Video Article

Microdialysis of Ethanol During Operant Ethanol Self-administration and Ethanol Determination by Gas Chromatography

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Abstract

Operant self-administration methods are commonly used to study the behavioral and pharmacological effects of many drugs of abuse, including ethanol. However, ethanol is typically self-administered orally, rather than intravenously like many other drugs of abuse. The pharmacokinetics of orally administered drugs are more complex than intravenously administered drugs. Because understanding the relationship between the pharmacological and behavioral effects of ethanol requires knowledge of the time course of ethanol reaching the brain during and after drinking, we use *in vivo* microdialysis and gas chromatography with flame ionization detection to monitor brain dialysate ethanol concentrations over time.

Combined microdialysis-behavioral experiments involve the use of several techniques. In this article, stereotaxic surgery, behavioral training and microdialysis, which can be adapted to test a multitude of self-administration and neurochemical centered hypotheses, are included only to illustrate how they relate to the subsequent phases of sample collection and dialysate ethanol analysis. Dialysate ethanol concentration analysis via gas chromatography with flame-ionization detection, which is specific to ethanol studies, is described in detail. Data produced by these methods reveal the pattern of ethanol reaching the brain during the self-administration procedure, and when paired with neurochemical analysis of the same dialysate samples, allows conclusions to be made regarding the pharmacological and behavioral effects of ethanol.

Video Link

The video component of this article can be found at https://www.jove.com/video/4142/

Protocol

1. Stereotaxic Surgery

1. All procedures follow the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.
2. Using a stereotaxic apparatus, well-handled Long-Evans rats, anesthetized with isoflurane, are implanted with a 21-gauge cannula (for later microdialysis probe insertion) (Plastics One, Roanoke, VA) above the brain region of interest, and a tether bolt is built into the head stage (used to support microdialysis equipment).
3. Monitor rats carefully during and after surgery. Make sure that all animals receive at least one week of post-surgical care and recovery and are healthy before beginning the following procedures. A JoVE video of the stereotaxic surgery method is available.[1]

2. Operant Training

1. After a week of post-surgical recovery, animals are trained to lever press for 10% sucrose solution in a Med Associates operant chamber (MedAssociates, Inc., Vermont, USA) outfitted with a lickometer, retractable lever, and sipper tube (as previously described in Howard *et al.*, 2009).[2] Software from MedAssociates is used to design all operant programs (MedAssociates, Inc., Vermont, USA).
2. Once trained to respond for sucrose, begin rats on an appropriate training schedule during which ethanol is gradually added into the solution over multiple drinking sessions.
3. For example, our lab currently uses an 8-day training schedule where ethanol is faded into the solution culminating in a 10% ethanol/ 10% sucrose drinking solution. Control animals only receive 10% sucrose or no drinking solution. The specific type of operant schedule to be used can vary widely, but the general procedures described below for microdialysis sampling can still be carried out. [3]

3. Pre-microdialysis Procedures: Tethering

1. The night before the second to last training session (in our example this is the 7th training session), animals are habituated to the tether spring, which attaches to the tether bolt on their head stage. The spring attaches to the microdialysis swivel and counter balance lever arm, which is suspended next to the cage with a ring stand and clamps so that the animal can move freely within its cage. Tethered animals spend the night in the operant room, in their home cage with *ad libitum* food and water, to habituate them to the tethering set-up.
2. The following day, the rat completes its operant program with the tether in place, to habituate the animal to the feeling of the tether while performing its behavioral tasks. Mount the tethering apparatus to the wall of the operant chamber near the top to allow suspension of the tether and swivel above the center of the roof of the operant chamber. All of this is placed within the sound attenuating chamber. After the session, return the rat to its home cage in the operant room to await microdialysis probe insertion.

