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Measuring the decrement times of volatile anesthetics in Drosophila melanogaster using gas chromatography-mass spectrometry

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Determining the decrement times of anesthetics in *Drosophila melanogaster* using gas chromatography/mass spectrometry

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Abstract:

Model organisms are widely used in research, especially in the context of complex situations. One model organism that has been widely used is the common fruit fly, *Drosophila melanogaster* (*D. mel*). *D. mel* are most commonly used in the context of genetics, but they have also been widely used in research focusing on general anesthetics. One value that has not been measured in *D. mel*, however, as it relates to general anesthetics, is the decrement times. This is what the present research set out to determine. The anesthetics studied in this research were isoflurane and sevoflurane. Flies were exposed to 40 µL of anesthetic in a centrifuge tube for 10 minutes, after which the flies were allowed a recovery period of a set time. In the case of the control group, this time was 0 minutes. The anesthetic was then extracted using dichloromethane (DCM) and enflurane was added as an internal standard. The decrement times of male flies with isoflurane were found to be around 30 seconds for the 50% decrement, 2 minutes for the 80%, and 3 minutes for the 90% decrement. For female flies the values found with isoflurane were found to be about 30 seconds for the 50% decrement and 4 minutes for the 80% decrement. The values found for the 50% decrement time of sevoflurane were about 5 minutes for the female flies and 2 minutes for the male flies, but the values found in female flies with sevoflurane were not consistent. Though the data with sevoflurane were inconsistent, those collected with isoflurane give valuable insight into general anesthetics. This information can be used in future work involving drosophila flies and anesthetics to gain a deeper understanding of how inhalational anesthetics work.

Introduction:

The first publicized use of anesthetics was nearly 200 years ago in 1846, and since then the use of anesthetics has become more common and safer for the patient.[15,17] There are still many dangers of anesthetics, however, especially associated with prolonged and early exposure to anesthetic gases.[4,5,12,14,16] Although many discoveries have been made, there are still many aspects of anesthesia that are not well understood. One way to further understand anesthesia is through the use of model organisms. A recent discovery that used model organisms found that stimulation or inhibition of certain neurons in the hypothalamus of mice had a correlation with quality and duration of sleep.[6] To be used as a model organism, research must indicate that the organism responds to given stimuli in a way similar to how humans respond. This data can then be used to better understand the stimuli in humans. The fruit fly, *Drosophila melanogaster* (*D. mel*), is commonly used as a model organism, especially in the context of genetics, and has been said to be the model organism for genetics.[13] While genetics is the arena where *D. mel* has the most acclaim, the organism can be a model in many other applications.

One application for which *D. mel* has been used as a model is with anesthetics. Karunanithi et al. explored how *D. mel* are similar to humans in the way their minds and bodies react to anesthetics.[7] They present a basic overview of anesthesia and how it affects different levels of consciousness and discuss a few experiments with anesthetics and *D. mel*, such as using propofol-laced food in order to monitor activity of flies with different levels of anesthesia exposure. In 2017, MacMillan et al. explored the effects of isoflurane and sevoflurane anesthetics on many different aspects of health in *D. mel* including fecundity, starvation, and temperature tolerances.[9] MacMillan et al. found that *D. mel* generally recover more quickly from sevoflurane than isoflurane after similar exposure times. It was also found that with increased exposure time, the recovery time was more sex-dependent in isoflurane than in sevoflurane and

that the fecundity of female flies was not significantly affected by either sevoflurane or isoflurane.

D. mel have been used in many different experiments to determine how anesthesia affects different organisms and how its effects can change based on genetics and sex.[3,7,8,9,10,18] Many properties of anesthetics have been measured in the organism. One property of anesthetics that is very important to those who work in the field of anesthesiology is the minimum alveolar content (MAC). The minimum alveolar content is a measure of how saturated the alveoli in the lungs must be with an anesthetic before approximately half of all humans will not react to a surgical incision.[11] Zalucki et. al. found the MAC value in *D. mel* to be 0.2-0.6% depending on the criteria used to determine if the fly is conscious.[18] Consciousness was determined through a startle response, but the MAC was found to be higher if the test involved what the authors described to be more complex behavior, such as reacting to a light beam rather than reacting to a vibration.

