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ORIGINAL PAPER

Clinically Relevant Concentration Determination of Inhaled Anesthetics (Halothane, Isoflurane, Sevoflurane, and Desflurane) by ^{19}F NMR

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Abstract Biophysical studies of protein–anesthetic interactions using nuclear magnetic resonance (NMR) spectroscopy are often conducted by the addition of micro amounts of neat inhaled anesthetic which yields much higher than clinically relevant (0.2–0.5 mM) anesthetic concentrations. We report a ^{19}F NMR technique to measure clinically relevant inhaled anesthetic concentrations from saturated aqueous solutions of these anesthetics (halothane, isoflurane, sevoflurane, and desflurane). We use a setup with a 3-mm NMR tube (containing trifluoroacetic acid as standard), coaxially inserted in a 5-mm NMR tube containing anesthetic solution under investigation. All experiments are conducted in a 5-mm NMR probe. We also have provided standard curves for four inhaled anesthetics using NMR technique. The standard curve for each of these anesthetics is helpful in determining the prerequisite amount of aqueous anesthetic solution required to prepare clinically relevant concentrations for protein–anesthetic interaction studies.

Keywords Anesthetics · Clinically relevant concentration determination · Standard curve · ^{19}F NMR

Introduction

For biophysical studies involving protein–anesthetic interaction, anesthetic concentrations can be determined by different analytic methods (e.g., water/gas partition coefficient [1], infra red spectroscopy [2], gas chromatography [3–5]). Clinically used inhaled anesthetics are generally fluorinated compounds and ^{19}F NMR methodology could be extremely helpful in determining the concentration of these anesthetics for biophysical studies [1, 6]. A gas-tight syringe is commonly used for the addition of anesthetics to a test tube for anesthetic–protein interactions by NMR spectroscopy. However, the addition of neat inhaled anesthetics below 1 µl becomes difficult and/or unreliable resulting in much higher than clinically relevant concentrations. Clinically relevant concentrations of most commonly used inhaled anesthetics are in the range of ~0.2 to 0.5 mM [7]. In order to generate clinically relevant concentrations of these inhaled anesthetics, saturated aqueous solutions [8] can be used and the concentration of anesthetic can be determined by ^{19}F NMR. Herein, we report a detailed ^{19}F NMR method to determine clinically relevant concentrations of inhaled anesthetics using a 5-mm (outer diameter) NMR tube coaxially arranged with a 3-mm (outer diameter) NMR tube containing trifluoroacetic acid (TFA) as a standard. We also report ^{19}F NMR generated standard curves for these anesthetics (halothane, isoflurane, sevoflurane, and desflurane) over clinically relevant concentration ranges which can be used for any biophysical study involving protein–anesthetic interactions.

Methods

Four inhaled anesthetics, halothane (Sigma), isoflurane (Anaquest), sevoflurane (Abbott), and Desflurane (Baxter) were used in this study. Deuterated SDS_{D25} was purchased from Cambridge Isotope Laboratories, Inc., both 5- and

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3-mm NMR tubes were purchased from Wilmad Lab Glass. Airtight microsyringe was purchased from SGE LE Syringe, Australia.

Standard TFA solution preparation: 186 µl neat TFA (MW 114.03 and density 1.535 g/cc) was added to 24 cc of H_2O in a volumetric flask and a final volume of 25 cc was obtained by adding water (TFA concentration 100.15 mM). An aliquot of 50 µl of the 100.15 mM TFA stock solution was added to 950 µl of H_2O in a tightly sealed flask resulting in a diluted TFA solution (5.007 mM). This TFA solution was diluted ten times to make a 0.5 mM TFA solution, which was used as a standard for the concentration determination of the four aqueous anesthetic solutions (halothane, isoflurane, sevoflurane, and desflurane).

Saturated solutions of four anesthetics were prepared in a similar fashion. For each anesthetic, 200 µl neat anesthetic was added to 2 cc H_2O, shaken well and allowed to equilibrate <1 min. The required volume (75, 125, and 175 µl) of aqueous solution was added to the SDS solution in a 5-mm NMR tube (Fig. 1) for ^{19}F NMR studies.

For experiments with neat halothane, SDS solution was prepared by the addition of 200 µl H_2O with 8.2 mg SDS_{D25} and gently shaken to obtain a clear solution; to this 100 µl PBS (pH 7.2) buffer and 50 µl D_2O was added for NMR field locking purpose. The solution pH was adjusted by the addition of HCl. Final volume of the SDS solution was set to 450 µl and pH of the final solution was 7.2 before halothane addition.