4. Pre-microdialysis Procedures: The Microdialysis Probe is Inserted the Day Before the Microdialysis Experiment, after the Rat has Completed Behavioral Training for the Day

1. The day before the microdialysis experiment, after the rat has completed an operant session while tethered, anesthetize the rat with isoflurane and remove the obturator from the guide cannula. Slowly (over ~ 5 min) insert a microdialysis probe, perfused with artificial cerebral spinal fluid (ACSF), through the cranial guide cannula into the brain region of interest. We use laboratory-constructed probes and microdialysis procedures modeled after Doyon *et al.*, 2003, and Pettit and Justice, 1991.[3, 4]
2. Use the previously discussed tethering apparatus to suspend the inlet and outlet lines of the probe above the animal.
3. Turn the probe flow rate down to 0.2 µl/min. Once again, the rat spends the night in the operant room.

5. Microdialysis Procedures: Collection of Samples During Self-administration Session with Appetitive and Consummatory Phases Separated

1. At least 2 hr before the experiment begins, turn up the probe to its working flow rate. We use either 1.0 or 2.0 µl/min depending on the brain region. Check that the probe flow rate is consistent, and at least 90% of the set flow rate by using a Hamilton syringe to measure volume over time.
2. Dialysis samples (5 or 7 min) are taken before, during, and after the operant session. The sampling interval depends on the brain region, neurotransmitter being analyzed, dialysate concentration of the analyte, and sensitivity of the analytical chemistry equipment to be used for the analysis. The behavioral phases are as follows:
    1. **Baseline:** At beginning of experiment, collect dialysis samples in the animal's home cage (4 samples).
    2. **Transfer:** After collection of home cage baseline samples, transfer the rat to the operant chamber. Transfer of the tethered rat requires extreme care to make sure that the microdialysis fluid transfer line does not become tangled, and that the rat remains calm. Immediately after the transfer, activate the operant program as you change from the baseline/transfer sample to the first wait sample.
    3. **Wait:** Continue to collect samples as the rat waits for the lever to extend into the chamber (3-4 samples depending on the brain region).
    4. **Drink:** After the lever is presented and pressed, a bottle of drinking solution (10% sucrose/10% ethanol or 10% sucrose) is made available to the rat for around 20 min (3-4 samples).
    5. **Post-drink:** After drink period, the bottle retracts, but the rat remains in the operant chamber for around 20 min (3-4 samples).

6. Microdialysis Procedures: Preparation of Microdialysis Sample for Ethanol Analysis

1. Two samples before animals self-administer the ethanol solution, and all samples after, are evaluated for ethanol concentration. Pipette either a 1 or 2 µl aliquot from the sample of interest into a 2 ml glass vial. Then seal the vial with an air tight septum (9 mm Red Poly Screw Cap, PTFE/Sil Septa, Agilent Technologies). The volume of the ethanol analysis aliquot (1 or 2 µl) depends on the total sample volume, and the sample volume required for any additional analyses.
2. If the samples will be used for later neurochemical analysis, store the sample appropriately after pipetting the aliquot for ethanol quantification.
3. For example, our lab analyzes the samples for dopamine. To accomplish this, place the samples on dry ice during the experiment and then, store the samples at -80 °C after the experiment. We use 2 µl aliquots for ethanol analysis of a 5 min sample collected using a flow rate of 2.0 µl/min for probes placed in the nucleus accumbens. This allows at least 7 µl remaining for later analysis of dopamine by high performance liquid chromatography with electrochemical detection.
4. For samples taken from the prefrontal cortex, which has much lower extracellular dopamine concentrations, we use a 1 µl aliquot for ethanol analysis taken from a 7-min sample using a flow rate of 1.0 µl/min.

7. Post-microdialysis Procedures

1. After the conclusion of the microdialysis experiment, anesthetize the rat and remove the probe. Replace the obturator if the animal is not immediately euthanized. Collect the brain within three days of the experiment. Otherwise, visualization of the probe tract may not be possible.
2. The brain should be removed in accordance with approved animal use protocols. We use sodium pentobarbital (150 mg/kg, ip) overdose, followed by cardiac perfusion with saline and then formalin in saline. Submerge brain in a formalin-saline solution for at least 12 hr before sectioning.
3. Coronally section the brain into 100 µm slices. Stain slices with cresyl violet, and examine for correct probe placement.[5, 6]

