Ethyl Linoleate in Meconium: A Biomarker for Prenatal Ethanol Exposure


**Background:** Fetal alcohol syndrome, fetal alcohol effects, alcohol-related neurodevelopmental disorder, and alcohol-related birth defects, all terms referring to the spectrum of consequences of in utero exposure to ethanol, are a major public health burden. There is currently no laboratory test to identify newborns exposed to ethanol in utero. Meconium was analyzed for ethyl linoleate, a metabolite of ethanol, as a biological marker for fetal ethanol exposure. **Methods:** Samples of meconium were obtained from 248 infants and analyzed for fatty acid ethyl esters. Detailed maternal alcohol, tobacco, and drug use histories were obtained within 1 month of giving birth. **Results:** The detection of ethyl linoleate in meconium was called a positive test. The mean number of drinks reported per week in the month before pregnancy, the first trimester, and overall were significantly higher in the positive group (unadjusted: 9.2 ± 1.9 vs. 4.3 ± 1.4, p = 0.004; 7.3 ± 1.7 vs. 3.6 ± 1.2, p = 0.03; and 6.1 ± 1.3 vs. 3.0 ± 1.0, p = 0.006). A positive test was not associated with marijuana, cocaine, or tobacco use. Sensitivity and specificity of the test were 72% and 86%, respectively, to distinguish women who reported 1 or more drinks/week in the third trimester from women who denied use, and 68% and 48% to distinguish women who used >1 drink/week in the month before pregnancy. **Conclusions:** The presence of ethyl linoleate in meconium is the first reported biological marker for maternal ethanol use during pregnancy. Because of the inherent inaccuracy associated with the use of self-reporting, the establishment of true values of sensitivity and specificity will require validation where the presence, quantity, and timing of exposure to alcohol is known. Further validation of this marker will permit identification and intervention of at-risk infants.

**Key Words:** Ethanol, Biological Marker, Prenatal Ethanol Use, Fatty Acid Ethyl Esters, Ethyl Linoleate.

**ALCOHOL USE during pregnancy is a significant public health problem.** Approximately 60% of adult women in the United States report drinking at least occasionally (Midha and Clark, 1994), and fully 20% of these women continue to drink some alcohol during pregnancy (Stratton et al., 1996). Although the majority of women stop drinking or drastically reduce their drinking once pregnant (Serdula et al., 1991; Day et al., 1993), some women continue to drink heavily during pregnancy. In the 1988 National Maternal and Infant Health Survey, 0.6% of the respondents had 6 or more drinks per week during pregnancy, and 0.2% reported an average consumption of 2 or more drinks per day (Center for Disease Control and Prevention, 1988). Although the percentage of women who drink heavily during pregnancy is relatively small, the absolute numbers represent a staggering burden to society. Heavy drinking during pregnancy is the cause of fetal alcohol syndrome, the leading known cause of mental retardation (Abel and Sokol, 1987). In addition, drinking during pregnancy can result in a spectrum of effects, including alcohol-related birth defects, alcohol-related neurodevelopmental defects, and subtle effects on a variety of behavioral, educational, and psychological tests (Stratton et al., 1996). Together, these effects are estimated to cost anywhere from $75 million (Abel and Sokol, 1987) to $9.7 billion per year (Harwood and Napolitano, 1985).

Identifying the alcohol-exposed newborn is difficult. In one study, the diagnosis of fetal alcohol syndrome was missed in 100% of newborns who were identified later in childhood (Little et al., 1990). Early identification of affected individuals is desirable in order to minimize secondary disabilities, such as mental health problems or trouble with the law (Streissguth et al., 1996). Determination of alcohol use during pregnancy is one way to identify infants at risk for alcohol-related problems. Currently, the only tool available to clinicians is the maternal report of alcohol use. However, it is widely recognized that the history is likely to underestimate the true amount of alcohol consumed (Ernhart et al., 1988; Lorio et al., 1991; Chasnoff et al., 1990). There are several short screening tools to determine risk drinking, but these reliably identify only heavy drinkers (≥14 drinks/week) (Sokol et al., 1989; Russell et al., 1994). Thus, development of a biological marker for fetal exposure to alcohol would be a major advance.

