalation of isoflurane was made. The optimal composition of eluant for TFAA analysis by ion-exchange chromatography was determined to be a solution of 2mM Na_2CO_3 and 4mM $NaHCO_3$. Rabbits were divided into 4 groups; A group: 1 hr inhalation of 2% isoflurane, B group: 2 hr inhalation of 2% isoflurane, C group: 2 hr inhalation of 3% isoflurane, and D group: 2 hr inhalation of 4% isoflurane. The maximum biliary TFAA concentration in groups A, B, C and D averaged 17.5 ± 1.4 μM (mean ± S.D.) at 105 min, 37.1 ± 11.5 μM at 315 min, 54.5 ± 9.4 μM at 585 min and 34.4 ± 12.1 μM at 555 min after termination of inhalation. Cumulative excreted amounts of biliary TFAA for 20 hours were 746 nmols in A group (8 hours), 3,421 nmols in B group, 6,582 nmols in C group and 3,267 nmols in D group. Half-lives of biliary excreted TFAA were 63 min, 325 min, 478 min and 546 min, respectively. These results showed that ion-exchange chromatography is more convenient for detecting TFAA as a metabolite of isoflurane than isotachophoresis, gas chromatography, paper chromatography and thin layer chromatography and that TFAA was determined as a biliary metabolite of isoflurane in the rabbit.

Key words: *Anesthetic, isoflurane; Chromatography, ion exchange; Biotransformation, trifluoroacetic acid.*

Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) is an isomer of enflurane. The blood/gas distribution coefficient of isoflurane is as low as 1.4 and it is regarded to be a promising inhalation anesthetic with rapid induction and recovery[2,18]. Isoflurane is considered to be stable both chemically and biologically[18]. Of the inhaled isoflurane, 95% is excreted unchanged in the expired gas, and urinary excreted fluoride as metabolite is less than 0.2% of the total body intake of isoflurane during the three days of the experiment[4]. Its structural formula implies its biodegradation into trifluoroacetic acid (TFAA)[3]. Hitt et al had shown that a metabolite in the urine of isoflurane inhaled rats and man was TFAA by thin-layer chromatography[3].

Another fluorinated anesthetics, halothane[7] and fluoxene, also undergo dehalogenation and produce TFAA in the body. It is important to analyze TFAA for studies on metabolism of these anesthetics.

Paper chromatography[10], thin-layer chromatography[14] and gas chromatography[6] have been applied in the analysis of TFAA. However, these methods for assay of TFAA require complicated sample preparation, well trained technicians and much time for analysis. TFAA in the urine or bile of halothane anesthetized patients and rabbits was analyzed by isotachophoresis with a simple technique[5,8,15], but this method is not sensitive enough to analyze TFAA as a metabolite of isoflurane.

The present study determined the optimal condition for TFAA analysis by ion-exchange chromatography and the quantitative biliary excretion of TFAA in rabbits during and after isoflurane inhalation.

MATERIALS AND METHODS

1. Reagents: TFAA, sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$) were obtained from Katayama Chemicals and dodecyl benzen sul-

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fonic acid from Kao Soap Chemicals. Other reagents were commercial products of the analytical grade.

2. Equipment and operating conditions: An ion-exchange chromatography (Yokogawa Electric IC-100) equipped with an electric conductivity detector was used in this study. Anion exchange resin packed in 250 mm × 4 mm stainless column (Yokogawa Electric SAX-1) was used for the immobile phase. Column temperature was 40°C. A mixture of Na_2CO_3 and $NaHCO_3$ was used as eluant at a flow rate of 2 ml/min for analysis.

3. Determination of the optimal composition of eluant for TFAA: Twelve different composition ratios of Na_2CO_3 and $NaHCO_3$ tested were as follows 2mM Na_2CO_3 only, 3mM Na_2CO_3 only, 4mM Na_2CO_3 only, 5mM Na_2CO_3 only, 2mM Na_2CO_3 and 2mM $NaHCO_3$, 3mM Na_2CO_3 and 3mM $NaHCO_3$, 4mM Na_2CO_3 and 4mM $NaHCO_3$, 5mM Na_2CO_3 and 5mM $NaHCO_3$, 2mM Na_2CO_3 and 4mM $NaHCO_3$, 3mM Na_2CO_3 and 6mM $NaHCO_3$, 4mM Na_2CO_3 and 8mM $NaHCO_3$, and 5mM Na_2CO_3 and 10mM $NaHCO_3$. Using these eluants, the elution time of TFAA and another ions (fluoride ion, chloride ion, nitrite ion, phosphate ion, bromide ion, nitrate ion and sulfate ion in the mixture solution) were measured. This mixture consisted of 5 ppm of sodium fluoride, 10 ppm of sodium chloride, 15 ppm of sodium nitrate, 30 ppm of sodium biphosphate, 10 ppm of sodium bromide, 30 ppm of sodium nitrite and 40 ppm of sodium sulfate was used for testing separation of ions. The optimal composition for separation of TFAA from other anions was determined.