For experiments with aqueous anesthetic solutions, three sets of solutions, [(8–8.6 mg SDS_{D25}, 50 µl D_2O, 325 µl PBS solution, 75 µl aqueous anesthetics solution); (8–8.6 mg SDS_{D25}, 50 µl D_2O, 275 µl PBS solution, 125 µl aqueous anesthetics solution), and (8–8.6 mg SDS_{D25}, 50 µl D_2O, 225 µl PBS solution, 175 µl aqueous anesthetics solution)] were prepared. In each case, the final volume of the SDS solution was adjusted to 450 µl and the pH of the solution was 7.2 before the addition of the aqueous anesthetic solution. In this experimental setup, both the 5 and 3 mm NMR tubes remained coaxially.

All NMR experiments were performed with a 500 MHz Bruker instrument operating at 470.56795 MHz for ^{19}F NMR studies at 22°C. A relaxation delay of 15–20 s was used in order to prevent any saturation effect of the TFA or anesthetic peaks as longitudinal relaxation (T_1) time of TFA is 135 ms [9] and T_1 relaxation time of halothane is around 660 ms [1]. All experiments were performed in a 5 mm ^{19}F probe and D_2O was used for field locking purposes. We used two coaxially arranged NMR tubes of different diameters for determination of anesthetic concentration. The thickness of the 5 mm NMR tube was 0.38 mm, the inner diameter of the 5 mm tube is 4.24 mm. The thickness of the 3 mm NMR tube was 0.29 mm, the inner diameter of 3 mm NMR tube was 2.42 mm (Fig. 1). The 3 mm NMR tube contained 200 µl of either 5 or 0.5 mM TFA solution as standard. Anesthetics, either neat or in aqueous solution, were added to the 5-mm tube containing SDS solution. Both NMR tubes were sealed tight with Teflon in order to prevent evaporation of inhaled anesthetics. It is important to note that NMR tubes were always handled at the top of the tube to minimize temperature change which might enhance evaporation of anesthetics from solution by disturbing the solution–gas equilibrium. TFA contains a CF_3 moiety similar to inhaled anesthetics (halothane, isoflurane, sevoflurane, and desflurane) under consideration (Fig. 2). The ^{19}F signal volume from the CF_3 moiety of TFA is used to calibrate the concentration of halothane, isoflurane, and desflurane. In the case of sevoflurane, ^{19}F peaks from the two CF_3 moieties; hence peak volumes of these CF_3 moieties are halved for concentration calibration with TFA.

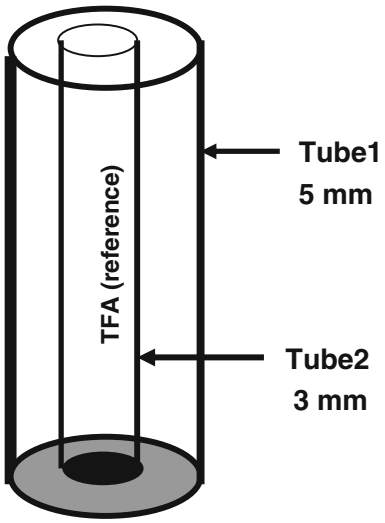

Fig. 1 Schematic diagram of the two coaxial NMR tubes (3 and 5 mm) used for anesthetic concentration determination studies