8. Analysis of Samples for Ethanol Concentration

1. The collected samples as well as external standards (0.3125 - 20 mM ethanol) are run on our gas chromatograph with flame ionization detection system. This system is comprised of a Varian CP 3,800 gas chromatograph with a flame ionization detector, a Bruker (Varian) 8,400 headspace autosampler heated to 50 °C, and an HP Innowax capillary column (30 m x 0.53 mm x 1.0 µm film thick), with helium as mobile phase.
2. Chromatograms are recorded and analyzed with chromatography software such as the Varian Star Chromatography Workstation software that will be specifically discussed here.
3. To prepare the system for ethanol analysis, heat the autosampler plate using a recirculating water bath, and open the two additional gas tanks (air and hydrogen; helium is always left running to preserve the wax column).
4. Record the gas tank pressures, as well as the number of samples you intend to run so that you can keep count of the number of times the fiber and septum have been used so that they can be changed when appropriate (fiber-500 injections; septum-100 punctures).
5. Initiate the start-up method, which prepares the system to run samples (program parameters in Table 2). Wait for the system to report that it is "Ready".
6. Initiate a 20-min "burn," which prepares the fiber for sample analysis by subjecting it to a high temperature to desorb any contaminates it has absorbed while at rest.
7. Standards are made by diluting 238 µl of 95% ethanol with water to a final volume of 100 ml using a volumetric flask in a chemical fume hood. This creates a 40 mM ethanol solution. We use a 1:1 serial dilution to create 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 mM standards. For each concentration of standard, use the same volume aliquot that will be taken from the dialysis samples. Pipette the aliquot into a 2 ml glass vial, and then seal the vial with an air tight septum.
8. All ethanol samples are heated in the autosampler until the entire liquid aliquot has been vaporized. We heat our samples for about 30 min.
9. This section describes the Star Workstation software that we use with our gas chromatograph. Other software may be used, but the description below may not be applicable.
10. To run samples, create a sample list noting which sample is in which autosampler slot, and how many times the sample should be punctured. Be sure to route the data files to your selected folder. Then, select the method of choice for your samples, and begin the run. We use the running parameters noted in Table 2.
11. Our dialysate ethanol program absorbs the sample for 3 min and desorbs into the helium stream, which feeds into the wax column, for 1 min. However, programs can be written to accommodate your specific needs.
12. The sample components separate in the wax column, and then go through the flame ionization detector where the carbon containing compounds, such as ethanol, combust and release ions. This results in increased electron flow from the detector's anode to the cathode creating a current, which is converted to voltage and recorded resulting in chromatograms like the one shown below (**Figure 1**). The change in voltage is proportional to the amount of carbon passing through the detector across time.7
13. After the system is finished running the samples you will need to check the integration of each peak. For Star Workstation software, click on the blue peak icon in the tool bar. Click on each peak's color and drag the arrows to adjust the baseline. Re-integrate each peak before moving onto the next group of peaks. The peak analysis software can be any general chromatography software.
14. For Star Workstation software, click on the batch report icon in your tool bar. Drag each sample from your specified folder to the batch report to print the sample reports. Reporting software can be customized with most common chromatography software systems.
15. The reports can be programmed to show the information of your choice. We currently use peak height, but the reports can also be programmed to use peak area.
16. To shut down the system, initiate the shut-down method (parameters noted in Table 2), turn off the water bath, and close the hydrogen and air tanks when the column oven temperature reaches 30 °C.

9. Ethanol Data

1. Plot the peak height as a function of each known external standard ethanol concentration (**Figure 2A**). Use the linear function given by these points to calculate the ethanol concentration from the peak height given by each dialysate sample.
2. By plotting the concentration of ethanol in the dialysate over time, we can see the pattern of ethanol levels in the brain region of interest during our behavioral session. Example data, shown below (**Figure 2B**), are represented as the concentration of ethanol in the dialysate across time during the self-administration session.