Ethanol is metabolized by both oxidative and nonoxidative pathways. The nonoxidative pathway conjugates ethanol to free fatty acids forming fatty acid ethyl esters (FAEEs) (Lange et al., 1981). FAEEs have been detected in numerous animal tissues, including fetuses and placentas after maternal ethanol administration (Bearer et al., 1992b). In humans, FAEEs have been identified in both cord blood (Bearer et al., 1992a) and meconium (Bearer et al., 1996, 1997; Mac et al., 1994). Compared with ethanol and the oxidative metabolites, FAEEs have a prolonged half-life in tissue, with 1 hr in rabbit adipose tissue (Laposata et al., 1989) to 1 week in mouse placenta (Bearer et
al., 1992b). In adults, FAEEs have been tested as a biological marker for alcohol-related fatalities (Laposata et al., 1989).

Traditionally, efforts to determine fetal drug exposure have focused on neonatal and/or maternal urine analysis, but these samples reflect exposure only in the 2 to 3 days before delivery. In addition, neonatal urine is difficult to collect. Therefore, analysis of meconium, the first fecal material passed by the neonate, has been proposed as an alternative (Ryan et al., 1994; Callahan et al., 1992). Meconium begins to form in utero around the 13th week of gestation, and accumulates thereafter. Thus, its analysis provides a longer record of exposure than urine, although the half-life of FAEEs in meconium has not been determined. It is easy to collect and can be collected cumulatively over a period of days. In this study, we investigated the usefulness of FAEEs in meconium as a biological marker for ethanol use during pregnancy.

METHODS

Subjects

Postpartum women were recruited from a large urban teaching hospital to participate in a 2-year longitudinal study on the neurobehavioral effects of prenatal cocaine exposure. Women were excluded if they had primary psychiatric diagnoses, low intellectual status, used methadone or heroin, tested positive for HIV, or had other significant health problems (diabetes, cancer). Low intellectual status was defined as a standard score of <50 on the Peabody Picture Vocabulary Test, combined with a block design mean score of <5 and a picture completion mean score of <5 on the Weschler Adult Intelligence Scale. Informed consent was obtained as approved by the Institutional Review Board of MetroHealth Medical Center. Random samples of meconium were obtained from 248 infants. Meconium was scraped from diapers, collected into Falcon tubes (15 ml, polypropylene, Becton-Dickinson), and frozen at -70°C.

Maternal Prenatal Substance Abuse Assessment

The maternal postpartum interview (Streissguth, 1986a,b; Streissguth et al., 1978), was used to estimate maternal alcohol, tobacco, and drug use the month before and during each trimester of pregnancy. For each time period, mothers were requested to recall both the amount and frequency of alcohol, tobacco, and drug use. To estimate the amount of alcohol used, the number of drinks consumed per drinking day was computed based on the amount of beer, wine, or hard liquor consumed per drinking day, with 12 oz of beer, 4 oz of wine, or 1 oz of hard liquor equivalent to one drink (0.5 oz of absolute alcohol). For tobacco, the number of cigarettes smoked per day, for marijuana, the number of joints smoked per day, and for cocaine, the number of rocks smoked per day was recorded. The frequency of use of alcohol, cocaine, and marijuana was estimated on a Likert-type scale ranging from 0 (less than once per month) to 7 (daily use). The frequency of use was multiplied by the amount used per day to compute an estimated dose per week for the month before pregnancy and for each trimester (referred to as dose/week). This score was then averaged for a total score for the prenatal exposure for each drug. For tobacco, it was assumed that the same number of cigarettes was smoked each day. The number of cigarettes smoked per day per trimester was averaged for all time periods to obtain an overall average. The postpartum interview was conducted as soon as possible after the birth of the child, generally within 1 month after birth.

Infant and Maternal Characteristics

Maternal age, gravidity, parity, infant birth weight, gestational age, birth length, head circumference, and APGAR scores at 1 and 5 min were taken from the hospital birth records. Socioeconomic status was calculated using the Hollingshead Index.