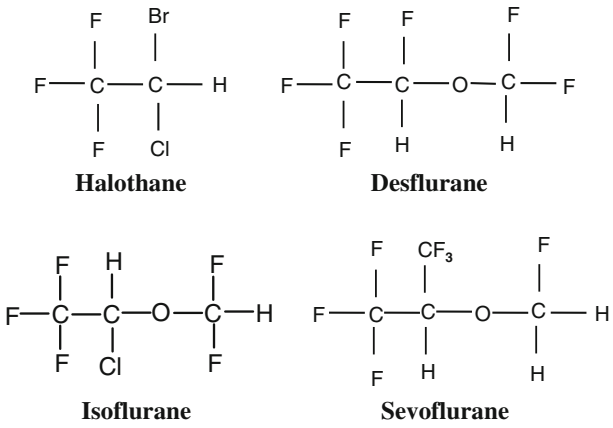

Fig. 2 Structure for four inhaled anesthetics under study

Results and Discussion

First, we have used this experimental technique to determine the halothane concentration due to the addition of neat halothane. Figure 3 represents the ^{19}F NMR spectra of: (A) SDS solution only; (B) SDS solution in 5 mm tube and 5 mM TFA in a 3-mm NMR tube; (C) 1 µl neat halothane in SDS solution in a 5-mm tube which is coaxially arranged with a 3-mm NMR tube containing 5 mM TFA; (D) 2 µl neat halothane in SDS solution in a 5-mm tube which is coaxially arranged with 3-mm NMR tube containing 5 mM TFA; and (E) 3 µl neat halothane in SDS solution in 5-mm tube which is coaxially arranged with a 3-mm NMR tube containing 5 mM TFA. NMR data as well as CF_3 peak volumes were processed using "Mestrc23" software. Since the outer diameter of the NMR tubes containing standard TFA (in 3-mm tube) and SDS_{D25} solution (in 5-mm tube) are different, peak volume correction is necessary for concentration determination. The volume of the solution within ℓ cm in the 5 mm NMR tube is proportional to

$$\pi\left[(2.12)^2 - (1.5)^2\right] \times \ell = 2.24\pi \times \ell\,\text{cc}. \quad (1)$$

The volume of solution within ℓ cm in the 3 mm NMR tube is proportional to

$$\pi(1.212)_2 \times \ell = 1.46\pi \times \ell\,\text{cc}, \quad (2)$$

where ℓ is the length sample in both tube in the NMR coil inside the probe. The ratio of the volume of the two solutions in a 3-mm tube:5-mm tube is $1.46\pi\ell$:$2.24\pi\ell$ = 1:1.53. In order to calculate the halothane concentration (Fig. 3) with reference to the standard TFA signal, the CF_3 peak volume from the anesthetic is divided by 1.53. The concentration of halothane, after addition of 1, 2, and 3 µl neat halothane is calculated to be 6.53, 11.53, and 14.05 mM, respectively (Fig. 3).

In order to determine the concentration due to the addition of aqueous anesthetic, the same methodology is applied. We measured the concentration of halothane, isoflurane, sevoflurane, and desflurane after the addition of various amounts (75, 125, and 175 µl) of aqueous anesthetic solutions (Fig. 4) to create a standard curve (Fig. 5). The ^{19}F NMR spectra for four anesthetics are shown in Fig. 4: panel I (halothane), panel II (isoflurane), panel III (sevoflurane), and panel IV (desflurane). The total volume of the final solution is kept constant in all four cases and amount of saturated aqueous solution in each case was the same. The concentration of the respective anesthetic was calculated based on the same procedure as outlined for neat halothane concentration determination. The concentrations of halothane, isoflurane, sevoflurane, and desflurane are plotted with the addition of the same amount of saturated aqueous anesthetics (Fig. 5). It is evident from Fig. 5 that in all four cases the addition of 65 to 90 µl (marked by two arrows to indicate the upper or lower range) of aqueous saturated solution in a total of 450 µl solution would generate a clinically relevant anesthetic concentration (marked by two dotted lines to indicate the concentration range).

Anesthetic and Protein Interaction

The effect of anesthetics on the central nervous system is believed to alter synaptic transmission, but the molecular