4. Analysis of TFAA as a metabolite of isoflurane: Male rabbits weighing 2-2.5 kg were given intramuscular injections of 20 mg/kg pentobarbital. Under regional anesthesia with 1% mepivacaine, tracheostomy was performed. Following intramuscular injection of 10 mg/kg gallamine, a Harvard respiration pump (Model 607) was connected to the animals and they were ventilated at a rate of 50 times/min and at a tidal volume of 5 ml/kg, using a non-rebreathing system. The femoral artery and vein were cannulated and arterial pressure was monitored. To maintain constant blood pressure, lactated Ringer's solution was continuously administered at 20—25 ml/hr via the femoral vein. During the experiment, pentobarbital and gallamine were intravenously administered as necessary. Following abdominal incision and ligation of the bile cyst, an external biliary drain was made by cannulation of the common bile duct.

Body temperature was maintained at 37°C with a heating pad (Gorman Rupp Model K-IC-3). Isoflurane concentration in the inspired gas and arterial blood was checked by gas-chromatography (Shimadzu GC4A) during and after administration of isoflurane. The bile was collected from external biliary drain every 30 min and was analyzed by ion-exchange chromatography.

Rabbits were divided into 4 groups: A group, 1 hr inhalation of 2% isoflurane (n=3); B group, 2 hr inhalation of 2% isoflurane (n=3); C group, 2 hr inhalation of 3% isoflurane (n=3); and D group, 2 hr inhalation of 4% isoflurane (n=3). To measure the concentration of biliary TFAA, the bile was diluted 10-fold with deionized water and 100 microliters of the sample was injected into the ion-chromatographic analyzer through a cation exchange filter.

5. Quantitative analysis of TFAA: The concentration of TFAA in this study was calibrated by using conductivity of authentic TFAA in 10-fold diluted bile.

RESULTS

1. Analytical condition of ion-exchange chromatography for TFAA analysis;

a) Determination of optimal composition of eluant: Fig. 1 shows that the elution times of TFAA ion (CF_3COO^-), nitrate ion (NO_3^-) and sulfate ion (SO_4^{--}) were close. When Na_2CO_3 and $NaHCO_3$ concentrations were reduced, the peaks of the respective ions were gradually separated. However, this required much time and the height of the peaks decreased. Of the 12 different compositions of eluants, 2mM Na_2CO_3 and 4mM $NaHCO_3$ 2mM Na_2CO_3 and 2mM $NaHCO_3$ or 3mM Na_2CO_3 only were relatively satisfactory for separation and economy of time. Among these three eluants, the composition of 2mM Na_2CO_3 and 4mM $NaHCO_3$ showed the shortest elution time of TFAA and better separation of TFAA from SO_4^{--}. Therefore, the eluant of 2mM Na_2CO_3 and 4mM $NaHCO_3$ (pH 9.83) was selected in the following experiments as optimal composition.

b) Analytical sensitivity of TFAA in bile: Fig. 2 shows calibration equation made by conductivity at various concentrations of authentic TFAA in 10-fold diluted with deionized water. The minimum analytical concentration of TFAA in the bile by this method was 0.5 μM. Using the equation, concentration of TFAA in the bile was calculated.

2. Identification and quantitative analysis of isoflurane metabolites;

a) Identification of TFAA in bile: Fig. 3 shows a chromatogram of 10 μM TFAA. The elution time of TFAA was 7.5 min. Fig. 4 shows a chromatogram of bile obtained prior to isoflurane inhalation. Fig. 5 shows a chromatogram of bile after inhalation of isoflurane, and the elution time of the peak was 7.5 min. Fig. 6 shows a chromatogram of a mixture of 10 μM TFAA and bile after isoflurane inhalation. The peak at the elution time of 7.5 min was single and was not separated. Peak height eluated at 7.5 min was nearly equal to the half-height of the solution in Fig. 3 plus the half-height of the bile in Fig. 5.