10. General Maintenance: The Fiber Should be Changed Every 500 punctures, and the Septum Every 100 Punctures

To change the fiber

1. On the gas chromatograph key pad press Menu, select 8,400, press enter; select change syringe, press enter. The autosampler will position itself to allow the fiber (SPME Fiber Assembly, 75 µm Carboxen-PDMS, Supelco Analytical, Bellefonte, PA) to be removed.
2. Open the door covering the fiber. Unscrew the lock nut, and move the latch to allow the fiber assembly to be removed. Take the fiber assembly out. Unscrew the nut that holds the fiber in the assembly, and then unscrew the fiber.
3. Replace the old fiber with a new one, and reassemble the fiber assembly. Replace the assembly in the autosampler, re-latching and screwing the assembly into place. When you are finished, press "change done" on the key pad, and the autosampler will ready itself for use.

To change the septum

1. First, unscrew the cap covering the septum (Hi-Temp .450 Dia. Generic Conditioned Septa, Varian) and then remove the old septum. Seat the new septum down into the fitting. Re-screw the cap and tighten it with the wrench. Then, use an injection needle to puncture the septum so that the fiber will not break the first time the septum is punctured.

11. Representative Results

Figure 1 shows example chromatograms for three concentrations of ethanol standards and for a rat dialysate sample collected at the end of the ethanol self-administration session. Ethanol peaks should be relatively symmetric, have consistent retention times, and a signal to noise ratio > 10. Failure to meet these criteria means that your system requires maintenance. Quality chromatography and correctly prepared standards produce a linear standard curve (R2 ≥ 0.99; **Figure 2A**) that is used to calculate the ethanol concentration of dialysate samples collected from ethanol self-administering animals over the course of their self-administration session (**Figure 2B**).

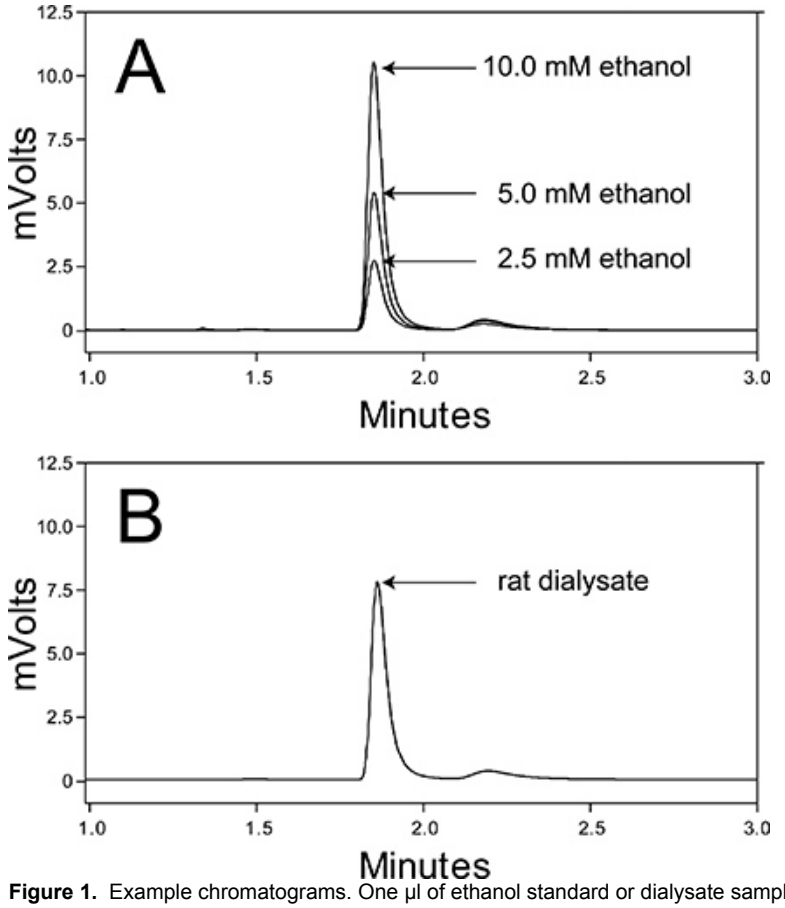

Figure 1. Example chromatograms. One µl of ethanol standard or dialysate sample was loaded into a gas chromatograph vial and analyzed as described in the text. A) Overlay of peaks generated from 2.5, 5.0 and 10 mM ethanol standards. B) Peak generated from a dialysate sample from a rat that has self-administered ethanol.