One property of anesthetics that has not been measured in *D. mel* is the decrement times. The decrement time of an anesthetic is defined as the amount of time necessary for a given percentage of that anesthetic to have left the organism.[11] In 1997, Bailey calculated the decrement times of isoflurane, sevoflurane, enflurane, and desflurane in humans.[2] Bailey found that after 6 hours of exposure, enflurane has the longest 90% decrement time, at about 100 minutes, with isoflurane at 86 minutes, sevoflurane at 65 minutes, and desflurane being considerably lower than the rest at 14 minutes.

Knowing the decrement times of different anesthetic gases in *D. mel* will further establish the organism as a model for volatile anesthetic properties in humans. In this experiment, the

decrement times of isoflurane and sevoflurane in male and female *D. mel* will be measured using gas chromatography-mass spectrometry (GC/MS).

Materials and Methods:

The method for this experiment was largely new, though various other previously published experiments were consulted in choosing an internal standard, administration of anesthetic, and deciding the GC/MS method.[1,9,10] The flies used were wild type drosophila flies ordered from Carolina (catalog number 172100). They were kept in culture vials and fed drosophila fly medium with yeast placed in the media and hydrated using sterile water. Flies were allowed to propagate and were transferred to new vials every month or so. The vials were stored in a Fisher Scientific 307C Isotemp Low Temperature Incubator/Freezer set to 21.0 ˚C. No special attention was paid to using virgin female flies, thus flies were removed from vials and tested when necessary.

Due to the fact that male and female drosophila flies have been found to have different recovery times, which would imply different decrement times, the flies were first sorted male and female.[9] This was done by removing flies from the culture vial to a new vial without medium. Flynap was then administered to the flies until they were all asleep, not exceeding two minutes of exposure. The flies were then placed on a notecard under a dissection microscope and sorted into two piles based on sex. Images of male and female flies have been included. It should also be noted that female flies are typically larger than male flies, but because this can vary based on genetics, all flies were sexed by flipping the fly over and checking the genitalia.

Image 1. Male drosophila fly. Notice the darker genitals and smaller size.

Image 2. Female drosophila fly. Notice the lighter, smooth genitals and larger size.

After the flies were sorted, they were placed in empty culture vials overnight, to allow them to recover and to ensure that the Flynap had no effect on responses to the anesthetics being tested. The following day, the flies were removed from the culture vials into a conical centrifuge tube. A foam disc was then inserted into the top of the tube, to ensure that the flies would not be able to escape. Into this foam disc was injected 40 µL of isoflurane or sevoflurane, depending on which anesthetic was being tested. The cap was placed on the centrifuge tube to keep the anesthetic inside the tube, and the flies breathed the anesthetic for 10 minutes. At 10 minutes, the

flies were transferred to a glass centrifuge tube, and were either allowed to recover, or, in the case of the control group, immediately killed by pouring dichloromethane (DCM) into the vial. At this point, an enflurane internal standard was also added to the vial. The internal standard was added as soon as possible to ensure any mistakes made during the procedure also affected the concentration of internal standard. The internal standard solution was made by diluting 40 µL of enflurane in DCM to a total volume of 10 mL.

The flies were then squished using a glass stir rod, to extract all of the anesthetic from their bodies by the DCM. This solution was then removed from the vial and diluted to 5 mL, in order to be sure that every trial was the same volume, no matter how much was removed into the GC/MS vial.

Before data was collected, the response factor of the internal standard being used, enflurane, was calculated with both isoflurane and sevoflurane. These were run in equal concentrations and the following equation was used to calculate response factor (F):

$$F * \frac{Area_{IS}}{[IS]} = \frac{Area_A}{[A]}$$

Where "IS" represents the internal standard, and "A" represents the analyte, which was either isoflurane or sevoflurane, depending on the trial. Two trials were run for both isoflurane and sevoflurane, and these values can be found in table 1.

