Prevalence of Fatty Acid Ethyl Esters in Meconium Specimens

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Background: Fetal alcohol syndrome (FAS), alcohol-related birth defects (ARBDs), and alcohol-related neurodevelopment disorders (ARNDs) in neonates are often the result of maternal alcohol consumption during pregnancy. Facial characteristics are associated with FAS, but ARBDs and ARNDs are more difficult to diagnose. Fetal exposure to alcohol can cause central nervous system dysfunction, pre- and postnatal growth problems, cardiac defects in neonates, and attention deficit disorders and mental retardation in older children. To date, diagnosis of fetal alcohol effect has depended largely on maternal interview, although clinical tests are becoming more widely used. Fatty acid ethyl esters (FAEEs) are formed in the body by esterification of ethanol with free fatty acids and trans-esteri-
fication of glycerides and have been detected in the meconium of newborns. This report estimates the prevalence of fetal alcohol exposure in two populations by detecting FAEEs in meconium.

Methods: We analyzed the prevalence of FAEEs in the meconium of two separate groups of neonates by use of solid-phase extraction and analysis by gas chromatography–mass spectrometry in the chemical ionization mode. In the first study, meconium samples were taken anonymously from babies born in a large, regional perinatal center in Hawaii. In the second study, specimens were obtained from infants admitted to six different newborn intensive care units within the state of Utah.

Results: In the first study, 73 of 436 (16.7%) meconium specimens tested were considered positive for FAEEs. When broken down into quartiles, the mean total FAEEs measured were 1059, 3133, 6628, and 62115 ng/g. In the second study, 35 of 289 (12.1%) specimens were considered positive. When broken into quartiles, the mean total FAEEs were 1139, 3067, 7674, and 50 143 ng/g. The overall FAEE profiles of the two study sets were remarkably similar.

Conclusion: In an adequate meconium specimen, a total FAEE concentration >10 000 ng/g may indicate that the newborn has been exposed to significant amounts of alcohol during pregnancy.

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Fetal alcohol syndrome, alcohol-related birth defects, and alcohol-related neurodevelopment disorders in neonates are a result of maternal alcohol consumption during pregnancy. Even at low intake, alcohol can cause adverse effects in newborns and later problems in childhood. To date, the determination of fetal alcohol exposure has depended largely on maternal interview, but laboratory tests are becoming more widely used.

Fatty acid ethyl esters (FAEEs) are formed in the body by esterification of ethanol with free fatty acids and can be detected in the serum and blood of alcohol users. They have been detected in human organs damaged by ethanol abuse and can be used as postmortem markers for premortem ethanol use. FAEEs persist and accumulate in adipose tissue after ethanol has been eliminated from the body.

Meconium, the first fecal matter passed by the newborn, is often analyzed for drugs to which a neonate has been exposed during the latter half of gestation. FAEEs in meconium have been reported to be potential biomarkers of fetal exposure to alcohol, and their detection may serve as a diagnostic tool to assess the extent of ethanol exposure to the fetus.

Materials and Methods

Calibrators

Acetone, n-hexane, and isopropanol were ACS reagent grade or better and were obtained from Fisher Scientific. The internal standard [heptadecanoic acid ethyl ester (E17:0)] and ethyl esters of palmitoleic acid (E16:1), palmitic acid (E16:0), linoleic acid (E18:2), oleic acid (E18:1), stearic acid (E18:0), and arachidonic acid (E20:4) were obtained from Sigma. The powders were weighed, diluted in hexane to make 1 g/L stock solutions, and stored at −20 °C. Heptadecanoic acid (E17:0) ethyl ester in hexane (10 mg/L) was used as the internal standard.

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**SPECIMENS**

Two completely different geographic and cultural populations were used as study sets for this research. In the first study, meconium samples (n = 436) were collected anonymously from babies born in a large, regional perinatal center in Hawaii. The samples were either frozen before shipping or sent immediately to US Drug Testing Laboratories, where they were analyzed for drugs of abuse and FAEEs. FAEEs are sensitive to heat and light; therefore, when samples were received, they were immediately aliquoted and stored frozen at −20 °C. Institutional Review Board approval for specimen collection was under RP 99-36 from the Kapi‘olani Health (now Hawaii Pacific Health) Research Institute.

In the second study, specimens were obtained from infants admitted to six different newborn intensive care units within the state of Utah (n = 289). Initially, the specimens were refrigerated after collection, but some were frozen. All specimens were shipped on dry ice to US Drug Testing Laboratories for analysis. Approval for the meconium collection and analysis was under Institutional Review Board 7919-00, University of Utah, and informed consent was received.