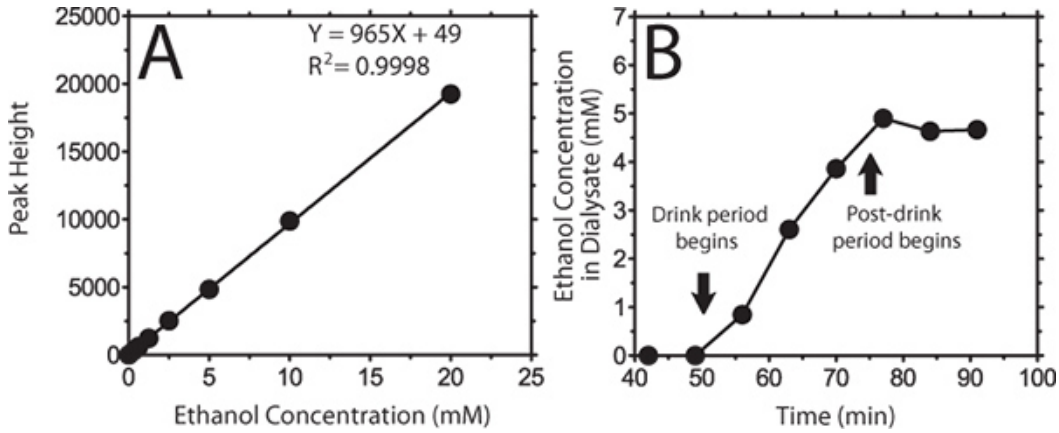

Figure 2. Graphical results from example experiment shown in **Figure 1**. A) Ethanol standard curve. B) Time course of dialysate ethanol concentration across an ethanol self-administration session.

Hardware	Parameter	Start-up setting	Running setting	Resting setting
8,400 Bruker (Varian) Autosampler				

	Injection mode	Spme	Spme	n/a
	Solvent penetration depth	0%	20%	n/a
	Sample penetration depth	20%	20%	n/a
	Absorption time	0.01 min	3 min	n/a
	Desorption time	19 min	1 min	n/a
	Clean mode solvent source	I	I	n/a
	Clean mode adsorption and desorption time	0.01 min	0.01 min	n/a
	Water bath (heats autosampler)*	50 °C	50 °C	Off
CP-3800 Varian Gas Chromatograph				
Injector Oven	Oven power	On	On	On
	Oven temperature	250 °C	220 °C	30 °C
Column Oven	Stabilization time	0.10 min	0.10 min	0.10 min
	Temperature	65 °C	65 °C	30 °C
Column	Mobile phase flow rate	8.5 ml/min	8.5 ml/min	n/a
	Column Pressure	~ 6 psi	~ 6 psi	≥ 0.1 psi
FID detector	Oven power	On	On	On
	Temperature	220 °C	220 °C	120 °C
	Electronics	On	On	Off
	Time constant	Fast	Fast	Fast
	Range	11	11	11
	Autozero	Yes	Yes	Yes

Table 2. Gas chromatograph with flame ionization detection system parameters. This table shows the parameters for the three programs used to prepare (start-up settings), run (running settings) and maintain the system while not in use (resting settings).

Discussion

Applications and limitations

Drug self-administration is used in rodents to model drug addiction. Many drugs of abuse that are modeled in this fashion can be administered intravenously, in which the drug is delivered directly to the central compartment. This allows for close monitoring of dose over a self-administration session. Since ethanol is usually orally self-administered, it is much harder to monitor drug levels due to individual differences in absorption and metabolism. By using microdialysis to sample from the brain region of interest, we are not only able to monitor the pattern of ethanol reaching the region, but we are also able to simultaneously monitor neurotransmitter changes in the same region over time during each self-administration phase.

