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Short Communication

Determination of methanol in whole blood by capillary gas chromatography with direct on-column injection

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ABSTRACT

A gas chromatographic procedure was developed for the determination of methanol in small-volume whole blood samples. Samples (100–200 μl) were prepared by protein precipitation, with direct injection of the supernatant on a wide-bore capillary column. The recovery of methanol and acetonitrile (the internal standard) was approximately 90% and did not vary with sample volume. The assay was linear from 2 μg/ml (the limit of detection) through 1000 μg/ml and was highly reproducible (intra-day coefficient of variation <2.5%). Assay performance was assessed following exposure of rats to methanol. The results indicate that the present procedure is suitable for studies of methanol disposition in small rodent species.

INTRODUCTION

The use of methanol as an extender of automotive fuel is likely to increase significantly over the next several years [1]. Consequently, human exposure to methanol would be expected to increase, primarily by inhalation of methanol vapor [2]. Although the disposition and toxicity of methanol following acute exposure to high doses by the oral route are well known [3], little information is available concerning methanol disposition and toxicity during chronic exposure to small quantities of the alcohol.

The quantitation of hydrophilic, volatile compounds contained in a biological matrix poses several analytical problems. Isolation of the analyte is difficult, since extraction into a water-immiscible solvent is not possible. In addition, analyte may be lost as vapor during sample processing. Furthermore, for compounds such as methanol that lack a significant chromophore, the choice of detection system providing the requisite sensitivity to quantitate nanogram quantities of analyte is limited. Gas chromatography with head space analysis is often employed [4], but is cumbersome and, under certain conditions, associated with poor reproducibility. Derivatization of aliphatic alcohols prior to chromatography al-

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so has been used [5], but requires an additional step to the analysis. The present study was undertaken to determine the feasibility of directly quantitating methanol in rat whole blood by gas chromatography with flame ionization detection following a simple protein precipitation procedure.

EXPERIMENTAL

Materials

Methanol and acetonitrile (HPLC-grade) were obtained from Burdick and Jackson (Muskegon, MI, USA) and were used without further purification. Aqueous solutions of analytes and reagents were made with HPLC-grade water. All other reagents were obtained from commercial sources.

Assay procedure

Aliquots of rat whole blood (200 µl) were added to 100 µl of water containing acetonitrile (0.3 µl/ml) and heparin (200 U/ml) in 0.5-ml polypropylene centrifuge tubes. The tubes were maintained in an ice water bath during the entire preparation procedure to prevent evaporative loss of both methanol and acetonitrile, and were vortex-mixed vigorously immediately following addition of blood to lyse erythrocytes. Proteins in whole blood were precipitated by the addition of zinc sulfate (10% in water, 100 µl) with vigorous mixing, followed by addition of sodium hydroxide (0.5 *M*, 100 µl), again with vigorous mixing. The tubes were centrifuged (15 000 *g*, 1 min) and cooled on ice. Sample supernatants were collected and transferred to tightly sealed injection vials with PTFE-lined septa. Aliquots (1 µl) of these samples were injected on-column for analysis.

Chromatography

Analysis was conducted on a Shimadzu GC-14A gas chromatograph (Shimadzu Scientific, Norcross, GA, USA) equipped with a flame ionization detector, an on-column capillary injector, an autosampler and a reporting integrator. Chromatographic separation of methanol and acetonitrile (the internal standard for the assay) from contaminants in prepared whole blood was achieved on a 15 m × 0.54 mm I.D. wide-bore fused-silica capillary column, with Carbowax (1.2 µm film thickness) as the bonded stationary phase (Alltech Assoc., Deerfield, IL, USA). The carrier gas (helium) was delivered to the column at a rate of 12 ml/min, which produced a back-pressure of 0.25 kg/cm^2. Hydrogen and air were delivered to the flame ionization detector at 55 and 450 ml/min, respectively. Isothermal chromatography was conducted with a column oven temperature of 35°C; injector and detector temperatures were maintained at 120°C. Peak-area ratios (methanol to acetonitrile) were determined by the reporting integrator. Following the analysis of each sample (4 min post-injection) the column oven temperature was elevated rapidly (40°C/min) to a final temperature of 100°C to remove water and endogenous contaminants from the column. The column oven

was maintained at the elevated temperature for 3 min prior to cooling for analysis of subsequent samples.

Assessment of assay performance parameters

Linearity and accuracy. Standard curves were constructed by adding stock solutions of methanol (20 μl) to heparinized whole blood (180 μl) obtained from untreated rats. The final blood methanol concentration ranged from 1 to 1000 μg/ml. Standards were prepared and analyzed as described above.

