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A New Method for the Determination of Blood Ethanol Levels in Rodents

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POHORECKY, L. A. AND J. BRICK. *A new method for the determination of blood ethanol levels in rodents.* PHARMAC. BIOCHEM. BEHAV. **16**(5) 693–696, 1982.—A procedure is described for the estimation of ethanol in blood of rodents. The procedure is based on the same principle as the breath analyzer method used with human subjects. Using a specifically designed mask, samples of rebreathed air are collected under equilibrium conditions. These are injected directly into a gas chromatograph for the quantitation of ethanol. We determined the conversion factor for calculating blood ethanol levels from those determined in breath to be 1:2857. The method was validated by comparing blood (from the jugular vein) and breath values obtained on the same animal after the administration of various doses of ethanol either intraperitoneally or intragastrically.

Blood ethanol Breath ethanol Repeated sampling Rats

CONVENTIONAL methods for the determination of effective *in vivo* levels of ethanol and acetaldehyde in experimental animals are based on the estimation of these compounds in blood samples. On the other hand, in studies employing human subjects, *in vivo* ethanol levels usually have been deduced from analysis of ethanol concentration in expired air. Several studies have validated measurements of ethanol and acetaldehyde levels in expired air [6,17] and of rebreathed air in specific [3,7]. Using mice, Gostomzyk and Streffen [8] demonstrated the rapid equilibration of ethanol between lungs and blood, validating the use of expired air in lieu of blood [3]. Of further significance is the fact that breath ethanol levels correlate better with arterial than with venous blood ethanol levels.

The feasability of measuring ethanol and acetaldehyde in expired air of rodents has been previously demonstrated [5, 10, 15]. In the first two studies animals were placed in air tight chambers from which air samples were then taken for analysis. Forsander and Sekki [5] extracted the ethanol and acetaldehyde in expired air before analysis by gas chromatography. Redmond and Cohen [15] injected samples of chamber air directly into a gas chromatograph. In neither case was there an attempt to relate breath ethanol and acetaldehyde levels to those in blood. Working with mice, Grieve and Littleton [10] employed a procedure similar to the one described here, but they did not provide extensive validation of their method.

An interesting, but not widely exploited method for the repeated sampling of rats, was described by Lester and Lin [13]. The procedure consisted of producing a subcutaneous air pocket on a rat's back. From it, air samples were then taken for the determination of ethanol.

We describe here a method for measuring *in vivo* levels of ethanol in rats. Our method closely resembles the approach used for analysis of ethanol levels in man. The method is simple, fast, inexpensive and can be used repeatedly on the same subject with minimal stress or discomfort to the animal. It consists of gas chromatographic quantitation of ethanol in samples of rebreathed air. The special mask employed for the collection of rebreathed air is described.

METHOD

Male Holtzman Sprague Dawley rats (400±25 g) were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Rats were housed in single cages in a temperature (21°C) and light (12:12 light:dark cycle, lights on at 0700) controlled room. Animals were implanted with jugular cannulas one week prior to the experiment. The cannulas, made out of PE-50 polyethylene tubing, were flushed daily with heparinized saline. On the day of experiment, animals received 0.5–3.0 g/kg ethanol (10–20% solution, w/v), and blood and breath samples were taken at several time periods. Two-50 μl samples of blood were removed from the jugular cannula immediately after which the breath samples were taken. Animals were unrestrained while the blood samples were taken, but were held firmly for the sampling of breath, as described below. For the studies requiring the intragastric administration of ethanol, animals were also implanted with gastric cannulas made of PE-100. The cannulas exited the animal at the scapular region.

For the analysis of ethanol in blood, we used a previously published gas chromatographic head space method [14,15]. Fifty microliters of freshly collected blood were added to precooled 5 ml tubes containing 0.45 ml of water and 0.5 g of sodium chloride [19]. The tubes were immediately capped with rubber stoppers and vortexed. Ethanol standards were similarly prepared, except that an appropriate dilution of 100% ethanol was added instead of the blood sample. All tubes were incubated in a shaking water bath at 55°C for 10 minutes. A 1.0 ml sample of the head space atmosphere was then removed with an air-tight syringe, and injected into a

