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Liquid chromatography-tandem mass spectrometry method for measuring vitamin E acetate in bronchoalveolar lavage fluid

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Abstract

We investigated the suitability of isotope-dilution liquid chromatography coupled with tandem mass spectrometry for identifying vitamin E acetate (VEA) in bronchoalveolar lavage (BAL) fluid. This new method demonstrates high accuracy, selectivity, and sensitivity, with mean recoveries higher than 90%, coefficients of variation ranging from 1.5% to 4.5%, and a limit of detection of 1.10 ng/mL. Calibration curves were linear ($R^2 > 0.99$). The linear range and detection limit of the method were adequate for identifying VEA in 48 of 51 BAL fluid samples collected from people with lung injury resulting from e-cigarettes, or vaping, product use. We conclude that this method is an effective tool for studying VEA accumulation in lungs caused by using e-cigarettes, or vaping, products that contain VEA.

Keywords

Vitamin E acetate; Isotope dilution LC-MS/MS; Bronchoalveolar lavage fluid

1. Introduction

The use of e-cigarettes, or vaping, products (EVPs) dramatically increased in the United States over the last decade, especially in youth [1–3]. Furthermore, data from the 2018 National Health Interview Survey indicate that more than eight million U.S. adults used these products on a regular basis [4–5]. The increased use of EVPs is partially explained by the perception that these devices are less harmful than cigarettes because no smoke is formed [6–7]. Commercially available e-liquids are sold in a variety of flavors and nicotine concentrations [8]. In addition, e-liquids can be customized to further meet the consumer's

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Maria Morel Espinosa: Investigation, Methodology, Validation, Writing - original draft. **Benjamin C. Blount:** Conceptualization, Resources, Writing - review & editing. **Liza Valentin-Blasini:** Conceptualization, Supervision, Writing - review & editing.

5. **Publisher's Disclaimer:** Disclaimer

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

preferences. Accessibility to e-liquid components makes it easy to create individualized e-liquid mixtures and for secondary “informal” sources to market e-liquid formulations. Vaping of cannabis has also increased substantially, with low tetrahydrocannabinol (THC) cannabis (“hemp extracts”) legal across the country, and high THC cannabis (>0.3%) legal for non-medical use in 11 states and Washington, DC. [9–10]. Furthermore, 33 states and District of Columbia allow high THC cannabis products to be sold for medical use, and these products are widely advertised for online purchase [11].

During the summer of 2019, people who self-reported using EVPs in the prior three months began presenting to U.S. emergency departments with rapidly worsening respiratory symptoms [12]. The number of reported cases of this newly identified EVP-associated lung injury, termed e-cigarette, or vaping, product use-associated lung injury (EVALI) increased from July 2019 to February 2020 to 2807 reported hospitalized cases, with 68 deaths [12]. In an attempt to identify causative agents, the U.S. Food and Drug Administration (FDA) and various state health departments analyzed vaping liquids provided by EVALI patients. The New York State Department of Health Wadsworth Center Laboratory analyzed a variety of products from 34 EVALI patients reporting using THC-containing products and found that each patient had used at least one THC product that also contained vitamin E acetate (VEA) [13]. Furthermore, FDA investigators found VEA in 49% of case-related THC-containing samples analyzed [14]. However, a significant fraction of EVALI cases denied use of THC products and/or gave investigators only products that contained no VEA [15–16]. Thus, a one-to-one link of vaped VEA to disease could not be established based solely on product analysis.