b) Biliary concentration of TFAA (Fig. 7): Time

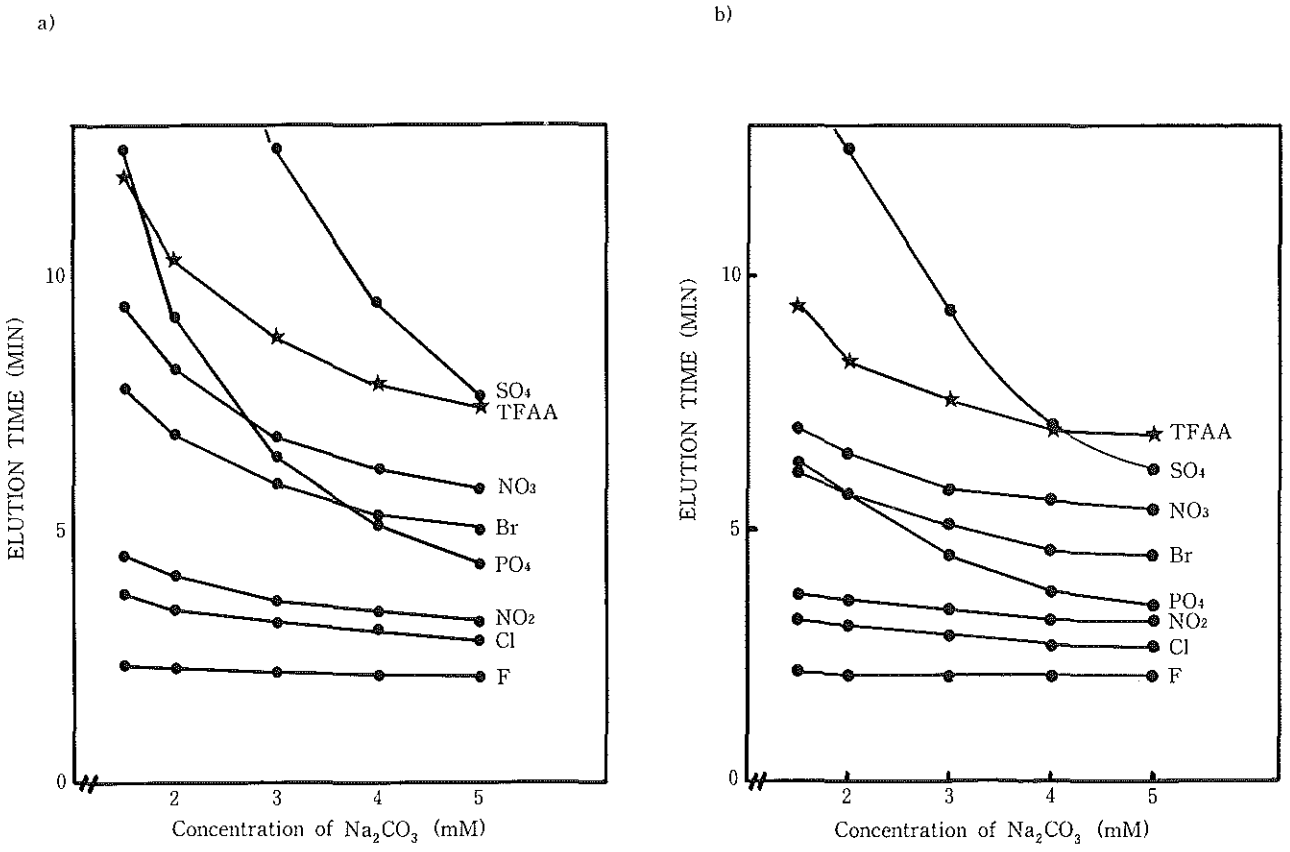
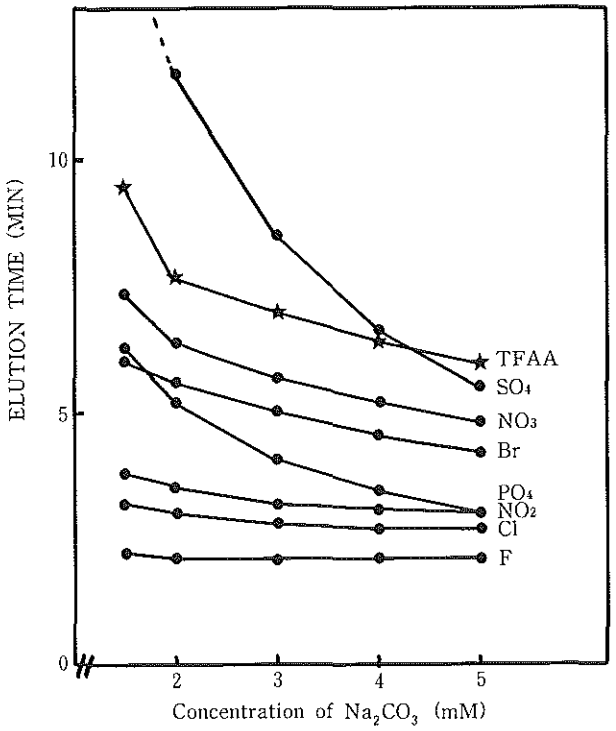


Fig. 1. Effect of composition rate on elution time; Using various composition ratios of Na_2CO_3 and $NaHCO_3$ for eluant at a flow rate of 2 ml/min, elution time of TFAA and anions were measured by ion-exchange chromatograph. Analytical temperature was 40°C.
a) The composition ratio of Na_2CO_3 and $NaHCO_3$ was 1:0. b) The composition ratio of Na_2CO_3 and $NaHCO_3$ was 1:1. c) The composition ratio of Na_2CO_3 and $NaHCO_3$ was 1:2.