Reproducibility. Five replicate methanol standards were prepared and analyzed as described above at each of two concentrations: 50 and 500 μg/ml. The peak-area ratios were used to determine the intra-day coefficient of variation of the assay at each concentration. Inter-day variability was determined by analyzing two quality control samples (25 and 250 μg/ml) daily for five consecutive days.

Influence of blood volume. Potential changes in reported methanol concentrations with varying blood volume were assessed by analyzing varying volumes (100, 150 or 180 μl) of rat whole blood containing a fixed amount of methanol (1 μg).

Recovery of methanol and acetonitrile during sample preparation. Four replicate samples of methanol in whole blood (50 μg/ml) were prepared and assayed as described above. Detector response (in terms of peak area for either methanol or acetonitrile) was normalized for the mass of material injected assuming 100% recovery of the analyte molecules during preparation.

Assessment of assay performance following exposure of animals to methanol

As a test of assay performance under relevant experimental conditions, timed blood samples were obtained from rats pretreated with methanol. Silicone rubber cannulas were implanted in the right jugular vein of two diethyl ether-anesthetized female Sprague Dawley rats 24 h prior to the experiment. A solution of methanol in saline (1.5 ml/kg, 167 mg/ml) was injected through the jugular vein cannula over 30 s. Blood samples (0.2 ml) were withdrawn from the cannula at timed intervals for 7 h post-injection. Immediately upon withdrawal from the animal, blood was placed in tubes containing 100 μl of water with acetonitrile and heparin as described above. The tubes were maintained in an ice water bath to inhibit evaporative loss of methanol and acetonitrile from the sample, and were capped and vortex-mixed vigorously immediately following addition of the blood sample. All samples were frozen within 60 min of collection and were prepared and analyzed as described above within 48 h. A series of standards for construction of a standard curve was prepared at the same time as the blood samples, and was stored under the same conditions.

RESULTS AND DISCUSSION

Representative chromatograms of blank rat whole blood and rat blood con-

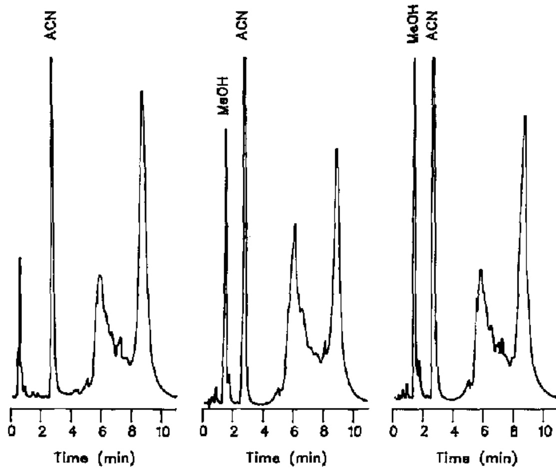

Fig. 1. Representative chromatograms for (left) whole blood obtained from a naive rat, (center) whole rat blood with methanol (MeOH, 10 μg/ml) and acetonitrile (ACN) added *in vitro*, and (right) whole blood obtained from a rat 3 h following intravenous injection of 250 mg/kg methanol (calculated methanol concentration = 49.1 μg/ml).

taining methanol are presented in Fig. 1. Under the chromatographic conditions described above, the retention times of methanol and acetonitrile were approximately 1.5 and 2.8 min, respectively. Both analytes were well resolved from each other and from contaminants in blood, which eluted primarily during the period when the column oven temperature was elevated. The large peak eluting at 0.5 min in blood obtained from naive animals was due to residual diethyl ether used to anesthetize the animals for collection of large blood samples by cardiac puncture. In contrast, methanol-treated rats were conscious during blood sampling. It should be noted that low concentrations of methanol are present normally in rat blood (Fig. 1, left panel). Analysis of blank rat blood from several animals suggested that "ambient" concentrations of methanol were approximately 1 μg/ml. This endogenous peak co-eluted with authentic methanol under all operating conditions (column oven temperature and carrier gas flow-rate) for three different chromatographic systems: a glass column (2 m × 2.5 mm I.D.) packed with 15% THEED on 100–120 mesh Chromosorb W AW; a fused-silica wide-bore capillary column (15 m × 0.53 mm I.D.) with RSL-200 (1.2 μm thickness) as the bonded stationary phase, and the system described in this communication. While these observations do not confirm the identity of this endogenous material, they suggest that the co-eluting peak is likely to be methanol. In any event, the presence of this peak in blood obtained from untreated rats results in a limit of detection of 2 μg/ml, assuming a signal-to-noise ratio of 2.

Standard curves prepared in blank rat whole blood were linear ($r^2 > 0.99$) through a methanol concentration of 1000 μg/ml. Relative errors (difference between observed peak-area ratios and ratios predicted from the regression line) did not exceed 15%. A representative regression equation for the relationship be-

tween peak-area ratio and methanol concentration is $y = 0.0160x + 0.0290$ ($r^2 = 0.999$).

