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Short communication

Quantitation of fatty acid ethyl esters in human meconium by an improved liquid chromatography/tandem mass spectrometry

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ABSTRACT

This paper reports the development and validation of an improved assay for quantitation of fatty acid ethyl esters (FAEEs) in human meconium using liquid chromatography/tandem mass spectrometry (LC–MS/MS). FAAEs (ethyl laurate, ethyl myristate, ethyl palmitate, ethyl palmitoleate, ethyl stearate, ethyl oleate, ethyl linoleate, ethyl linolenate, and ethyl arachidonate) and the internal standard (I.S.), ethyl heptadecanoate, were separated by reverse phase HPLC and quantified by MS/MS using electrospray ionization (ESI) and multiple reaction monitoring (MRM) in the positive ionization mode. The absolute recovery of FAEEs varied from 55 ± 10% for 0.33 nmol/g (100 ng/g) of ethyl linoleate up to 86 ± 8% for 1.55 nmol/g (500 ng/g) of ethyl miristate. The LODs and LOQs varied from 0.01 to 0.08 nmol/g and from 0.02 to 0.27 nmol/g, respectively. The assay has been successfully applied to examine the FAEE levels in 81 meconium samples from babies born to mothers reporting alcohol consumption, to varying degrees, during pregnancy.

1. Introduction

Prenatal alcohol exposure may have dramatic consequences in exposed fetuses. Meconium levels of FAEEs, nonoxidative metabolites of ethanol, highly correlate with prenatal alcohol exposure [1–3], and their relevance was demonstrated in a recent study where increased levels of five FAEE were significantly associated with poor mental and psychomotor development in the first 2 years of age [4]. However, a critical review of meconium FAEE concentrations identified several limitations when used as a diagnostic tool of prenatal alcohol exposure, including analytical problems [5].

LC–MS/MS methods have the advantage of being more specific in simultaneously quantifying several analytes in complex biological matrices. A LC–MS/MS method for quantifying FAEEs in meconium was recently reported elsewhere [6]. However, we considered that several aspects of the method could be improved. For example, the authors used 1 g of meconium, and the retention times reported for five of the ten FAEEs quantified in the study were above 10 min. This results in a long sample run time which could potentially be reduced.

We report the development and validation of an improved LC–MS/MS spectrometry assay to quantify the FAEEs levels in meconium of women exposed to alcohol during pregnancy.

2. Experimental

2.1. Chemicals and reagents

FAEEs, including ethyl laurate (E12:0), ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl palmitoleate (E16:1), ethyl stearate (E18:0), ethyl oleate (E18:1), ethyl linoleate (E18:2), ethyl linolenate (E18:3), and ethyl arachidonate (E20:4) were purchased from Cayman (Ann Arbor, MI, USA). Ethyl heptadecanoate ester (E17:0)

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was obtained from TCI America (Portland, OR, USA) for use as the internal standard (I.S.). Each FAEE standard solution was supplied at a concentration of 500 mg/mL in 99.9% ethanol. All other chemicals of analytical or HPLC grade were obtained from J.T. Baker (Phillipsburg, NJ, USA). Blank meconium was obtained during delivery from women who reported no alcohol consumption during pregnancy and had periodic obstetric assessments at the Cheil Hospital & Women's Healthcare Center (Seoul, Korea). Distilled water, prepared from demineralized water, was used as needed.

2.2. Preparation of standard and quality control solutions

Standard solutions were diluted with 99.9% ethanol to prepare 10 and 100 μg/mL working solutions, which were stored at −20 °C until analysis. Using the working solutions, calibrators were prepared in ng/g concentrations in meconium. Calibrators containing analyte concentrations ranging from 50 to 500 ng/g were prepared daily for each analytical batch. Quality control (Q.C.) samples of 100 and 400 ng/g were prepared for each analyte in drug-free meconium and stored at −20 °C.