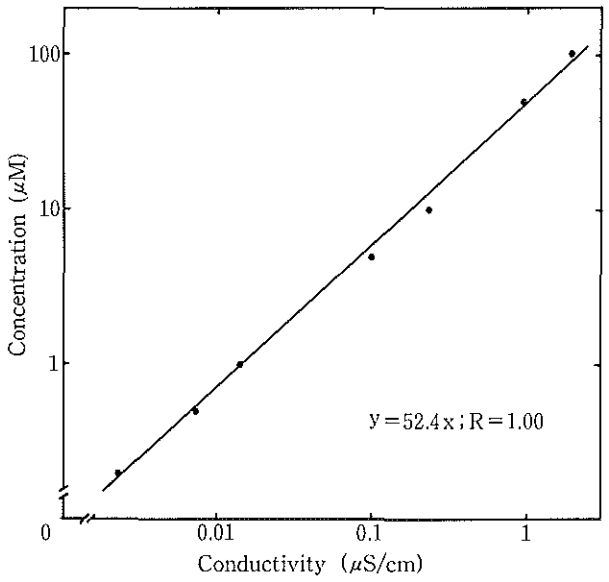


Fig. 2. Calibration equation made by conductivity at various concentration of authentic TFAA in 10-fold diluted bile with deionized water.

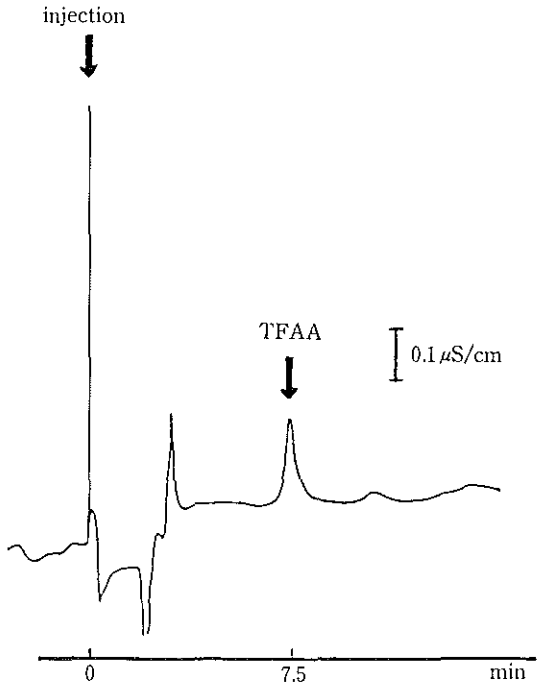


Fig. 3. Ion-exchange chromatogram of TFAA. A 100μl of 10 μM TFAA in deionized water was injected to the column of the ion-exchange chromatography. The eluant used was a mixture of 2mM Na_2CO_3 and 4mM $NaHCO_3$ at flow rate of 2 ml/min. Analytical temperature was 40°C. Elution time of TFAA was 7.5 min. Other detail was described in MATERIALS AND METHODS.

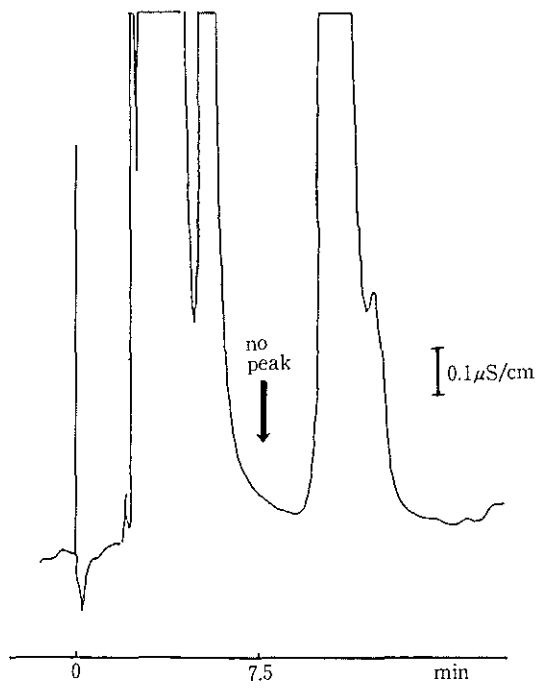


Fig. 4. Typical ion-exchange chromatogram of bile of untreated rabbit. A 100μl of 10-fold diluted bile with deionized water was injected. Analytical condition of ion-exchange chromatography was described in Fig. 3 legend. No anion was observed at elution time of 7.5 min.

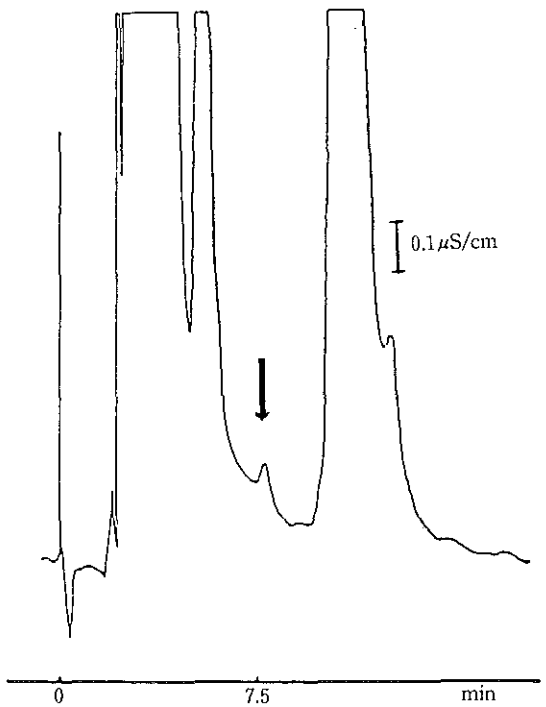


Fig. 5. Typical ion-exchange chromatogram of bile of rabbit after 2 hr inhalation of 3% isoflurane. A 100 μl of 10-fold diluted bile with deionized water was injected. Anion was eluated at elution time of 7.5 min. under the condition of ion-exchange chromatography as described in Fig. 3 legend.