Neurochemical alterations and drug-induced responses in the brain are associated with drug abuse and dependence; thus, the ability to concurrently measure neurochemical and drug concentrations during specific self-administration phases provides a very powerful and unique tool. One issue to keep in mind for correlating dialysate concentrations of analytes with behavior is the physical characteristics of the microdialysis plumbing. Specifically, the time it takes for fluid to be transferred from the lumen of the microdialysis probe to the collection tube is critical, and shorter times are better. In our lab, we construct the probes so that the time is about 90 sec. Using these conditions, we have found that there is an accumbal dopamine increase at the initiation of ethanol consumption, which decreases over the course of the drink and post-drink periods as the dialysate ethanol concentration increases.[2,3,8,9] These experiments, when combined with data from pharmacological studies, have allowed us to parse out the strictly pharmacological effects of ethanol and self-administration associated environmental cues on changes in neurotransmitter concentrations.

It should be noted that this particular application of combined behavioral-microdialysis techniques is suited to the current research interests of our laboratory. It is designed to evaluate the temporal pattern of ethanol reaching the brain in comparison to the pattern of neurotransmitter changes in the same region, so that we can relate these measures to synchronous self-administration behaviors. The derived dialysate ethanol concentrations are not corrected for *in vivo* probe recovery, and are only a fraction of the brain ethanol tissue concentration. If quantitative microdialysis of ethanol is required, the extraction fraction of ethanol that diffuses from the extracellular space into the probe should be experimentally determined. See previous publications from our laboratory for methods and further discussion.[10,11,12]

Although this protocol illustrates the use of gas chromatography along with solid phase microextraction of ethanol from the headspace of microdialysis samples, other methods for analysis of the ethanol content of the microdialysis sample could be used. However, alternative methods may suffer from some disadvantages. For example, less sensitive analytical methods may require a larger sample volume which necessitates sampling times greater than the 5-7 min illustrated here. The type of system discussed here uses a solid phase microextraction

that concentrates the ethanol in the vapor phase in the sealed sample vial by allowing absorption to the fiber placed in the vial headspace. This improves the detection limit compared with direct headspace sampling which typically allows 50-100 µl of the vapor to be injected. Another major advantage of the headspace method is that the sample injected for analysis is extremely clean and free of salts. Direct injection of the liquid microdialysis sample may also be used with higher sensitivity, but this will require more instrument down time due to regular maintenance required for cleaning out the injected salts.

Trouble-shooting and other notes

1. Before your experiment begins, give yourself plenty of time to check that your microdialysis set-ups are functioning properly and to trouble-shoot any issues. We suggest that you have an extra set-up, perfused with ACSF, ready to switch out with a malfunctioning set-up to save yourself time, as most operant sessions tend to occur at a specific time every day.
2. Be sure that the microdialysis probes are inserted over a period of about 5 min to minimize the tissue damage produced by shearing of the tissue as the probe penetrates the brain.
3. When transferring the rat into the operant chamber it is helpful to have a second person assist so that the transfer line (part of the microdialysis set-up) does not becoming tangled, and so that the operant program can be initiated on time.
4. Make sure that you give the autosampler water bath plenty of time to reach the appropriate temperature. Our system typically requires two hr to reach 50 °C. Also, assure that your samples are fully vaporized before analyzing the samples. We typically visually confirm that this has occurred before initiating sample analysis.
5. Standard good analytical chemistry practices should be followed. These include, but are not limited to, validation of individuals, equipment, and procedures. In brief, we employ the following guidelines: Individual users should demonstrate the ability to generate reproducible peak height values for standard concentrations and linear standard curves (R2 ≥ 0.99) across multiple days. To verify the GC-FID system is working correctly, standards should be always be analyzed before any dialysate samples are injected. The standard curve generated should have an R2 ≥ 0.99.
6. Most of the equipment we use was purchased through Varian, Inc., which was acquired by Agilent Technologies in 2010. At this time, Bruker Scientific Instruments obtained Varian's laboratory gas chromatography instruments business. For future purchases, consult either company.
7. The present protocol shows operant behavior with rats, but the viewer should be aware that more disruption of behavior is likely to occur with the smaller mouse model. Another issue to be aware of is that placement of a microdialysis probe into brain regions other than the medial prefrontal cortex or nucleus accumbens may disrupt operant behavior to a greater degree than shown here. It is important to closely examine numerous behavioral parameters to determine if the damage caused by probe placement produces severe changes in behavior.

Disclosures

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