FAEE Analysis

The analyses were performed by investigators blinded to the questionnaire results. One gram of meconium was used for analysis. For samples <1 g, the whole sample was used. Ethyl heptadecenoate was added as an internal standard. FAEEs were extracted with acetone/hexane and isolated using silica gel chromatography. The isolated FAEEs were identified and quantitated by gas chromatography using a flame ionization detector and comparison to authentic standards. Recovery of ethyl myristate, ethyl palmitate, ethyl palmitoleate, ethyl heptadecanoate, ethyl stearate, ethyl oleate, ethyl linoleate, ethyl linolenate, and ethyl arachidonate were all >95%. Recovery of ethyl laurate varied from 20% to 60%. Results from 10 samples were confirmed by gas chromatography/mass spectrometry. The limit of detection for ethyl linoleate was 1.0 pmol/g meconium. A women and her newborn had a positive test if a meconium sample from the newborn yielded a gas chromatogram with a peak at a retention time ≥ 0.02 min that of an ethyl linoleate standard.

Statistical Analysis

Characteristics of the infants and mothers in the positive and negative test groups were compared by Mantel-Haenszel χ² test or Student’s t test. Alcohol, cocaine, marijuana, and tobacco use were compared between positive and negative test groups by analysis of variance, with repeated measures reported for the month before, first, second, and third trimesters as the within-subject factor and test groups as the between-subject factor. Variables are expressed as means ± standard error of the mean (SE). The alcohol use adjusted for marijuana is achieved by adding marijuana to the analysis of covariance model as a covariate with alcohol as the dependent variable and with the test (+ or − FAEE) as the grouping variable (see Table 2). Alcohol, marijuana, and cocaine dose per week and tobacco dose per day were examined separately as dependent variables. Subsets of the positive and negative groups based on exclusive use of one or more substances were compared by Student’s t test. Analyses were performed with SAS computer software. Statistical tests were two-tailed. Significance was defined as a p-value < 0.05.

RESULTS

FAEE Analysis

Ethyl laurate (12:0EE), ethyl myristate (14:0EE), ethyl palmitate (16:0EE), ethyl stearate (18:0EE), ethyl oleate (18:1EE), ethyl linoleate (18:2EE), ethyl linolenate (18:3EE), and ethyl arachidonate (20:4EE) were detected in some meconium samples. The predominant forms of FAEEs were ethyl linoleate > ethyl oleate > ethyl linolenate. Representative chromatograms of meconium from four different newborns are shown in Fig. 1. Chromatograms A to C represent positive tests, whereas chromatogram D is negative.

Association of the Test to Maternal and Infant Characteristics

There were no differences in maternal age, socioeconomic status, gravidity, parity, and ethnicity between women whose infants had ethyl linoleate present in their
meconium and those who had none (Table 1). There were no differences in gestational age, birthweight, birth length, head circumference, and Apgar score at 1 and 5 min between infants who tested positive or negative. Although differences in infant characteristics between heavy drinkers and abstainers have been reported (Day et al., 1980), there may be no differences between the alcohol-exposed and nonexposed infants in this sample, because the nonexposed group was composed of high-risk infants who had been exposed to cocaine and marijuana. Both these drugs have been associated with poor fetal growth (Zuckerman et al., 1989; Singer et al., 1994).

Association of the Test to Maternal Trimester History of Alcohol and Other Substance Use

The standard to which the test was compared was maternal retrospective self-report of alcohol use. Previous studies have shown that outcomes are more significantly associated with self-reported drinking before recognition of pregnancy (Streissguth et al., 1980). This finding may be interpreted as alcohol effects on early fetal development, or that self-report was more reliable when free from the stigmata of drinking while pregnant. Therefore the influence of maternal self-report of substance use independently by trimester on the association with the test was examined.

The results are shown in Table 2. Significant differences in mean dose of alcohol per week between women with a positive or negative test were found for alcohol use during the month before pregnancy (9.2 ± 1.9 vs. 4.3 ± 1.4, p = 0.004), the first trimester (7.3 ± 1.7 vs. 3.8 ± 1.2; p = 0.03), and average use over pregnancy and the month before (6.1 ± 1.3 vs. 3.0 ± 1.0; p = 0.006) (Table 2).