VEA does not occur naturally; rather it is the shelf-stable synthetic form of vitamin E that is typically added to orally consumed dietary supplements and dermally applied skin products [17]. When taken orally, VEA is hydrolyzed to vitamin E within the intestine [18–19]. VEA has been administered orally and topically for years without adverse health effects. However, the safety of inhaled VEA has not been evaluated. Starting in late 2018, VEA began to be added to vaping liquids [27, 28], presumably to lower production costs while maintaining the golden color and viscous appearance of pure THC oil [20–22]. Use of these VEA-containing vape products has been associated with lung injury cases [23,24,29]. Furthermore, mice exposed to vaped VEA develop a pattern of lung injury that closely resembles that seen in EVALI patients [25,30,31]. Given, several plausible mechanisms by which vaped VEA could cause EVALI [32–35], the challenge was to identify a way to measure VEA and other harmful substances that could be accumulating in the lung epithelial lining fluid from EVALI patients as a result of repeated inhalational exposure. Bronchoalveolar lavage (BAL) fluid was already being collected from some EVALI patients to assess for other underlying causes of illness and to enable characterization of lipid-laden macrophages. Thus, measurement of VEA in residual BAL fluid could provide the missing information to better understand VEA accumulation and health effects. However, no methods existed for measuring VEA in BAL fluid. We developed and validated a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for detecting VEA in BAL fluid.

2. Methods

2.1. Instrumentation

Analyses were conducted with an Acquity ultra-pressure liquid chromatography (UPLC) system equipped with a gradient pump, autosampler, and column compartment (Waters, Milford, MA, USA). Acquity software was used for system control. The separation was performed using an Xterra C18 column (Waters) with a 100 μL injection loop. A Sciex 5500 triple-quadrupole mass spectrometer (Sciex, Redwood City, CA, USA) with electrospray interface was used for VEA detection.

2.2. Reagents and chemicals

Unlabeled VEA (VEA; CAS# 7695-91-2; chemical purity: 99.9%; analytical standard), labeled VEA [VEA- (trimethyl-d_9); deuterium enrichment: ≥ 98 atom %; chemical purity: ≥98%], and formic acid (CAS# 64-18-6; chemical purity: ≥ 98%; ACS reagent) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol Optima (CAS#67-56-1; LC/MS grade 99.9%;) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Deionized water with a specific resistance of 18 MΩ.cm or greater was generated in-house using an Aqua Solutions model RODI-C-11BL ultrapure water purifications system (Jasper, GA, USA). A commercial synthetic BAL fluid of Gambles' simulated lung fluid (not stabilized) was obtained from Pickering Laboratories (Mountain View, CA, USA).

2.3. Native and isotopically labeled standard solutions

We prepared a standard stock solution by dissolving native (unlabeled) VEA in methanol. This concentrated stock was stored at −20 °C until use. Intermediate stock solutions were prepared by diluting the concentrated native VEA stock with methanol. Isotopically labeled VEA was prepared in a similar manner to be used as an internal standard. Seven calibration standards were prepared daily by spiking 100 μL of commercial synthetic BAL fluid with the appropriate intermediate native VEA stock solution along with 40 μL of the intermediate internal standard solution. Final concentrations covered a range of 10–1000 ng/mL.

2.4. Quality control materials

Two quality control (QC) pools were developed at a low VEA level (25 ng/mL) and at a higher VEA level (250 ng/mL). QC materials were prepared from commercial synthetic BAL fluid, uniformly mixed in an amber bottle and stored until use. QC characterization involved 20 discrete measurements for each QC pool to define the mean VEA concentration in each pool as well as lower and upper limits for precision evaluation.

2.5. Sample collection and storage

BAL fluids were collected from 51 EVALI patients in 16 states using each institution's routine clinical processes and thus were not standardized. Samples were refrigerated or frozen after collection and shipped on dry ice to the U.S. Centers for Disease Control and Prevention (CDC). A CDC human-subjects research review judged this sample collection to be a non-research public health response activity.