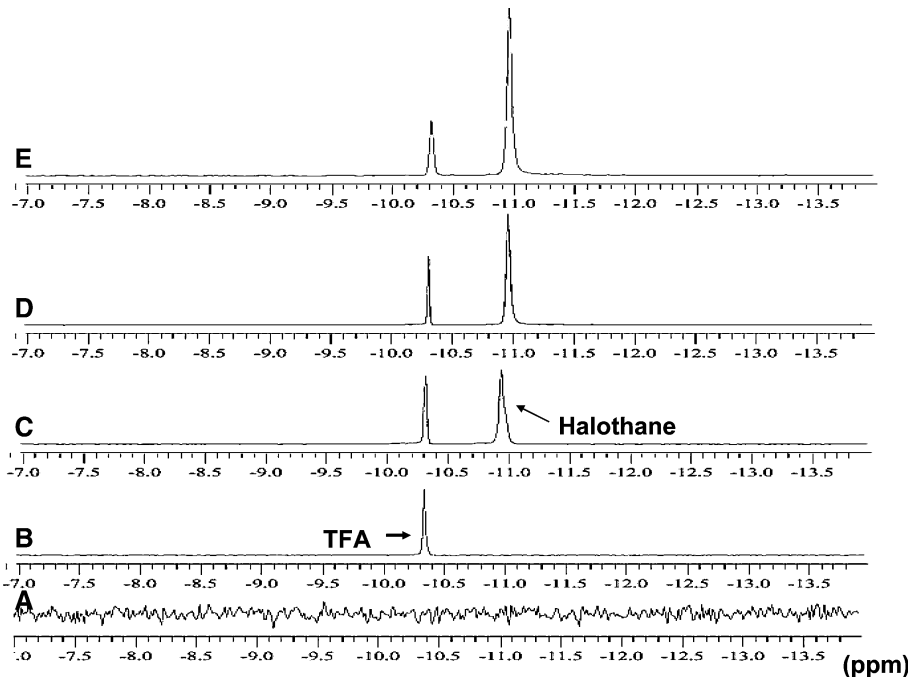

Fig. 3 ^{19}F spectra in different conditions: SDS only in 5 mm NMR tube (**a**), SDS only in 5-mm NMR tube and 5 mM TFA in 3-mm NMR tube (**b**), 1 µl neat halothane in 5-mm NMR tube and 5 mM TFA (reference) in 3 mm NMR tube (**c**), 2 µl neat halothane in 5-mm NMR tube and 5 mM TFA (reference) in 3-mm NMR tube (**d**), and 3 µl neat halothane in 5-mm NMR tube and 5 mM TFA (reference) in 3-mm NMR tube (**e**)

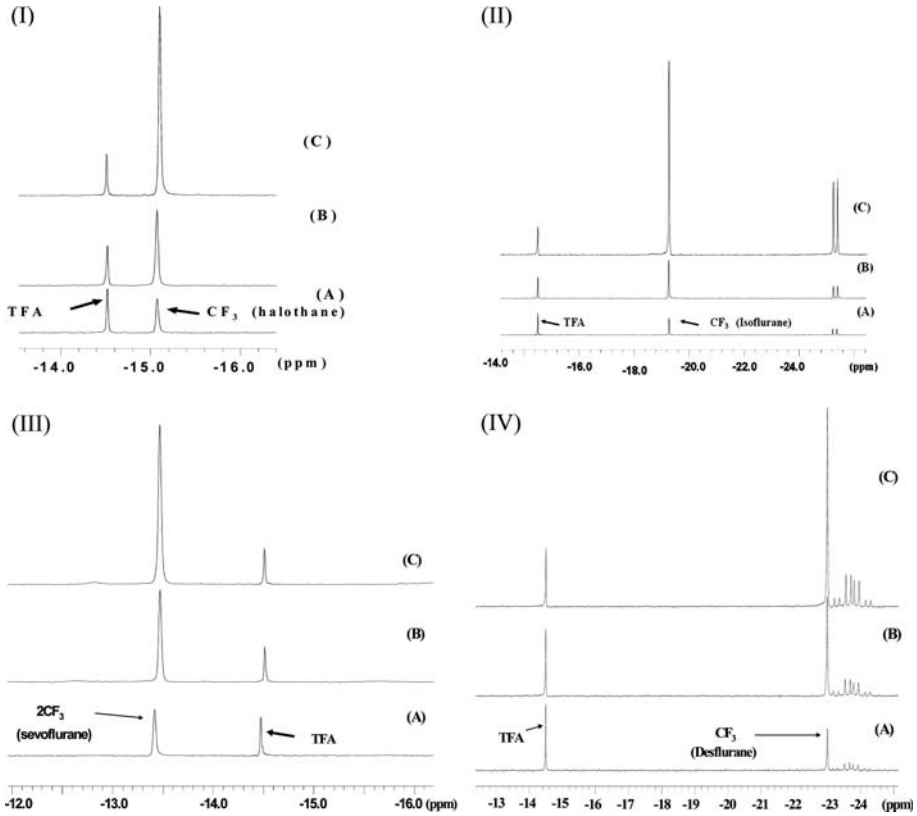

Fig. 4 ^{19}F spectra of aqueous halothane (I), isoflurane (II), sevoflurane (III), and (IV) desflurane in similar conditions. (A) 75 µl aqueous respective anesthetics solution was added in total 450 µl SDS only in a 5-mm NMR tube and 0.5 mM TFA was used as reference in a 3 mm NMR tube; (B) 125 µl aqueous anesthetic solution was added in total 450 µl SDS only in a 5-mm NMR tube and 0.5 mM TFA was used as reference in a 3-mm NMR tube. (C) 175 µl aqueous anesthetic solution was added in total 450 µl SDS solution in a 5-mm NMR tube and 0.5 mM TFA was used as reference in a 3-mm NMR tube