No significant correlation between other substance use and test score was found, except for the use of marijuana in the third trimester (Fig. 2). However, this difference was not significant when alcohol use was added to the analysis of covariance model as a covariate with marijuana as the dependent variable and test (+ or − FAEE) as the grouping variable (F = 2.77, df = 1, p = 0.10). There were no other significant differences in the mean dose per week of marijuana or cocaine in any trimester, nor was there a difference in the average number of cigarettes smoked per day in women with positive or negative tests. The trend of the reported alcohol use with time between the positive and negative test groups was significantly different only for alcohol use (ANOVA with repeated measures, F = 5.44, df = 1, p = 0.02).

When marijuana use is added to the model as a covariate, significant differences in amount of alcohol used between the positive and negative test groups persist in the month before pregnancy, the first trimester, and the overall average (Table 2). Because meconium does not begin to form until the end of the 1st trimester, the FAEEs in meconium are unrelated to the ethanol consumed before pregnancy and in the first trimester. It is more likely that the loss of significance in the last two trimesters is due to maternal underreporting of alcohol use and not false-positive tests.

To further examine the impact of other drug use on the relationship between self-reported alcohol use and the test outcome, the mean dose of alcohol was calculated for women excluding those who reported use of marijuana or cocaine (Alcohol Only; Table 2). The mean alcohol dose per week is significantly higher in the women who did not use cocaine or marijuana (Alcohol Only group) who tested positive in the overall mean dose (1.0 ± 0.4 vs. 0.2 ± 0.1, p = 0.01), the month before pregnancy (1.9 ± 0.5 vs. 0.6 ± 0.2, p = 0.03), and the second trimester (0.7 ± 0.4 vs. 0.1 ± 0.1, p = 0.04).

Sensitivity and Specificity of the Test

To estimate the sensitivity and specificity of the test, we identified two subgroups of women: those most likely to have abstained from alcohol use during pregnancy and those who admitted to alcohol use during the third trimester. Women who denied ethanol use in the month before pregnancy and throughout pregnancy are most likely to be true abstainers. The specificity of the test to identify these women was 51%. Women who admitted to drinking alcoholic beverages in the second and third trimesters are most
likely to actually use alcohol in the third trimester. The association of a positive test with maternal report of any ethanol use in second and/or third trimester was statistically significant, as shown in Table 3. For women who reported any ethanol use in the third trimester, the sensitivity of the test was 68%. For women who reported any ethanol use in the second trimester, the sensitivity of the test was 66%. For women who reported any ethanol use either in the second or third trimester, the sensitivity of the test was 66%, and for women who reported ethanol use in both the second and third trimesters, the sensitivity of the test was 68% (Table 3).
Because the range of alcohol consumed varied from 0.125 drinks/week to 100 drinks/week, we further examined the correlation of the test with maternal history of >1 drink/week and >3 drinks/week in the third trimester. These results are shown in Table 3. Increasing the threshold to 1 drink/week or 3 drinks/week did not consistently increase the sensitivity of the test.

To compare the sensitivity and specificity of the meconium test to that reported for several short screening questionnaires (Russell et al., 1994), the sensitivity and specificity of the test to distinguish between maternal self-reported dose/week of women in the month before pregnancy was calculated (Table 4). At ≥14 drinks/week, the meconium test had a sensitivity of 74% and a specificity of 43%, compared with that reported for the questionnaires with 79% and 83%, respectively (Russell et al., 1994). However, even at ≥1 drink/week, the meconium test had a sensitivity of 68% and specificity of 48%.

DISCUSSION

Our study is the first report of a biological marker specific for in utero ethanol exposure. The presence of ethyl linoleate in meconium was strongly associated with multiple measures of maternal ethanol use in a disadvantaged, predominantly African-American population. It was not associated with self-report of tobacco, marijuana, or cocaine use.