2.6. Sample preparation

Room temperature BAL fluid samples were vortexed to suspend particulate material. A 100 μL aliquot was transferred to an autosampler vial and spiked with labeled internal standard. The sample was diluted with 860 μL methanol and queued for injection into the LC-MS/MS system. If the prepared BAL fluid sample turned cloudy it was transferred to a microcentrifuge tube and centrifuged for 10 min at 15,000 rpm. Supernatant was transferred to an autosampler vial and queued for analysis. Samples with initial results <10 ng/mL were re-analyzed to improve sensitivity by concentrating using a TurboVap system. BAL fluid (100 μL) was transferred to a glass conical tube and vortexed after spiking with labeled internal standard and adding 2 mL of methanol. Samples were dried under an N_2 flow at 60 °C for 20 min, resuspended with 100 μL of methanol, vortexed, and transferred to an autosampler vial for analysis. This second sample preparation procedure resulted in a more concentrated injection that improved assay sensitivity.

2.7. Chromatography

VEA analysis was carried out on an Xterra MS C18 analytical column (125 Å pore size, 3.5 μm particle size, 2.1 mm inner diameter × 100 mm length) with a mobile phase of methanol–water (90%:10%) with 0.1% formic acid at a 0.4 mL/min flow rate under isocratic conditions. The column temperature was kept at 40 °C, and samples were injected using a loop injection mode and a 100-μL injection loop. A 10-μL injection volume under these conditions results in a VEA peak with a retention time of 10 min with a total run time of 15 min.

2.8. Mass spectrometry

The mass spectrometer was operated in positive electrospray ionization (+ESI) mode. The source was used at 350 °C with curtain and ion source gases at 20 psi and 45 psi, respectively. Mass spectral data were acquired in multiple reaction monitoring mode, cycling between transitions for VEA (473 → 207, 473 → 165) and VEA-trimethyl-d_9 (482 → 216) with a dwell time of 250 ms for each transition. Collision energy and other mass spectral parameters were optimized for maximum transmission of transitions of interest (Table 1).

2.9. Data analysis

We evaluated all VEA and VEA-trimethyl-d9 data for accuracy of integration and manually reintegrated them, if necessary. The default auto-integration tool of Analyst software 1.6.2 software (Applied Bio-systems, Foster City, CA, USA) was utilized for data evaluation. Visual inspection was conducted for consistency of integration among samples by verifying correct peak choice (retention time matching) and integration (extended or cut integration of peaks was manually corrected). Quantitation was based on a full set of seven calibrators run with each set of samples. Calibration curves were constructed using the peak area ratio of analyte to stable isotope-labeled internal standard versus known standard concentrations weighted by the reciprocal of concentration ($1/x$). Samples with levels exceeding the highest standard were diluted and reanalyzed.

3. Results and discussion

3.1. Method validation

3.1.1. Analytical specificity—We verified specificity by testing pooled human BAL fluid and non-vaping related BAL fluid samples. Fig. 1 shows baseline-resolved chromatograms and the absence of interfering matrix components in commercial synthetic BAL fluid for VEA quantitation and confirmation mass transitions at a 25 ng/mL [Fig. 1 (a–b)] and deuterated VEA used as an internal standard [Fig. 1 (c)]. Selected reaction monitoring (SRM) response ratios between quantitation and confirmation ion transitions further improved method specificity. The confirmatory SRM response was used to determine the presence of interferences in the sample analysis. Percent differences among calculated concentrations from the quantitation and confirmation transitions for all VEA containing samples were ≤ ± 10%. VEA results were within the acceptable threshold of ± 25%.

3.1.2. Dynamic range, linearity, and limits of detection—The chosen dynamic range for VEA spanned three orders of magnitude to capture broad levels in BAL fluid. Seven calibration standard solutions prepared in commercial synthetic BAL fluid were used to construct a 1/x weighted least-square model calibration curve. Residual analysis of seven independent calibration curves confirmed a linear behavior with linear correlation coefficient (R^2) value>0.999. Individual calibration curves had $R^2 \geq 0.998$. The limit of detection (LOD) was extrapolated based on Taylor's method [26]. The LOD is defined as $3S_{0,}$ calculated from the y-intercept (S_0) of a regression line of the standard deviations of the low-level calibration standards versus their known concentrations. The dynamic range, linearity, and LOD of this method are shown in Table 2.