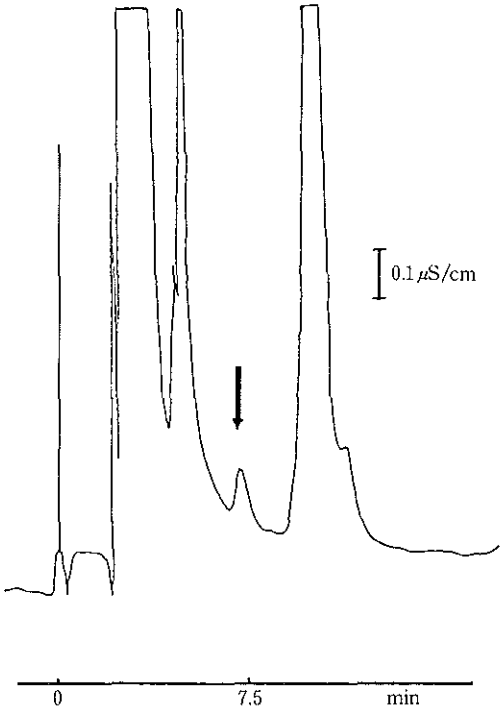


Fig. 6. Chromatogram of authentic TFAA and bile of rabbit after 2 hr inhalation of 3% isoflurane. A mixture of 50 μl of 10 μM TFAA (the same solution as in Fig. 3) and 50 μl of 10-fold diluted bile of rabbit after 2 hr inhalation of 3% isoflurane (the same bile as in Fig. 5) was applied under the condition of ion-exchange chromatography as described in Fig. 3 legend.