2.3. Sample preparation

Meconium 500 mg was transferred into 15-mL screw-capped polypropylene centrifuge tubes (Corning Inc., Lowell, MA, USA), and 20 μL of I.S. (25 mg/L), 250 μL distilled water and 500 μL acetone were added. The tubes were vortex-mixed for 1 min and 5 mL hexane was added. The tubes were placed in a horizontal shaker for 10 min. After centrifugation at 500 × *g* (2000 rpm) for 10 min, the organic layer was transferred to new polypropylene tubes and the solvent was evaporated to dryness at 45 °C under a nitrogen stream. The dried samples were then resuspended in 500 μL hexane, vortex-mixed for 15 s, and applied onto aminopropyl silica solid-phase extraction (SPE) columns (500 mg of adsorbent, 3 mL volume, 57–90 μm particle size; Sep-Pak, Waters, Milford, MA, USA), which had been preconditioned with 3 mL hexane. The FAEEs were recovered by elution with hexane (4.5 mL). The eluent was evaporated to dryness at 45 °C under a steam of nitrogen, resuspended in 100 μL mobile phase, and vortex-mixed for 10 min. An aliquot of 20 μL volume was injected into the LC–MS/MS system.

2.4. LC–MS/MS system

The LC–MS/MS system comprised a PerkinElmer (Series 200, Shelton, CT, USA) HPLC system interfaced to a triple quadrupole mass spectrometer equipped with ESI probe (API 2000, Applied Biosystems/MDS Inc., Concord, ON, Canada). Chromatographic separation was performed on an XBridge C8 column (150 mm × 1.0 mm, 3.5 μm; Waters, Milford, MA, USA), maintained at 25 °C, using a mobile phase of 0.1% formic acid in 90% acetonitrile (v/v). The flow rate was 50 μL/min.

The ESI source was operated in the positive ionization mode. The mobile phase was infused through an integrated syringe pump into the ESI probe at a rate of 10 μL/min. The pseudomolecular ions $(M+H)^+$ were selected in the first quadrupole for MRM of individual FAEEs and I.S. Optimized MS parameters were as follows: capillary voltage at 5.5 kV; source temperature at 300 °C; and collision gas was nitrogen at a collision cell pressure of 0.019 Pa. The following MRM transitions (*m*/*z*) were: ethyl laurate 229.4 → 201.4, ethyl myristate 257.4 → 229.4, ethyl palmitate 285.4 → 257.4, ethyl palmitoleate 283.5 → 219.4, ethyl oleate 311.5 → 265.4, ethyl linoleate 309.5 → 263.4, ethyl linolenate 307.4 → 261.5, ethyl stearate 313.5 → 287.4, ethyl arachidonate 333.5 → 281.5, and ethyl heptadecanoate (I.S.) 299.4 → 271.5. The declustering potentials (V) were 60, 60, 60, 40, 40, 10, 10, 60, 10 and 60, respectively. The collision energies were 16, 17, 18, 17, 17, 13, 17, 18, 14 and 18.

2.5. Assay validation

Linearity was assessed by weighted linear regression ($1/x^2$) of standard curves based on analyte-I.S. peak area ratios prepared in triplicate on three separate days. Intra- and inter-day precision (as relative coefficient of variations, %), and corresponding accuracies (as relative error, %), were determined by analysis of three replicates of each QC sample on 10 different days. The lower limit of quantification (LLOQ) was defined as the lowest concentration at which both precision and accuracy were less than 20%. The limit of detection (LOD) was the concentration with a signal-to-noise ratio of 3. Selectivity of the assay was assessed by comparing the chromatograms from meconium samples obtained from women who reported not having consumed alcohol during pregnancy with chromatograms for the corresponding spiked meconium samples. Recoveries of analytes and I.S. were determined by comparing the peak areas of analytes and I.S. in extracted QC samples with those in post-extraction blank meconium samples spiked with QC solutions. Stability was assessed by storing processed QC samples at room temperature for 12 h.

2.6. FAEEs in meconium samples

The analytical method was used to determine meconium concentrations of FAEEs obtained at delivery from women who reported alcohol consumption during pregnancy. The clinical protocol was approved by the Ethics Committee at Ilsan-Jeil Women's Hospital (Kyoungki-do, Korea), Kwangmyoung-Cheil Women's Hospital (Kyoungki-do, Korea), and Cheil Hospital & Women's Healthcare Center (Seoul, Korea). Written informed consent was obtained from all volunteers before entering the study. Meconium samples were collected within the first 6–12 h postpartum from the birth.

3. Results and discussion

3.1. Method development

The method reported herein represents an improved technique of an LC–MS/MS method recently published elsewhere [6]. The initial chromatographic conditions reported herein were based on the reference method. However, various mobile phase combinations were investigated to optimize sensitivity, speed and peak shape. A mobile phase with 0.1% formic acid in 90% acetonitrile (v/v) gave a better response than the reference method. The retention times reported in the reference method varied from 6.7 to 16 min (total sample run time: 21 min). Using the present technique retention times varied from 4.1 min for ethyl laurate to 8.8 min for ethyl stearate and 7.5 min for the I.S. (total sample run time: 15 min) (Fig. 1).