FAEES have previously been reported in the meconium of infants exposed to alcohol in utero (Mac et al., 1997). However, the distribution of the fatty acids present in the FAEES from that study were very different from our own results. The predominant FAE was ethyl laurate, followed by ethyl palmitate, and ethyl stearate. No other FAE was reported. Due to its volatility, our recovery of ethyl laurate was low and highly variable, making it unsuitable as an accurate biomarker. As seen in Fig. 1, ethyl palmitate was found in meconium samples, but to a much lesser degree
Table 4. Sensitivity and Specificity of Ethyl Linoleate in Meconium to Identify Drinking in the Month Before Pregnancy

<table>
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<th>n (&gt;)</th>
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Association of a positive meconium test with amount of drinking reported in the month before pregnancy.

than ethyl oleate, ethyl linoleate, or ethyl linolenate. We rarely found peaks corresponding to ethyl stearate. The basis for these discrepancies is not clear. They may be a result of population or dietary differences between the two study groups. Our group was predominantly disadvantaged African Americans. The ethnic and socioeconomic characteristics of the other group were not described. The method used for isolation of FAEEs also differed; we have used silica gel chromatography in comparison with use of amnonopropyl columns. We have attempted to use amnonopropyl columns to isolated FAEEs from meconium with little success.

It is unclear how the amount of ethyl linoleate in meconium relates to maternal drinking. It may relate either to the amount of ethanol consumed, or the length of the interval between the last drinking occasion and birth. The half-life of FAEE in meconium is currently unknown. Several lines of evidence suggest that the half-life of FAEEs may be protracted. First, meconium is metabolically inert; other drug metabolites are stable in meconium. Use of cocaine before recognition of pregnancy resulted in detectable concentrations of cocaine in only the meconium in the distal large intestine at 33 weeks gestation (Ostrea et al., 1994). In rats given cocaine subcutaneously during gestation days 1 to 7, cocaine was detected in their meconium at gestational day 20 (Ostrea et al., 1994). Second, the half-life of FAEEs in mouse placenta, a metabolically active tissue, was 1 week in our previous study (Bearer et al., 1992b). Third, in preliminary experiments, ethyl palmitate, ethyl oleate, ethyl linoleate, and ethyl arachidonate showed no appreciable degradation >96 hr when stored under nitrogen at 37°C (unpublished observations). In addition, the meconium analysis reported herein was based on wet weight of meconium. The water content of the meconium will vary between patients, but also as a function of the time spent in the diaper, a highly absorptive surface, before collection. Therefore, an accurate comparison of the amount of ethyl linoleate between samples based on wet weight is impossible. A more accurate quantitative measurement of the amount of meconium is needed, such as dry weight, or content of sphingomyelin. These methodologies are currently being investigated.

In our study, we used our limit of detection to categorize samples as positive or negative. This categorization resulted in a specificity of 51%. Several factors can account for this high false-positive rate. First, there may be some FAEEs in meconium as a result of normal metabolism. Ethanol, in small amounts, is synthesized in the body. It is also present in common dietary ingredients, such as vanilla extract. The background level of FAEEs in pregnant women with a full assurance of alcohol abstention needs to be determined to correctly identify a cut-off point for the amount of ethyl linoleate above which the presence of ethyl linoleate in meconium is indicative of maternal ethanol use. Second, the flame ionization detector used in this study is nonspecific in its identification of ethyl linoleate. It may be necessary to use detection systems with greater specificity, such as a mass spectrometer, to establish a cut-off level, thereby increasing the specificity of the test. These studies are currently in progress: Lastly, it has been shown that alcohol consumption is notoriously underreported. The history of alcohol use obtained for this study was retrospective and may not be a gold standard for identifying abstainers.

Nevertheless, the test was able to identify 72% of those women reporting third trimester alcohol use of ≥1 drink/week and 74% of women reporting month before pregnancy use of ≥14 drinks/week. This sensitivity compares favorably with the sensitivities of screening questionnaires used in the second trimester to identify women who drank ≥14 drinks/week in the month before pregnancy (range: 49 to 79%) (Russell et al., 1994). Use of a more sensitive detection system, such as a mass spectrometer, may increase the sensitivity of the test. These analyses are currently underway.

We conclude that ethyl linoleate and other FAEEs may be useful as biomarkers for prenatal ethanol exposure. Further analysis of the sensitivity and specificity of this test for prenatal ethanol exposure must be done under conditions of known ethanol exposure.

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