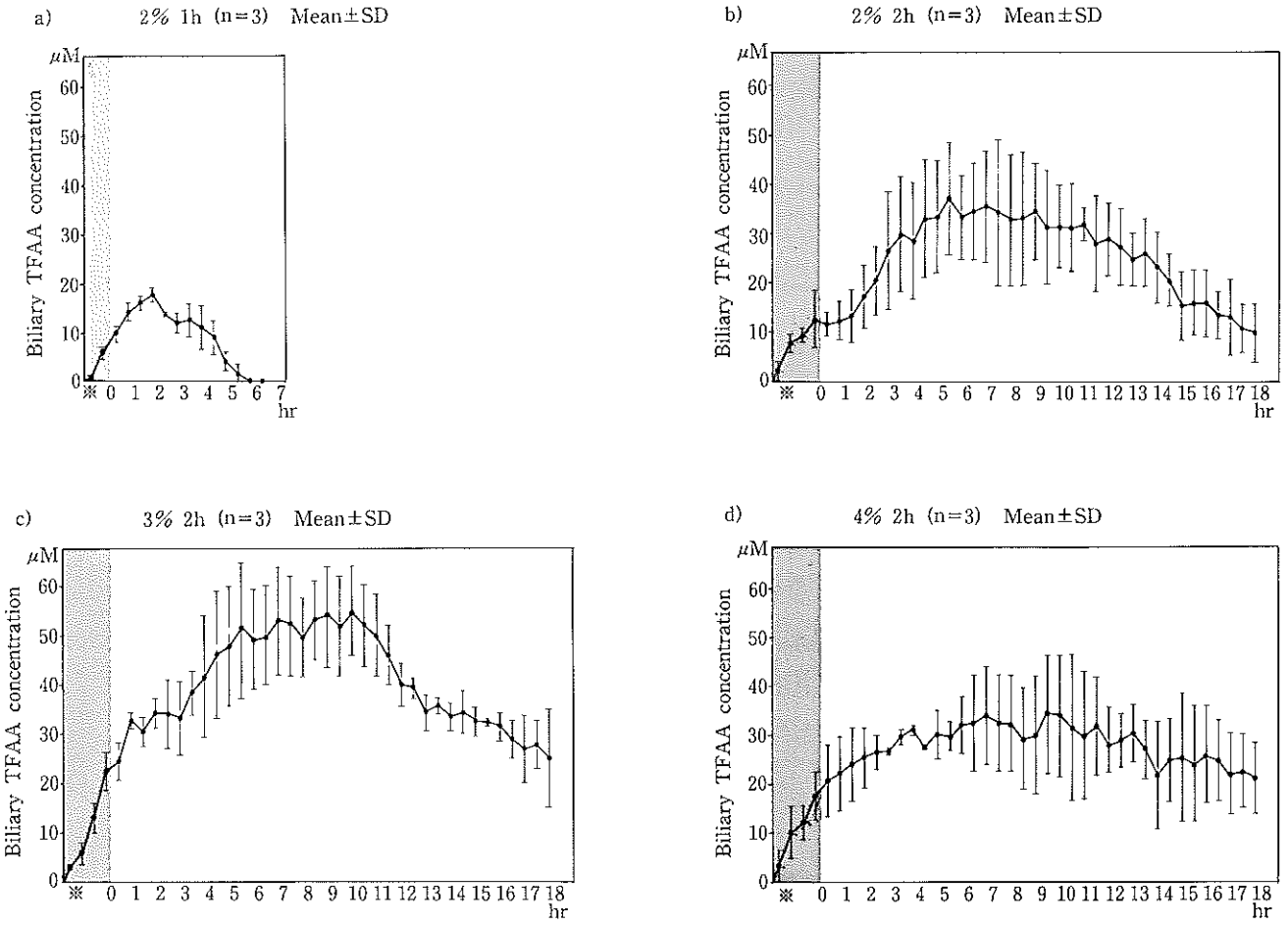


Fig. 7. Biliary TFAA concentration after inhalation of isoflurane. a) 1 hr inhalation of 2% isoflurane (A group) b) 2 hr inhalation of 2% isoflurane (B group) c) 2 hr inhalation of 3% isoflurane (C group) d) 2 hr inhalation of 4% isoflurane (D group). Shaded area(※) shows inhalation period of isoflurane.

0 was considered at the end of inhalation of isoflurane. Biliary TFAA concentration of A group was maximal at 105 min. Thereafter, the concentration decreased, and it became undetectable at 345 min. The maximum biliary TFAA concentrations in B, C and D groups were 37.1 ± 11.5 μM at 315 min, 54.5 ± 9.4 μM at 585 min and 34.4 ± 12.1 μM at 555 min, respectively. Thereafter, the concentration decreased.

c) The average amount of excreted bile measured every 30 minutes was 4.2 ± 4.4 ml as shown in Table 1.

d) Excretion rate of biliary TFAA (Fig. 8): Total amount of excreted TFAA/30 min was calculated from TFAA concentration and excreted bile volume measured every 30 min. The maximum biliary TFAA excretion rates of A, B, C and D groups were 110.3 ± 28.8 nmol/30 min at 105 min, 152.7 ± 20.1 nmol/30 min at 315 min, 255.8 ± 19.4 nmol/30 min at 315 min and 120.6 ± 15.2 nmol/30 min at 225 min, respectively. The values after 315 min were used for calculation of half-lives except for A group. Half-life of A group was calculated using the value of the rate after 105 min. The half-lives of A, B C and D groups were 63 min, 325 min, 478 min and 546 min, respectively.

e) Cumulative amounts of TFAA during the experiment: Cumulative amounts of TFAA excreted in the bile during this experiment of A, B, C and D groups were 745.8 ± 338.9 nmol, 3421.1 ± 294.1 nmol, 6581.9 ± 362.1 nmol and 3267.1 ± 450.6 nmol, respectively. The cumulative amounts of TFAA in bile of isoflurane anesthetized rabbits depends on inhaled concentration of isoflurane except D group.

DISCUSSION

1. Analytical condition of ion-exchange chromatography; At the optimal composition of eluant of 2mM Na_2CO_3 and 4mM $NaHCO_3$, the minimum analytical concentration of TFAA in bile was 0.5 μM. This analytical sensitivity is higher than that of isotachophoresis[8]. TFAA analysis by paper chromatography, thin-layer chromatography and gas chromatography have been employed, but these technics require very complicated sample preparation. Moreover, their sensitivity except gas-chromatography was lower than ion-exchange chromatography.

2. Identification and quantitative analysis of isoflurane metabolities;

a) Detection of TFAA in the bile of isoflurane in-

Table 1. Amount of bile measured every 30 minutes after inhalation of isoflurane. These amounts were used to calculate the excretion rate of biliary TFAA.