The reproducibility of the assay was assessed with both within-day (at methanol blood concentrations of 50 and 500 μg/ml) and between-day (at methanol blood concentrations of 25 and 250 μg/ml) protocols (five samples per concentration). At 50 μg/ml, the calculated methanol concentration in the five samples was 50.0 ± 1.22 μg/ml; at 500 μg/ml, the calculated concentration was 500 ± 6 μg/ml. The intra-day coefficients of variation therefore were quite low (< 2.5%) and did not appear to be concentration-dependent. The relative error (calculated *versus* known methanol concentration) did not exceed 4.0%, and averaged 1.64% (50 μg/ml) and 0.96% (500 μg/ml). Inter-day coefficients of variation were somewhat higher (approximately 8 and 10% at 250 and 25 μg/ml, respectively), and also appeared to be independent of methanol concentration. The fact that inter-day variability exceeds intra-day variation by nearly four-fold may be due to slight changes in the aqueous solution of acetonitrile with time. Preparation of fresh internal standard solution on a daily basis may reduce this variability.

The recovery of methanol and acetonitrile was determined by comparing peak areas of the two analytes from prepared blood samples with peak areas produced by injecting aqueous stock solutions of the analytes directly on-column. Four blood samples were analyzed for the determination of recovery and were compared to four separate injections of the stock solutions. On average, 300 μl (or 60%) of each blood sample was recoverable as supernatant following protein precipitation and centrifugation. Assuming that both methanol and acetonitrile distribute evenly throughout the sample (*i.e.*, achieve equal concentrations in the supernatant and the pellet following centrifugation) approximately 60% of the mass of each analyte should be present in the supernatant. Based upon this assumption, the recovery of methanol and acetonitrile was 89.0 ± 5.2 and 88.8 ± 6.8%, respectively. The mechanism underlying the 10% loss during sample processing is unknown, but may be due to vaporization of both compounds during mixing and centrifugation. It should be noted that the relative recovery of methanol (as compared to acetonitrile) was essentially 100%, suggesting that loss of methanol from the sample is parallelled by loss of acetonitrile. In any event, analyte loss during sample processing is minor and does not appear to affect the reproducibility of the assay.

Differences in sample blood volume (100, 150 or 180 μl) did not affect the peak-area ratio to any significant degree following addition of a fixed mass of methanol (analysis of variance, $p > 0.2$). Thus, over the range of blood volume examined, the presence of varying quantities of blood constituents appears to have no effect on the relative recoveries of methanol and acetonitrile during sample preparation. It is therefore likely that physiological changes (*e.g.*, hematocrit) and variations in available sample volume (*e.g.*, blood from neonatal animals as opposed to adults) will not affect the performance of the present assay.

Whole blood methanol concentration–time profiles following intravenous injection of a 250 mg/kg dose of the alcohol to two female rats are displayed in Fig.

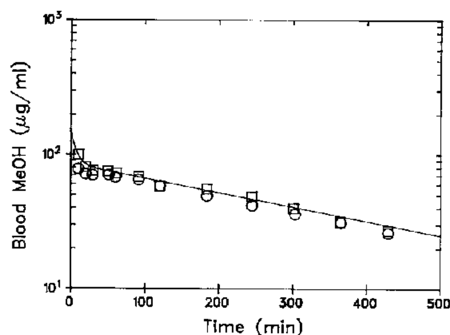

Fig. 2. Concentration–time profiles for whole blood methanol in two rats receiving an intravenous injection of 250 mg/kg. Points indicate calculated methanol concentration for each animal; lines represent the fit of a biexponential equation to the data by non-linear least-squares regression.

2. The dose-normalized blood concentrations of methanol are consistent with previous reports in the literature [6,7]. At the blood concentrations produced by this dose (< 100 μg/ml), the disposition of methanol appeared to be first-order, with a terminal elimination half-life of approximately 4 h. By the end of the sampling period (7 h), whole blood concentrations of methanol were approximately ten-fold higher than the limit of detection of the assay. Thus, the present assay appears to have the requisite sensitivity and precision to perform pharmacokinetic investigations of methanol in small rodent species.

CONCLUSION

A simple, rapid assay was developed for the direct determination of methanol in rat whole blood. Sample preparation involved precipitation of proteins from whole blood, with injection of the aqueous supernatant directly on-column. Assay precision was well within the requirements for accurate profiling of the disposition of the alcohol, and the limit of detection was sufficient to detect approximately 2 μg/ml in relatively small (200 μl) whole blood samples.

ACKNOWLEDGEMENT

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