3.1.3. Accuracy—We evaluated method accuracy and repeatability using spike-recovery results (Table 3). Recoveries were determined at three concentrations through six independent sample preparations and analyses. Pooled human BAL fluid and commercial synthetic BAL fluid were used as sample matrices. The overall mean recoveries for human BAL fluid and commercial synthetic BAL fluid were 90.0% and 97.8%, respectively. The average coefficient of variance for all spiked concentrations was 3.2% for both human and commercial synthetic BAL fluids.

3.1.4. Precision—Method precision was evaluated as repeatability and intermediate precision of 20 independent QC samples at two levels over 10 days (Table 2). Repeatability (within-run variation) for VEA was 2.31% and 3.55% for QC low and QC high, respectively. Intermediate precision (inter-day variation) was 3.28% for the low QC level and 8.92% for the high QC level.

Short-term stability of VEA in commercial synthetic BAL fluid at −20°C was tested for four weeks. Stability results were defined to be within 15% of the characterized QC mean when compared to each QC batch average. VEA quality control samples were stable under the described conditions. These results spanned our method application activities. However, long-term stability tests are needed to better describe the stability of VEA in human BAL fluid.

3.2. Method application

A total of 51 case-related BAL fluid samples from 16 states were analyzed for VEA using this newly developed and validated method in response to the 2019 EVALI outbreak. These samples were obtained by various clinical teams for the purpose of clinical care and were therefore the lavage procedures were not standardized. This led to variable efficiency of lavage in capturing epithelial lung lining fluid in the BAL fluid sample. Thus, we only report qualitative results for the EVALI BAL fluid samples. Results were reported as “detect” for all values above LOD or “non-detect” for those below LOD. VEA was detected in 48 of the 51 (94%) case patients samples analyzed. Concentrations ranged from “non-detect” to 19,900 ng/mL. These results suggest a strong association of BAL fluid VEA with EVALI lung injury [23]. The method we present in this article was used to identify VEA as the likely cause of the 2019 EVALI outbreak.

4. Conclusions

We present an analytical method that enabled detection of the inhaled toxicant VEA in the lung as sampled using BAL fluid. This method has high sensitivity, accuracy, repeatability, and precision as documented by the validation and characterization data presented. The method was applied to BAL fluid samples from EVALI cases and results clearly demonstrated that VEA in BAL fluid was strongly linked to EVALI. These findings were crucial for identifying inhaled VEA from EVPs as the likely cause of the 2019 EVALI lung injury outbreak. Thus, this method helped save lives by stopping the EVALI outbreak and establishes LC-MS/MS analysis of BAL fluid as a strategy for evaluating inhaled toxicants.