**EXTRACTION AND ANALYSIS**

After the meconium (0.5–1 g) was allowed to thaw, deionized water (1 mL), internal standard (500 ng), and acetone (5 mL) were added, and the specimen was centrifuged (10 min at 1125g). The supernant was removed, and hexane (5 mL) was added. After mixing, the hexane fraction was passed through a normal-phase solid-phase extraction cartridge (1). The FAEEs were finally eluted from the column in hexane (3 mL) and evaporated to dryness under nitrogen at 40 °C. The dried extract was reconstituted in hexane (60 μL), transferred to an autosampler vial, and analyzed by full-scan chemical ionization gas chromatography–mass spectrometry with acetone as the reagent gas.

A Varian Star 3400 bench-top gas chromatograph coupled to a Saturn II ion trap mass spectrometer was operated in the full-scan positive chemical ionization mode. The gas chromatography column was bonded-phase fused-silica [30 m × 0.25 mm (i.d.); 0.25 μm film thickness]. The injector was operated at 250 °C in splitless mode, and the injection volume was 3 μL. The oven was programmed from 50 °C for 1 min to 310 °C at a rate of 20 °C/min. The fragmentation ions for each FAEE are shown below. Chemical ionization was chosen for this analysis because electron impact ionization of these compounds yields identical fragments for the various FAEEs (2). In chemical ionization mode, a diagnostic ion for each compound is obtained. The monitored ions were as follows (parent ion in italics):

- Internal standard (E17:0): 299, 298, 300
- Ethyl palmitoleic (E16:1): 283, 282, 284
- Ethyl palmitic (E16:0): 285, 286, 284
- Ethyl linoleic (E18:2): 263, 245, 309

Ethyl oleic (E18:1): 311, 265, 247
Ethyl stearic (E18:0): 313, 312, 314
Ethyl arachidonic (E20:4): 333, 287, 268

**RESULTS**

**STUDY 1**

Of the 436 specimens analyzed, 73 (16.7%) were positive for FAEEs (>50 ng/g). E12 (lauric) and E14 (myristic) acid ethyl esters were most frequently detected in meconium, even in specimens taken from neonates born to alcohol-free mothers; they therefore were not included in the total concentration reported. In our experience, palmitoleic acid is not present in large amounts in the meconium of non-alcohol-exposed neonates; it therefore was included in the profile.

All the specimens discussed were reported as “positive” for FAEEs, with the implication that the newborns had been exposed to alcohol during gestation. Specimens were reported as positive if the cumulative concentration of FAEEs exceeded 50 ng/g. The concentration of 50 ng/g was chosen as an analytical limit of detection above which criteria for the correct identification of the FAEEs could be met.

Ninety percent of the positive samples from study 1 contained linoleic and palmitic acid ethyl esters, 51% contained oleic and palmitoleic acid ethyl esters, 20% contained stearic acid ethyl ester, and 18% contained linolenic acid ethyl ester. None of the specimens contained arachidonic acid ethyl ester, which is the most susceptible to light and heat of those in the profile.

After observing that no arachidonic acid ethyl ester was detected in the samples from study 1, we carried out a stability study for FAEEs in meconium. Specifically, after 1000 ng of each FAEE was added to meconium, the meconium was divided into aliquots, and the aliquots were stored (a) in the light at room temperature, (b) in the dark at room temperature, (c) in the refrigerator (2–8 °C), (d) in the dark at room temperature under argon, and (e) frozen at −20 °C.

The results of the FAEE stability study are shown in Fig. 1. Specimens stored at room temperature in the light lost 86% of the total FAEE concentration within 24 h, whereas specimens stored in the dark lost only 60% at room temperature. Samples stored in the freezer lost only 10% within 24 h and up to 18% within 3 days; samples stored in the dark at room temperature under argon lost ~40% over 3 days and stabilized at a 50% loss thereafter. The most stable specimens were those stored in the freezer, which lost 11% of the total FAEEs over 6 days but did not have a loss ≥50% for at least 43 days.

Obviously, specimens that are collected and stored at room temperature and are not immediately shipped on ice are at risk for FAEE degradation. It is possible that storage and shipping conditions contribute to variations in the concentrations of FAEEs detected. The longer chain FAEEs seemed to be more susceptible to heat and light than the esters with shorter hydrocarbon chains. Meco-
nium specimens must be stored frozen and shipped on ice to the laboratory.

STUDY 2
Of the 289 specimens analyzed, 35 (12.1%) were positive for FAEEs (>50 ng/g). The overall percentage of positives was less than in study 1, although this is not unexpected because the study set came from Utah, a state where only 32.1% of adults 18 years and older reported alcohol use in the past month compared with 49.5% in Hawaii. Of the positive samples, 80% contained linoleic acid ethyl ester, 82.8% contained palmitic acid ethyl ester, 65.7% contained oleic ethyl ester, 51.4% contained stearic acid ethyl ester, and 28.6% contained palmitoleic acid ethyl ester, but only one sample (2.8%) contained linolenic acid ethyl ester. However, in this sample set, 25.7% contained arachidonic acid ethyl ester. Some of the specimens from this set were refrigerated after collection and some were frozen, but all specimens were shipped on ice.