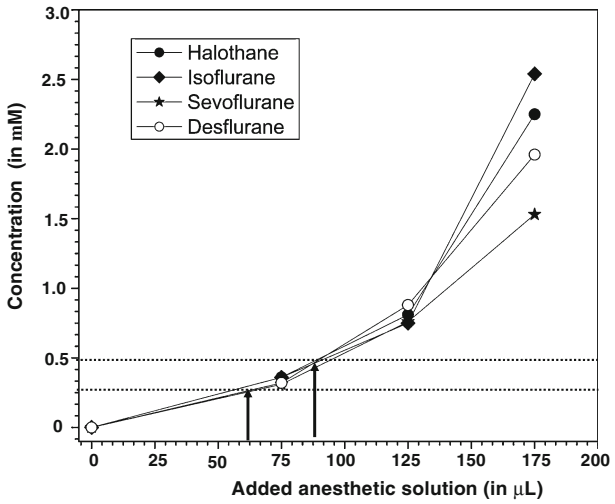

Fig. 5 Plot of concentration of different anesthetic with the addition of (75 µl, 125 µl and 175 µl) aqueous anesthetics solution, where total solution volume remains same (450 µl) in all four cases. Dotted lines indicate the clinically relevant concentration range of these anesthetics. The concentration plot is helpful to guide the amount of aqueous anesthetic need to be added for generating clinically relevant concentration (indicated by two arrows for lower and upper margin). It is important to note that although these standard curves provide the guidance for the amount of aqueous volume need to be added, however, it is always necessary to measure the anesthetic concentration using ^{19}F NMR as described here

processes are poorly understood [10]. There is evidence that this action may result because of anesthetic–protein interactions [11]. At clinically relevant concentrations, inhaled anesthetics inhibit the PDZ domain-mediated protein interaction between PSD-95 or PSD-93 and the *N*-methyl-D-aspartate receptor or neuronal nitric-oxide synthase [10]. Effects of different inhaled anesthetics on ion channel (e.g., K^+) [12, 13] as well as in different receptors [14] have also been reported. In these biophysical studies, the concentrations of halothane, isoflurane, and sevoflurane were determined by gas chromatography [10]. Heteronuclear single quantum coherence (HSQC) experiments [15] are useful in monitoring the protein–anesthetic interaction studies by ^{19}F NMR [16]. Recently Aβ peptide interaction studies with anesthetics have been reported and it has been shown that at higher than clinically relevant concentrations, anesthetics interact specifically with Aβ peptide to induce structural changes from monomeric to oligomeric forms [16]. These anesthetic-induced Aβ peptide structure modulations need to be performed at clinically relevant concentrations. The present experimental procedure using aqueous inhaled anesthetic to generate clinically relevant concentration of anesthetic concentration will be helpful in monitoring Aβ peptide–anesthetic interactions. However, the present experimental method is restricted to inhaled anesthetics containing a fluorine atom.

In conclusion, we have provided the standard curves for four inhaled anesthetics which are important to generate clinical relevant concentrations of these anesthetics for biophysical studies of anesthetic interactions with proteins, ion-channels, and receptors. A previous report [1] has demonstrated the determination of inhaled anesthetic concentration using ^{19}F NMR method with utmost care. However, in protein–anesthetic interaction studies, protein solution should not be disturbed by passing inhaled anesthetics and the NMR tube containing protein solution should not be spun. In our case, we are using a saturated solution and this experiment will give us an idea of how much of the saturated solution needs to be added to obtain clinically relevant concentrations. After addition of the aqueous anesthetic solution to the 5-mm NMR tube, we did not spin the NMR sample.

There has been a report [6] of neat isoflurane anesthetics concentration determination by a different setup where the sample tube is in a 5-mm tube and TFA solution is in a 10-mm tube. For the measurement of anesthetic concentration with this setup, a 10 mm ^{19}F probe is required and for protein–anesthetic interaction studies, the 5 mm NMR tube must be removed from the 10-mm NMR tube for HSQC and or homo and heteronuclear NOESY experiments. Hence the two tubes are separated for doing two different types of experiments. The separating of two tubes likely allows the evaporation of TFA from 10-mm tube as well as taking out 5-mm tube may disturb the equilibrium of anesthetic in aqueous and gaseous phases.

Conclusions

In our method, two coaxial tubes remain always together, for both protein–anesthetic interaction studies as well as determination of anesthetic concentration by ^{19}F NMR studies. We do not need to separate them until all necessary experiments are completed. This methodology can be extended to anesthetic concentration determination in liposomes and future studies for protein–anesthetics in liposomes.

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