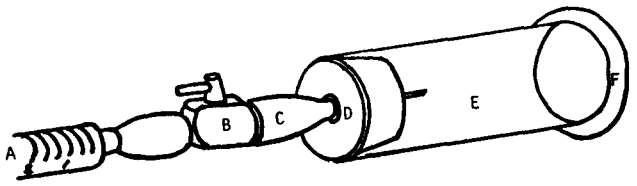

FIG. 1. Breath Sampling Assembly for Rats. The device consists of (A) sampling syringe, (B) a two-way valve, (C) a sampling needle, (D) a rubber stopper, (E) breathing chamber, and (F) a protective rim.

gas chromatograph (Gaw Mac Instrument Co., Madison, NJ). The apparatus was equipped with a 6 ft. long stainless steel column packed with 50–80 mesh Poropak 'N.' Column temperature was 140°C, and the injection port was 184°C.

Breath ethanol concentration were also quantitated by gas chromatography. Breath samples were obtained using a specifically designed mask. The mask consisted of a 3×9 cm cylinder made of 0.2cm thick polypropylene, total volume 30 cm^3. One end of the cylinder was closed off with a #5 rubber stopper. The rim of the other end of the cylinder was covered by a thin rubber tubing to protect the animal from the sharp edge of the tube and to provide a more air tight fit with the animal. An air-tight injection needle port was fitted through the center of the rubber stopper. Samples were taken with a gas-tight 1.0-ml Hamilton syringe equipped with a two-way air tight valve (Omnifit, Inc., Cedarhurst, NY) (Fig. 1).

For the sampling procedure the animal is held firmly in such a way that neither its trachea, nor its respiratory activity, are obstructed in any way. The animal's head can be conveniently grasped around the animal's ears and held firmly between the thumb and index finger. The mask, with the attached syringe and the sampling port in the open position, is then placed over the animal's snout covering the mouth in its entirety. After one has ascertained that the animal is breathing freely, a 6 second sampling of rebreathed air is initiated. After 6 seconds, the syringe is loaded with 1.0-ml of rebreathed air. The needle valve on the syringe is closed, and it is disengaged from the mask. The sample is then injected directly into a gas chromatograph. Between samples the syringe and the mask are continuously flushed with air. The described procedure is best performed by two persons. One person holds the animal and the mask, while the other person operates the sampling syringe, does the timing, and subsequently injects the sample into a gas chromatograph.

Initially several rats were used to ascertain the optimal time for sampling of the air in the mask. Breath samples were taken after animals had breathed into the mask for 1–8 seconds. Stable readings were reached after 4–5 seconds of breathing. Therefore we adopted a 6 second sampling time.

RESULTS

To validate our procedure, we first carried out a dose-response study. Rats (n=6/dose) were injected intraperitoneally with 0.5, 1.5 or 3.0 g/kg of ethanol. Blood and breath samples were collected on the same animal consecutively at 15, 30, 60 and 120 minutes after ethanol treatment. These results are presented in Table 1. Ethanol levels calculated from blood samples were highest at 15 minutes for all three doses.

TABLE 1

BLOOD ETHANOL LEVELS AFTER INTRAPERITONEAL ADMINISTRATION OF ETHANOL

Dose Ethanol g/kg	Time after Injection Admin. (min)	Ethanol Levels (mg%) Blood	Breath
0.5	15	80.5 ± 9.3	85.3 ± 8.7
	30	55.3 ± 6.9	66.5 ± 4.9
	60	41.7 ± 5.8	43.0 ± 5.3
1.5	15	179.8 ± 19.0	190.5 ± 21.0
	30	164.5 ± 17.8	187.3 ± 19.3
	60	157.7 ± 11.0	132.4 ± 15.1
	120	71.3 ± 7.7	80.2 ± 9.1
3.0	15	301.3 ± 33.4	350.9 ± 38.5
	30	279.5 ± 28.7	315.7 ± 29.3
	60	240.7 ± 23.2	270.5 ± 28.1
	120	188.1 ± 18.7	217.2 ± 25.0