EMPIRICAL DATA ANALYSIS
The total FAEE concentrations measured for both studies were divided into four quartiles and plotted (Fig. 2). There was a distinct difference between the first three quartiles and the final group; the profile between the two studies, however, was similar. The overall mean FAEE concentrations in both studies were plotted (Fig. 3). Again the correlation between the two studies was remarkable, with oleic and linoleic acid ethyl esters being present in the highest concentrations when detected in a specimen; however, they were not always present in positive samples.

Discussion
Fetal alcohol syndrome, alcohol-related birth defects, and alcohol-related neurodevelopment disorders in neonates are a result of maternal alcohol consumption during pregnancy. Even at low intake, alcohol can cause adverse effects in newborns and problems later in childhood (3, 4). To date, determination of fetal alcohol exposure has depended largely on maternal interview, although laboratory tests are becoming more widely used.

FAEEs, which are formed in the body by esterification of ethanol with free fatty acids (5), can be detected in the serum and blood of alcohol users (6, 7) and in human organs damaged by ethanol abuse; they can also be used as postmortem markers for premortem ethanol use (8). FAEEs persist and accumulate in adipose tissue after ethanol has been eliminated from the body (9).

Meconium, the first fecal matter passed by the newborn, is often analyzed for drugs to which a neonate has been exposed during the latter half of gestation (20 weeks) (10). The determination of FAEEs in meconium serves as a diagnostic tool to assess the extent of ethanol exposure to the fetus. Some research has centered on the investigation of ethyl linoleate (E18:2) as the biomarker most likely to identify alcohol-related birth defects (11). In our studies, E18:2 was present in 90% and 80% of the positive samples in studies 1 and 2, respectively. Chan et al. (12) showed that FAEEs can be produced in vitro in meconium and that their production may be subject to individual variability. Koren et al. (13) indicated that FAEEs do not cross the human placenta, indicating that their measurement in meconium represents a valid estimate of ethanol circulating in the fetus. In the present study we used a wider range of FAEEs than previous researchers...
and found that the alcohol exposure rate in the Hawaiian population (study 1) was 16.7%, which is in close agreement with other estimates based mostly on maternal interviews (14). However, the limited maternal information associated with these specimens did not correlate at all with the FAEE measurement. Because it is not possible to carry out a true dose–response study on pregnant women, the reliability of information from the mothers must be assessed at the time of interview. The different FAEE profiles may give more information concerning “binge drinking” or frequent alcohol consumption during pregnancy, but at this time such a correlation cannot be made.

In study 2, the alcohol exposure rate was 12.1%, somewhat lower than in study 1, but the population sampled is known to have a lower rate of adult alcohol use (Utah). Empirical analysis of the data divided into quartiles showed a definite distribution into two distinct populations in both studies. The fourth quartile showed a greatly increased total mean concentration of FAEEs over the other three quartiles, although the overall prevalences were different in the two groups. Those newborns with FAEE concentrations >10,000 ng/g would seem to be at higher risk of alcohol-related problems.

Previously, we have shown a FAEE profile for meconium specimens received consecutively into our routine laboratory and concluded that oleic acid ethyl ester (E18:1) is the most prevalent individual ester, followed by palmitic and palmitoleic (15). However, in study 1, 90% of the positive samples contained linoleic and palmitic acid ethyl esters, whereas only 51% contained oleic and palmitoleic acid ethyl ester. The overall profile (Fig. 3) showed that oleic acid ethyl ester, when detected, was present in the highest concentrations. This is in excellent agreement with our previous work (15). In study 2, more than 80% of the samples contained linoleic and palmitic acid ethyl esters, whereas 65% contained oleic acid ethyl ester. In this population, linoleic acid ethyl ester was present in the highest mean concentration (8589 ng/g), closely followed by oleic acid ethyl ester (7678 ng/g). Again, the profile was similar to that observed in study 1 and previously published work.

In the first study, none of the specimens contained arachidonic acid ethyl ester. Refaai et al. (8) have shown that its presence is indicative of recent alcohol use because it was measured only in the tissues of individuals with detectable blood ethanol at the time of autopsy. Because meconium represents a longer timeframe of drug exposure than urine, it is possible that alcohol use in these women was not recent. In the second study, 25.7% of specimens contained E20:4 at a mean concentration of 2413 ng/g. These specimens were shipped on dry ice; thus, differences in shipping and storage may account for the observed difference in arachidonic acid ethyl ester concentrations because arachidonic acid ethyl ester is the most susceptible to degradation from exposure to light and heat.

To minimize losses from exposure to heat and light, meconium specimens should be refrigerated or frozen immediately after collection and shipped on dry ice.

In conclusion, in two separate studies, meconium specimens were positive for FAEEs, biomarkers for exposure to ethanol, at prevalence rates of 16.7% and 12.1%, respectively. When the samples were divided into quartiles on the basis of total FAEE concentration, the fourth quartile had values >10,000 ng/g in both studies. It is likely that these newborns were exposed to significant amounts of alcohol during pregnancy.

References