Table 1 The values measured to calculate response factor (F) of isoflurane and sevoflurane with enflurane. Because equal volumes of internal standard (IS) and analyte were used, the ratio of analyte to IS was equal to F. The average of the two runs was then calculated for use later.

Trial	Analyte Peak Area	Enflurane Peak Area	Area Ratio (analyte/IS)	Response Factor (F)	Average F
Isoflurane 1	1257948	182306	6.900	6.900	6.945
Isoflurane 2	1311796	187665	6.990	6.990	-
Sevoflurane 1	115502	186218	0.620	0.620	0.626
Sevoflurane 2	120471	190880	0.631	0.631	-

The GC/MS used was a Shimadzu GCMS-QP2010 SE and the column used was a Rtx-5MS column of length 30.0 m, diameter 0.25 mm, and thickness 0.25 µm. Many methods and solvents were tested to find one suitable. The solvent used was dichloromethane (DCM), but because DCM elutes later than isoflurane, sevoflurane, and enflurane, the mass spectrometer sensor was turned off around the 2-minute mark to avoid bombarding the sensor. Typically, the solvent elutes first, thus there is a solvent cut-off time, meaning the sensor waits to turn on until after the solvent has eluted. But because the solvent eluted later than the compounds being analyzed, it was decided to turn the sensor off after the analytes were measured.

The method used was adapted from MacMillan et al. and was as follows.[11] The column oven temperature was 30.0 ˚C. The injection temperature was 200.0 ˚C. A split injection mode was used with a split ratio of 1:50.0. The total flow was set to 54.7 mL/min with a column flow of 1.01 mL/min. The column oven temperature started at 30.0 ˚C and held at that temperature for 4.00 minutes. The temperature then increased at a rate of 40.00 ˚C/min to a temperature of 140.0 ˚C, which was held for 5.00 minutes.

Data and Discussion:

After exposing the flies to anesthetics and extracting them with DCM, the sample was analyzed by the method described above. The data collected have been compiled below for both female and male flies and for isoflurane and sevoflurane.

Table 2 Data for isoflurane in female flies. Percent decrement is calculated based on the control group.

Trial	Number of flies	Isoflurane peak area	Enflurane peak area	Amount of isoflurane	Isoflurane/fly	Percent decrement
Control	30	15380	5983	1.48×10^{-3}	4.94×10^{-5}	0
0.5 min recovery	21	5106	5388	5.46×10^{-4}	2.60×10^{-5}	47.34
1 min recovery	30	7097	5447	7.50×10^{-4}	2.50×10^{-5}	49.31
2 min recovery	31	3751	5053	4.28×10^{-4}	1.38×10^{-5}	72.05
3 min recovery	32	4471	4496	5.73×10^{-4}	1.79×10^{-5}	63.73

4 min recovery	26	3350	6305	3.06×10^{-4}	1.18×10^{-5}	76.15

The amount of isoflurane was calculated by using the same equation used to calculate F. Thus, the value calculated for the 0.5-minute recovery trial was calculated as follows:

$$F = \frac{(Area_{analyte}/Area_{IS})}{([analyte]/[IS])}$$

$$[analyte] = (\frac{Area_{analyte}/Area_{IS}}{F})([IS])$$

The value used for the concentration of internal standard ([IS]) was 0.004, because the internal standard was prepared by diluting 40 µL to 10.00 mL with DCM. The value calculated for the concentration of anesthetic was then divided by the number of flies in that trial, to standardize the amount of anesthetic present.

Table 3 Data for isoflurane in male flies. Percent decrement is calculated based on the control group.

Trial	Number of flies	Isoflurane peak area	Enflurane peak area	Amount of isoflurane	Isoflurane/fly	Percent decrement
Control	29	21681	5739	2.18×10^{-3}	7.50×10^{-5}	0
0.5 min recovery	29	9010	4958	1.05×10^{-3}	3.61×10^{-5}	51.90
1 min recovery	21	2816	4505	3.60×10^{-4}	1.71×10^{-5}	77.15
2 min recovery	32	3978	4598	4.98×10^{-4}	1.56×10^{-5}	79.24
3 min recovery	25	1689	6202	1.57×10^{-4}	6.27×10^{-6}	91.64
4 min recovery	23	1414	5607	1.45×10^{-4}	6.31×10^{-6}	91.58

Table 4 Data for sevoflurane in female flies. Percent decrement is calculated based on the control group. Notice the very low percent decrement after 2 minutes of recovery.