Our technique also reduced the volume of aliquot injected onto the chromatographic system to 20 μL, compared to 50 μL in the reference method. Other conditions were similar or had minor variations.

3.2. Assay validation

Representative chromatograms of blank meconium and meconium spiked with 100 and 500 ng/g of individual FAEE are shown in Fig. 1. Details of the assay validation are shown in Table 1. The absolute recovery of FAEEs varied from 55 ± 10% for 0.33 nmol/g (100 ng/g) of ethyl linoleate to 86 ± 8% for 1.55 nmol/g (500 ng/g)

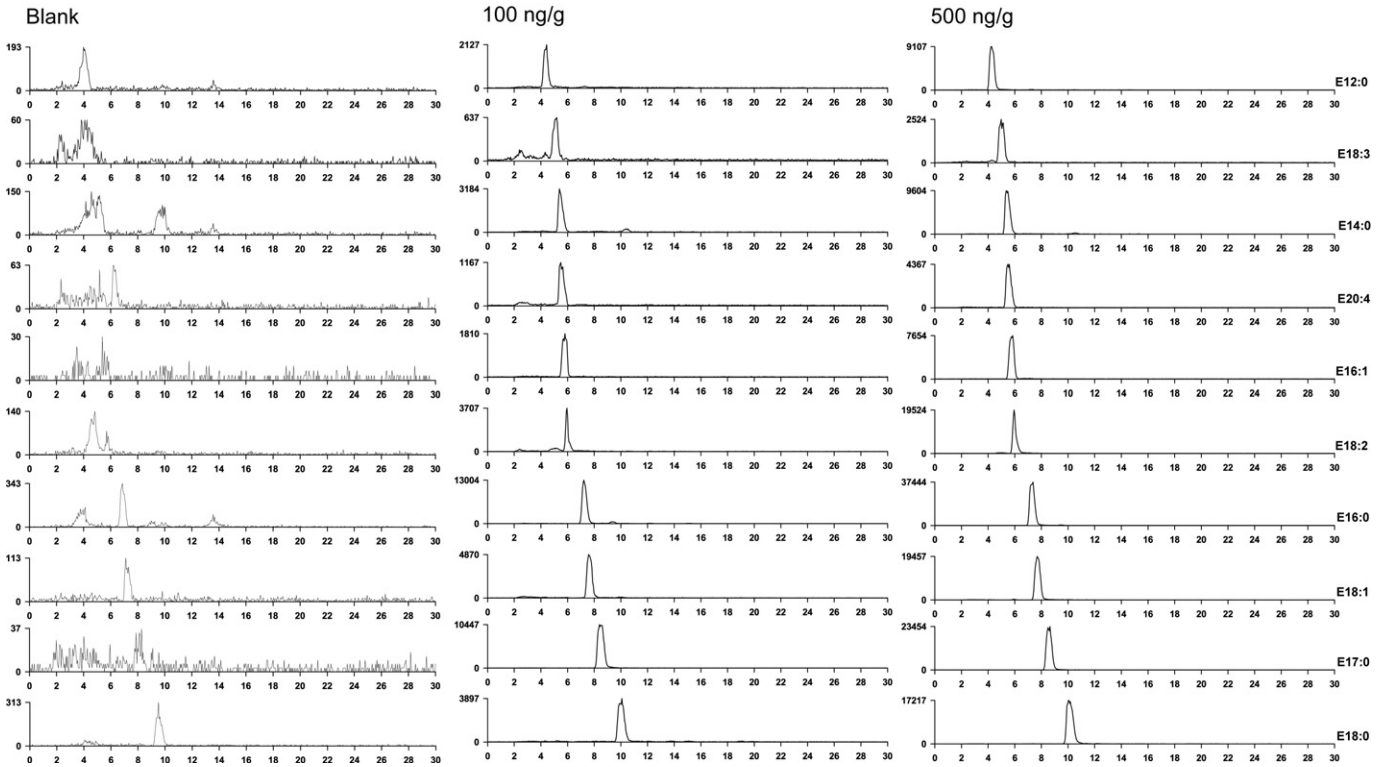

Fig. 1. MRM chromatogram of blank meconium (left) and meconium samples spiked with 100 ng/g (center) and 500 ng/g (right) which were processed and analyzed with the technique described herein. E12:0: ethyl laurate; E18:3: ethyl linolenate; E14:0: ethyl myristate; E20:4: ethyl arachidonate; E16:1: ethyl palmitoleate; E18:2: ethyl linoleate; E16:0: ethyl palmitate; E18:1: ethyl oleate; E17:0 (I.S.): ethyl heptadecanoate ester; and E18:0: ethyl stearate. For individual levels of FAAEs expressed in nmol/g, see Table 1.