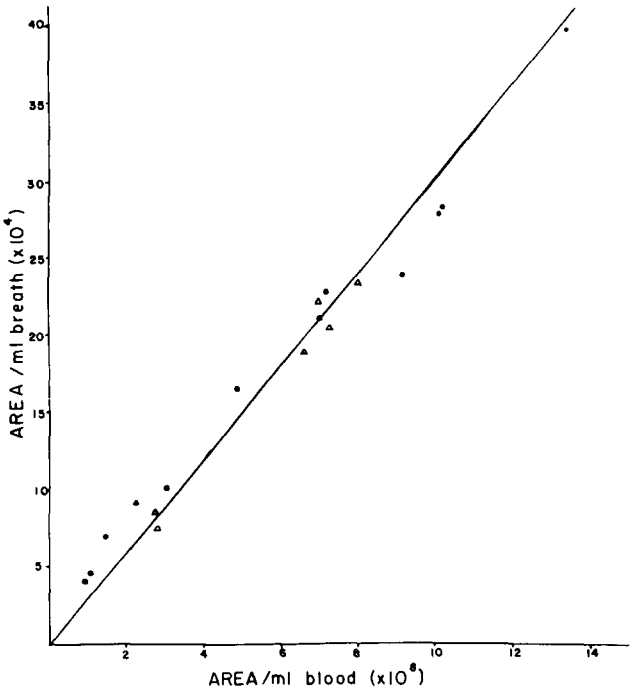

FIG. 2. Correlation of values obtained from ethanol determination in blood and breath samples on the same animals over several doses of ethanol. Area refers to the area under the ethanol peak on the gas chromatograph.

Thereafter they declined slowly. Blood ethanol levels were dose dependent.

In Fig. 2 are plotted the raw data obtained from breath and blood samples at each dose of ethanol. It is evident that the correlation is high. The calculated coefficient of correla-

TABLE 2

BLOOD ETHANOL LEVELS IN RATS AFTER INTRAGASTRIC ADMINISTRATION OF ETHANOL

Dose Ethanol g/kg	Time After Admin. (min)	Ethanol Levels (mg%) Blood	Breath
1.5	15	125.7 ± 15.5	143.3 ± 10.2
	30	145.5 ± 13.0	114.0 ± 11.9
	60	150.3 ± 11.2	128.1 ± 14.8
3.0	15	259.4 ± 27.0	301.3 ± 34.5
	30	245.8 ± 22.9	280.8 ± 27.0
	60	215.7 ± 22.5	269.7 ± 28.1
	120	150.1 ± 20.7	195.4 ± 26.9

tion is 0.92, and the ratio for breath to blood is 0.35±0.003. To convert breath values to mg% in blood, the following formula was used:

$$\text{mg\% in blood} = \frac{\text{mg ethanol in breath sample}}{\text{breath: blood ratio}} \times (100)$$
$$= \text{mg ethanol in breath} \times (100/0.035)$$
$$= \text{mg ethanol in breath} \times (2857)$$

The milligrams of ethanol in the breath samples are extrapolated from the standard curve. The value of 2857 is relatively close to the factor of 2300, used in calculating blood ethanol levels from results obtained with breath analyzers in human subjects [7,12]. The calculated ethanol values obtained in our study also are presented in Table 1. It can be seen that they correspond relatively well to those obtained from the direct analysis of ethanol in blood. Overall values calculated from breath analysis were slightly higher than those calculated from the direct analysis of blood. The highest values were seen at 15 minutes with both procedures. From the decline of blood ethanol levels, we calculated a clearance rate of 67.8±2.3 mg/100 ml/hr.

Our next step was to determine whether this method is also applicable when ethanol is given by a different route of administration. As much of our research also focuses on the chronic as well as acute effects of ethanol administration, we were concerned whether this method could be used when ethanol was administered intragastrically, for instance. Again the correspondence between values obtained from the analysis of blood compared well with those obtained by analyzing the breath.

Several differences in results are apparent when Table 1 and 2 are compared. For example, the breath ethanol level following intragastric administration of ethanol is generally lower than following intraperitoneal injection. Initially we were concerned that high levels of ethanol in the stomach would, via the alimentary tract, increase the measured ethanol in breath. From these results, that would appear not to be the case. That effect as well as the slightly higher levels of blood ethanol in intraperitoneally injected subjects is probably due to differences in the rate of ethanol absorbtion as a result of the different routes of administration.

DISCUSSION

We have described a new reliable method for measuring ethanol levels in rodents. We have validated the method by measuring simultaneously blood and breath ethanol levels on the same animal. Because of the high correlation found as well as other advantages, we propose the new method as an alternate procedure for measuring blood ethanol in levels *in vivo* in rodents.