time (min) / group	0 ~ 30	30 ~ 60	60 ~ 90	90 ~ 120	0 ~ 30	30 ~ 60	60 ~ 90	90 ~ 120	120 ~ 150	150 ~ 180	180 ~ 210	210 ~ 240	240 ~ 270	270 ~ 300	300 ~ 330	330 ~ 360	360 ~ 390	390 ~ 420	420 ~ 450	450 ~ 480
A group (mean ± SD ml)	*5.4 ± 1.1	*4.7 ± 2.2			5.1 ± 1.5	5.4 ± 3.1	5.2 ± 1.8	6.5 ± 3.2	6.8 ± 2.7	6.6 ± 2.5	6.0 ± 1.8	5.6 ± 1.6	4.4 ± 1.1	5.7 ± 1.4	4.8 ± 0.9					
B group (mean ± SD ml)	*4.5 ± 1.0	*3.9 ± 1.1	*4.0 ± 1.5	*3.6 ± 0.6	3.7 ± 1.1	3.8 ± 1.3	4.4 ± 1.3	4.6 ± 1.6	4.6 ± 1.8	4.6 ± 2.1	4.8 ± 2.3	4.7 ± 2.5	4.5 ± 2.0	4.2 ± 2.0	4.5 ± 2.1	4.6 ± 2.1	4.4 ± 1.8	4.2 ± 1.6	3.9 ± 1.3	3.8 ± 1.2
C group (mean ± SD ml)	*4.5 ± 1.4	*4.2 ± 1.3	*4.3 ± 1.0	*4.4 ± 1.1	5.0 ± 1.4	4.8 ± 1.3	4.8 ± 1.1	5.0 ± 0.7	5.4 ± 0.7	5.3 ± 1.0	5.4 ± 0.7	5.3 ± 0.6	5.3 ± 0.7	5.1 ± 1.0	5.2 ± 1.1	5.0 ± 1.1	4.7 ± 1.0	4.6 ± 1.1	4.5 ± 1.0	4.5 ± 1.0
D group (mean ± SD ml)	*5.2 ± 1.0	*3.2 ± 1.4	*3.7 ± 0.6	*3.2 ± 0.4	3.1 ± 0.3	4.1 ± 0.7	4.4 ± 0.7	4.3 ± 0.6	4.2 ± 0.6	4.2 ± 0.6	4.0 ± 0.6	3.9 ± 0.6	3.7 ± 0.4	3.7 ± 0.5	3.6 ± 0.5	3.5 ± 0.5	3.5 ± 0.3	3.5 ± 0.5	3.6 ± 0.6	3.6 ± 0.5

time (min) / group	480 ~ 510	510 ~ 540	540 ~ 570	570 ~ 600	600 ~ 630	630 ~ 660	660 ~ 690	690 ~ 720	720 ~ 750	750 ~ 780	780 ~ 810	810 ~ 840	840 ~ 870	870 ~ 900	900 ~ 930	930 ~ 960	960 ~ 990	990 ~ 1020	1020 ~ 1050	1050 ~ 1080
B group (mean ± SD ml)	3.9 ± 1.4	3.9 ± 1.3	3.9 ± 1.1	3.8 ± 1.1	3.7 ± 0.8	3.5 ± 0.7	3.4 ± 0.7	3.3 ± 0.9	3.2 ± 0.6	3.3 ± 0.6	3.1 ± 0.3	3.1 ± 0.3	3.1 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	2.9 ± 0.3	2.7 ± 0.3	2.7 ± 0.6	2.6 ± 0.6	2.3 ± 1.1
C group (mean ± SD ml)	4.3 ± 0.8	4.2 ± 0.8	4.2 ± 0.8	4.2 ± 0.9	4.2 ± 1.0	4.2 ± 1.0	4.2 ± 0.8	4.1 ± 0.7	4.0 ± 0.7	4.0 ± 1.0	3.8 ± 0.9	3.8 ± 0.9	3.7 ± 0.8	3.4 ± 0.6	3.4 ± 0.8	3.5 ± 0.7	3.5 ± 0.8	3.5 ± 0.8	3.4 ± 0.7	3.3 ± 0.8
D group (mean ± SD ml)	3.5 ± 0.5	3.3 ± 0.5	3.1 ± 0.4	3.1 ± 0.6	2.9 ± 0.5	2.7 ± 0.3	2.6 ± 0.2	2.5 ± 0.2	2.3 ± 0.2	2.4 ± 0.3	2.3 ± 0.2	2.2 ± 0.2	2.3 ± 0.2	2.4 ± 0.1	2.3 ± 0.2	2.3 ± 0.5	2.4 ± 0.3	2.3 ± 0.5	2.2 ± 0.6	2.1 ± 0.7

(* mark shows inhalation period of isoflurane)