of ethyl miristate. The intra- and inter-assay precisions varied from 7% to 21% and from 10% to 17%, respectively. The intra- and inter-assay accuracies ranged from −17% to 15% and from −4% to 14%, respectively.

Calibration curves were linear and intercepts were not significantly different from zero. The LODs and LOQs varied from 0.01 to 0.08 nmol/g and from 0.02 to 0.27 nmol/g, respectively. In comparison The LOQs for FAEEs reported by the reference LC–MS/MS method varied from 0.12 to 0.20 nmol/g [6].

3.3. FAEEs in meconium samples

Reducing the amount of matrix (meconium) was investigated after chromatographic conditions were optimized. The sensitivity

Table 1
Absolute recovery ($n = 3$), intra-day ($n = 3$) and inter-day ($n = 10$) precision and accuracy.

FAEE	Concentration [nmol/g (ng/g)]	Absolute recovery (mean, %)	Intra-assay precision (CV, %)	Inter-assay precision (CV, %)	Intra-assay accuracy (error, %)	Inter-assay accuracy (error, %)
Ethyl laurate (E12:0)	0.44 (100)	58 ± 3	7	12	6	13
	1.74 (500)	61 ± 7	10	10	−3	14
Ethyl myristate (E14:0)	0.39 (100)	80 ± 3	17	12	12	8
	1.55 (500)	86 ± 8	15	10	2	10
Ethyl palmitate (E16:0)	0.35 (100)	65 ± 5	16	17	15	−1
	1.40 (500)	62 ± 1	11	15	−3	10
Ethyl palmitoleate (E16:1)	0.35 (100)	80 ± 7	18	15	4	3
	1.41 (500)	72 ± 7	16	14	−6	5
Ethyl oleate (E18:1)	0.32 (100)	71 ± 11	12	17	4	9
	1.28 (500)	62 ± 7	18	13	−17	8
Ethyl linoleate (E18:2)	0.32 (100)	69 ± 5	21	15	−1	−2
	1.29 (500)	72 ± 8	19	15	−13	1
Ethyl linolenate (E18:3)	0.33 (100)	55 ± 10	18	13	4	−4
	1.30 (500)	58 ± 10	18	14	−11	5
Ethyl stearate (E18:0)	0.32 (100)	40 ± 11	12	13	4	12
	1.28 (500)	44 ± 3	12	12	−17	2
Ethyl arachidonate (E20:4)	0.30 (100)	66 ± 2	14	15	4	3
	1.20 (500)	62 ± 5	12	12	−9	6

and selectivity of the method was similar when processing 500 mg of meconium, compared to 1000 mg in the reference method for quantification of FAEEs [6].

The total FAEE concentration in meconium obtained from 81 women who reported alcohol consumption during pregnancy was 0–37.8 nmol/g. As was previously reported in a study using a LC–MS/MS [6], FAEEs levels exhibit high inter-individual variability.

The difficulties, advantages and disadvantages of using meconium FAEE concentrations as a single diagnostic tool of prenatal alcohol exposure were carefully analyzed elsewhere [5], and the authors concluded that FAEE levels alone do not appear to be an acceptable strategy in the absence of other data to corroborate such exposure. In this context, the method proposed herein has improved analytical characteristics over another LC–MS/MS previously reported [6]. However, its use as a single method for diagnosing alcohol abuse during pregnancy cannot be recommended. Further research is needed to clarify the role of FAEE levels in meconium as biomarkers of prenatal alcohol abuse, especially during the second half of pregnancy.

4. Conclusion

An improved LC–MS/MS method for the quantification of FAEEs in human meconium has been developed and validated. The major advantages of the assay over the previously published method include a reduced amount of meconium as the biological matrix, a shorter run time allowing high throughput analysis, a smaller injection volume, and improved sensitivity (LLOQ). The assay has been successfully applied to examine the FAEE levels in 81 meconium samples from children born to mothers reporting alcohol consumption during pregnancy.

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