Obtaining blood samples from experimental animals for the analysis of ethanol and/or acetaldehyde is usually a cumbersome and/or drastic procedure. In terminal studies, blood collected from the decapitated trunk is used for determination of ethanol at a single time point. However, to obtain repeated samples on one animal is considerably more difficult. A variety of methods have been devised for this purpose. All of these have more or less serious disadvantages. For rodents, the most common methods include collecting blood from the severed tip of the tail, by puncture of the retroorbital sinus (a procedure usually requiring brief anesthesia), by cardiac puncture (also requiring anesthesia), and from indwelling arterial or venous cannulas. These procedures, except for the last one, involve considerable stress to the animal at the time of sampling. Indwelling cannulas, on the other hand, are considerably more difficult operationally and technically. A skilled animal surgeon is essential, and the investment in time for surgery, limits the number of animals which can be reasonably handled for a given experiment. Further problems are the relatively short life span of the cannulas (about two weeks), and the necessary 5 days or so, of postoperative recovery. Therefore this method is not suitable for long term studies. In addition, indwelling cannulas require daily maintenance.

There are further precautions to be exercised with techniques based on the analysis of blood samples. With some procedures one must ensure rapid and complete lysis of cellular blood elements, to eliminate further metabolism of ethanol and acetaldehyde [2,11]. If deproteinized blood is used, there are problems with spontaneous generation of acetaldehyde (e.g., [2,16]). A more general problem associated with taking repeated blood samples from a single animal is the loss of both the cellular and plasma components of blood. Although the cellular elements can be restored to the animal [1], the loss of plasma constituents (ions, hormones, nutrients, etc.) cannot, and normally are not, restored or compensated for. Therefore an advantage of our method is that it obviates most, if not all, of the problems associated with present methods for ethanol and acetaldehyde analysis, as well as those associated with the removal of multiple blood samples.

A critical issue in many experiments is the ethanol level to which the brain is exposed. As the best approximation, ethanol is usually measured in peripheral venous, or mixed trunk blood. There is evidence to indicate that these measures generally underestimate the ethanol levels in arterial blood which best reflect the exposure of the brain. Forney *et al.* [3] for instance, demonstrated that ethanol levels in venous blood are lower than those in arterial blood. That the ethanol levels tend to be higher in arterial versus venous blood, especially during the absorptive phase, has been further confirmed in man [9] and also in rat [18]. This probably explains the higher values of ethanol obtained from the analysis of breath compared to jugular blood (Tables 1 and 2). Furthermore because many organs and tissues oxidize ethanol and acetaldehyde, their content in venous blood

tends to vary [5]. Therefore for both acetaldehyde and ethanol, arterial blood levels reflect with greater accuracy the levels of ethanol to which the brain is exposed.

The validity of our method rests on the attainment of equilibrium conditions during breath sampling. This is essential since at equilibrium, ethanol levels in blood, lungs, and the rebreathed air in the mask are in a constant proportion to each other. This of course, assumes unimpaired diffusion of ethanol between blood and alveolar air. Our procedure is valid even when the experimental treatment results in changes in respiratory rate. If the respiratory rate is accelerated equilibirium between blood and alveolar air ethanol would be reached faster, and the converse would be true if respiration is depressed. The sampling time can be appropriately adjusted to the particular conditions of an experiment.

There is only a small discrepancy between blood:breath ratios for man and those determined here for rats. The ratio determined here is also very close to the one determined by Lester and Lin [13] using air samples taken from subcutaneous air pockets. It should be noted however that the value of 2857 was determined under a specific set of conditions and may vary under different conditions (i.e., different age, sex, strain). Therefore before this method is employed, the ratio relating breath and blood ethanol should be redetermined. The principle, and the basic method as described here, should remain valid.

The advantages of our method are obvious. The method is reliable, quick, simple, inexpensive and noninvasive. It involves comparatively little stress to the animal, so experimental subjects can be sampled as many times as one wishes. In fact, the rate at which samples can be handled, is limited only by the efficiency of the gas chromatograph. This procedure entails no further processing of the samples, since expired air is analyzed directly. The method does have limitations. One must have a gas chromatograph, since the method is not suitable for the enzymatic quantitation of ethanol. Also the procedure, as described, involves two persons. Nevertheless, the method should be useful for a variety of experimental applications, especially those requiring repeated determinations. The method could also be adapted for other animal species. Finally, our preliminary data indicates that the method can also be used for the determination of acetaldehyde levels in blood. This particular procedure should avoid many, if not all, of the current problems associated with measuring acetaldehyde levels in blood samples.

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