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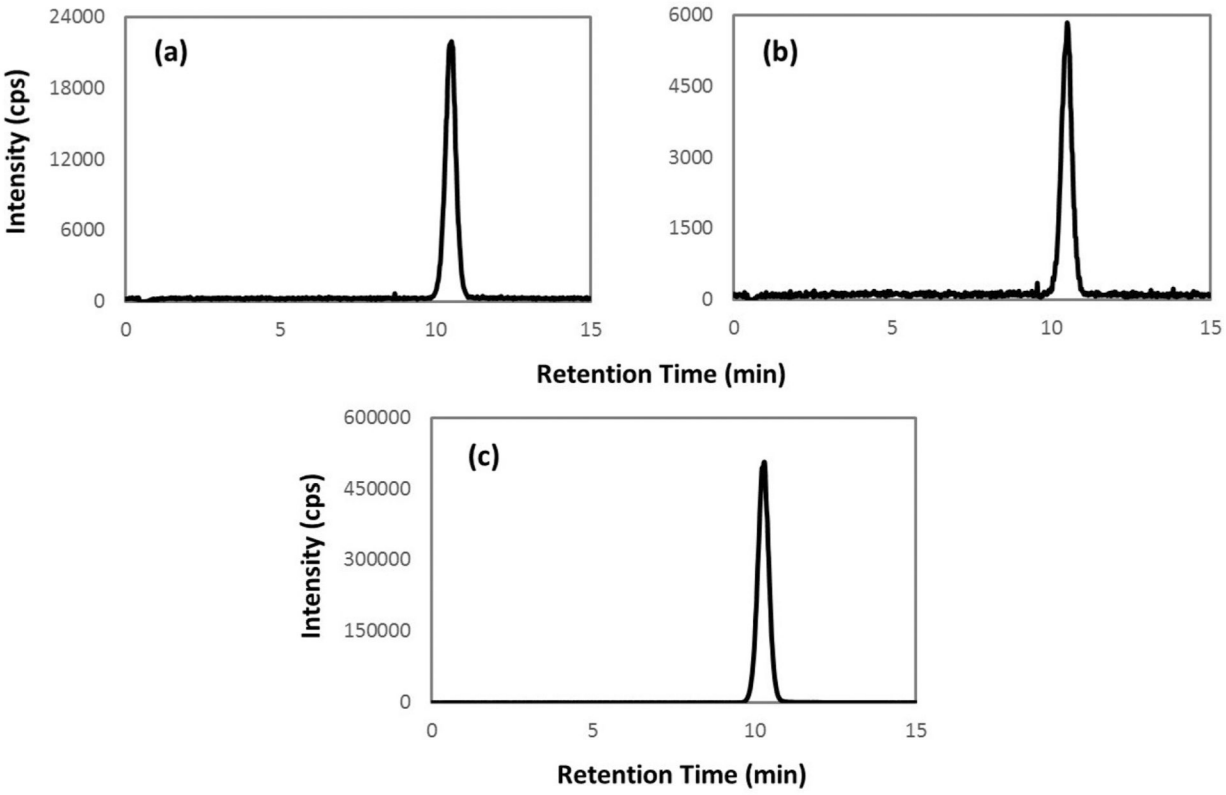

Fig. 1.

Selected reaction monitoring transitions of VEA spiked in synthetic BAL fluid (25 ng/mL). (a) VEA quantitation transition *m*/*z* 473 → 207, (b) VEA confirmation transition *m*/*z* 473 → 165 and (c) deuterated VEA transition *m*/*z* 482 → 216 (internal standard at 200 ng/mL).

Table 1

Multiple reaction monitoring specifications.

Analyte	Ion Transition	Dwell Time (ms)	[a] DP (V)	[b] CE (V)	[c] CXP (V)	Transition Type
Vitamin E acetate (VEA)	473 → 207	250	211	23	8	Quantitation
	473 → 165	250	211	39	42	Confirmation
Vitamin E acetate-trimethyl-d_9 (VEA-trimethyl-d_9)	482 → 216	250	191	21	22	Internal Standard

[a] DP – declustering potential.

[b] CE- collision energy.

[c] CXP – collision cell exit potential.

Table 2

Dynamic range, linearity, LOD, and precision for vitamin E acetate in synthetic BAL fluid.

Vitamin E acetate						
LOD (ng/mL)	**Dynamic Range (ng/mL)**	**Linearity (R^2; n = 7)**	**Precision (CV%; n = 20)**			
			Repeatability		**Intermediate Precision**	
			25 ng/mL	**250 ng/mL**	**25 ng/mL**	**250 ng/mL**
1.10	10–1000	0.999	2.31	3.28	3.55	8.92

Table 3

Analyte recovery for fortified lung fluid at three spike levels (based on six replicates for each spike level).

Vitamin E acetate					
Matrix	**Spike concentration (ng/mL)**	**Spike recovery (%)**	**CV %**	**Mean Recovery (%)**	**Average CV, %**
Synthetic	25	102	1.5	97.8	3.2
BAL fluid	250	93.1	3.7		
	500	97.9	4.5		
Pooled	25	85.1	3.8	90.0	3.2
human	250	92.7	3.1		
BAL fluid	500	92.2	2.6		