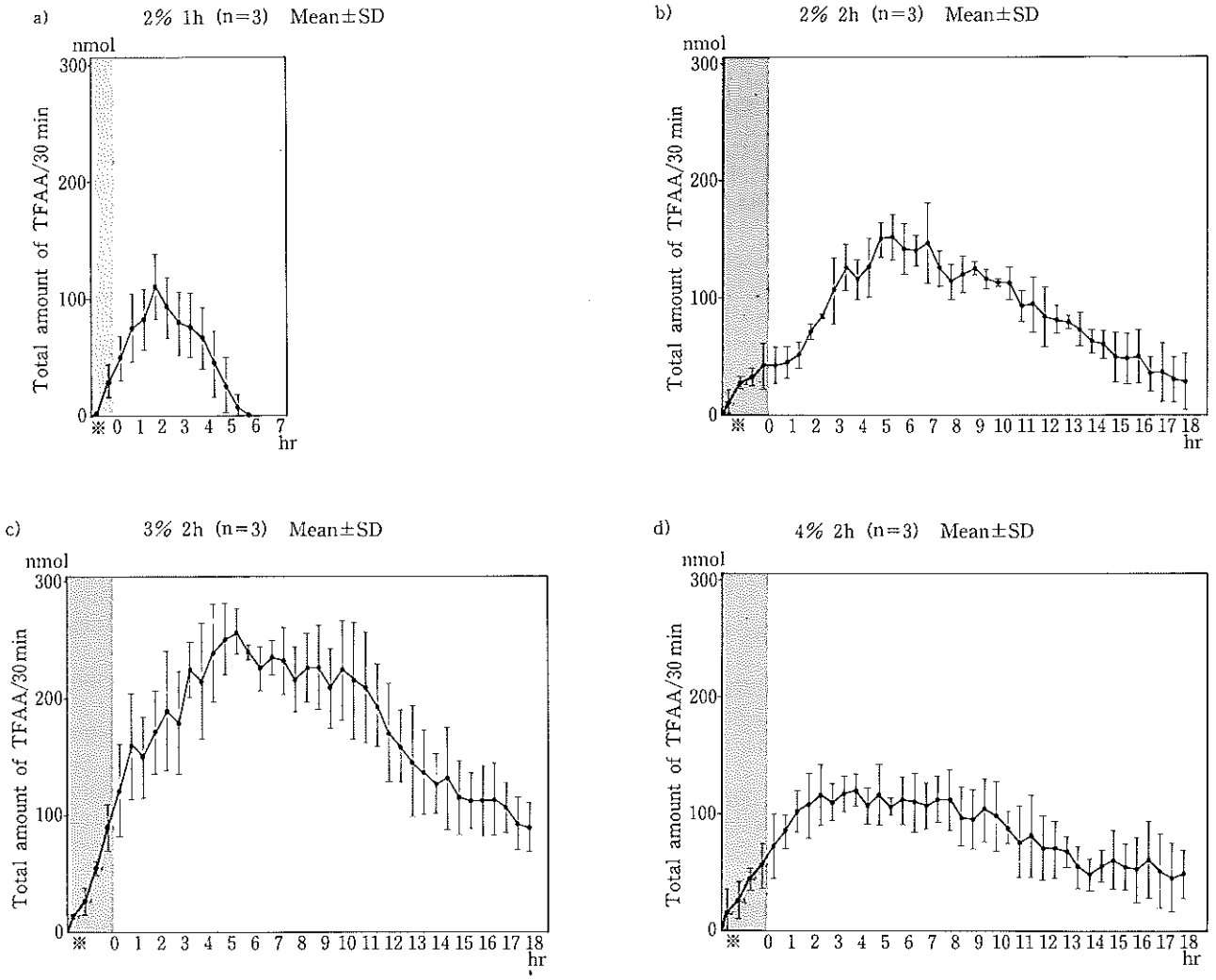


Fig. 8. Biliary TFAA excretion rate after inhalation of isoflurane. a) A group, b) B group, c) C group and d) D group. Y axis is indicated total amount of TFAA/30 min calculated from biliary TFAA concentration and volume of bile measured every 30 minutes.

haled rabbits: We detected a substance in the bile after inhalation of isoflurane which was not detected prior to inhalation by ion-exchange chromatography using an eluant of optimal composition. The elution time of this substance for ion-exchange chromatography was the same as that of authentic TFAA (7.5 min), and the height of this substance was increased when the inhalation time of isoflurane was longer or when the inhaled concentration was higher except D group. It is therefore considered that the substance detected in the bile after isoflurane inhalation is TFAA, a metabolite of isoflurane.

b) Pharmacokinetic study on metabolism of isoflurane: The greater the MAC (minimum alveolar concentration) hrs in A group, B group and C group, the greater was the rate of biliary excretion of TFAA. However, in D group, the excretion of TFAA was not proportional to MAC hrs. Stevens et al[12] reported that hepatic blood flow was proportional to blood pressure. During this experiment, systolic blood pressure was maintained at approximately 70—80 mmHg in A, B and C groups, but in D group blood pressure dropped to 50—60 mmHg. We therefor conclude that the supply of isoflurane and oxygen to the liver might be reduced and the production of TFAA in the liver might be decreased in D group.

Halothane is also biotransformed to TFAA[1,8,13,16,17]. Using isotachophoretic analyzer, Okida et al[9] measured biliary excreted TFAA in halothane anesthetized rabbits. They showed that the concentration of TFAA in the bile was higher at subanesthetic concentration of halothane than at anesthetic concentration. Sada[11] also reported that debromination of halothane at high concentration of halothane inhaled was smaller than that at low concentration of halothane. They suggested that hepatic metabolism of halothane to TFAA is inhibited by the agent itself, and therefore the excretion of TFAA in the bile is not proportional to MAC hours. However, our data show that excretion rate of TFAA in bile of isoflurane anesthetized rabbits depends on inhaled concentration of isoflurane except D group. Therefore, isoflurane may be metabolized to TFAA without such substrate inhibition like

halothane.

The excretion rate of TFAA in bile when 2% isoflurane (1.7 MAC) was inhaled for 2 hr was approximately 1/50 lower than that when 1.5% halothane (2.0 MAC) was inhaled for 2 hr at the end of inhalation[9]. Our results suggest that dehalogenation of isoflurane to TFAA was smaller than halothane.

In this study we showed that ion-exchange chromatography is convenient for measuring of small amounts of TFAA as a metabolite of isoflurane, and that dehalogenation of isoflurane to produce TFAA is smaller than that of halothane.

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