Trial	Number of flies	Sevoflurane peak area	Enflurane peak area	Amount of sevoflurane	Sevoflurane/fly	Percent decrement
Control	28	4247	5407	5.02×10^{-3}	1.79×10^{-4}	0
2 min recovery	30	4905	6011	5.22×10^{-3}	1.74×10^{-4}	3.04
5 min recovery	31	1740	4714	2.36×10^{-3}	7.61×10^{-5}	57.55
8 min recovery	34	2969	4167	4.56×10^{-3}	1.34×10^{-4}	25.30
10 min recovery	25	0	4707	0	0	100

Table 5 Data for sevoflurane in male flies. Percent decrement is calculated based on the control group.

Trial	Number of flies	Sevoflurane peak area	Enflurane peak area	Amount of sevoflurane	Sevoflurane/fly	Percent decrement
Control	31	7897	4598	1.10×10^{-2}	3.54×10^{-4}	0
1 min recovery	29	2081	4922	2.70×10^{-3}	9.32×10^{-5}	73.68
2 min recovery	27	4277	5918	4.62×10^{-3}	1.71×10^{-4}	51.69
3 min recovery	39	4560	9534	3.06×10^{-3}	7.84×10^{-5}	77.86
5 min recovery	32	4619	8626	3.42×10^{-3}	1.07×10^{-4}	69.80
7 min recovery	22	0	5304	0	0	100

The decrement times of isoflurane and sevoflurane in *D. mel* have not been measured and published to date. In order to measure this, male and female flies were separately exposed to 40 µL of isoflurane or sevoflurane for 10 minutes, killed, and the anesthetic was extracted using dichloromethane. The relative amounts of anesthetic were measured using gas chromatography-mass spectrometry, and it was found that the 90% decrement times for isoflurane for male flies was about 3 minutes. The 50% decrement times were also found to be around 30 seconds for female flies and slightly less than this for male flies, though an exact number for male flies was unknown due to the difficulty of extracting the anesthetic that quickly. The highest decrement times found for isoflurane in the female flies was the 80% decrement time at a little longer than 4 minutes.

The decrement times of sevoflurane are harder to determine based on the data collected. For female flies, the 50% decrement time was found to be a little under 5 minutes, but with a recovery time of 8 minutes, the decrement was found to be only 25%. A trial with a recovery time of 10 minutes using female flies found no sevoflurane peak, so it can be said that the 100% decrement time is somewhere between 8 minutes and 10 minutes. For male flies using sevoflurane, the data was similarly inconsistent. Like with the female flies, the only thing that can be said about the decrement times of sevoflurane in male flies is that the 100% decrement

time is somewhere between 5 minutes and 7 minutes, as no sevoflurane peak was found after a recovery time of 7 minutes.

One possible reason for the inconsistency in the data for sevoflurane is that sevoflurane may be metabolized by *D. mel* in some way that is unknown and that can occur at different rates under different circumstances. Though the environment during collection of data was never greatly changed, there may be some unknown factor that caused this change in metabolism.

Conclusion:

As mentioned before, the decrement times of anesthetics in *Drosophila melanogaster* have yet to be published, even though *D. mel* is a common model organism. Though the data collected using sevoflurane were inconsistent, the values found for isoflurane showed consistency and would be able to be used in future work involving drosophila flies and anesthetics to gain a deeper understanding of how inhalational anesthetics work.

This experiment is limited by the fact that the data must be interpreted in the context of the procedure conducted. That is, the flies were exposed to anesthetic for ten minutes, whereas in some experiments they may be exposed for much longer than that. The amount of anesthetic the flies were exposed to, however, saturates the centrifuge tube and makes adding any more anesthetic superfluous.[9] While this does present a challenge when applying this data to other situations, the data is nonetheless novel and can still be helpful in future research.

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