Analytical Methods

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/d4ay00697f

An LC-MS/MS method for the quantification of 3-bromotyrosine in plasma from patients diagnosed with eosinophilic esophagitis†

Morgan E. Thomas,[a] Jonathan E. Markowitz,[b] Ada C. Arwood,[a] Joshua M. Germany[a] and William M. Gilliland, Jr *[a]

Eosinophilic esophagitis (EoE) is a disease marked by a surplus of eosinophils, a type of white blood cell that causes inflammation and irritation. The current diagnostic and monitoring procedure for EoE is endoscopy with biopsy, which is invasive, expensive, and leads to tissue tearing in patients. A biomarker in plasma would offer a much less invasive form of disease monitoring for patients with EoE. Eosinophils have been shown to make eosinophil peroxidase, an enzyme that produces hypobromous acid, reacts with primary amines, and forms bromoamides. One product of this biochemical reaction is 3-bromotyrosine. We have optimized a selective, sensitive, and reproducible method to detect and quantify L-tyrosine and 3-bromotyrosine in human plasma using high-pressure liquid chromatography and tandem mass spectrometry (HPLC-MS/MS). Our sample preparation and analysis method requires fewer steps and provides a faster analysis than previous methods. Method validation yielded limits of quantification of 50 ng mL^{-1} for L-tyrosine and 10 ng mL^{-1} for 3-bromotyrosine. Calibration curves for quantification were linear from 50 to 500 ng mL^{-1} with an R^2 value of 0.9995 for L-tyrosine and 10 to 300 ng mL^{-1} with an R^2 value of 0.9998 for 3-bromotyrosine. Method variability was assessed resulting in relative standard deviations of 0.98–4.6% for 3-bromotyrosine ($n = 18$) and 0.20–0.58% for L-tyrosine ($n = 18$). Method applicability was tested with patients with a confirmed diagnosis of EoE, initially suggesting little to no correlation between eosinophil count and 3-bromotyrosine concentration in plasma. However, we do observe a relationship between eosinophil count and esophageal deformities. More research must be conducted to determine a more definitive correlation.

Received 16th April 2024
Accepted 4th September 2024

DOI: 10.1039/d4ay00697f

rsc.li/methods

1 Introduction

Eosinophilic esophagitis (EoE) is the most common eosinophilic gastrointestinal illness affecting both children and adults.[1–3] In pediatric patients, symptoms of the disease include irritability, food aversion, vomiting, and failure to thrive. The disease is characterized by eosinophil granules, a type of white blood cell that promotes inflammation. Endoscopic features include white exudates and longitudinal furrows,[4,5] and, as the disease progresses, esophageal remodeling can occur. Eosinophils produce leukotriene C_4 and platelet-activating factor, both of which have been shown to contract airway smooth muscle and elicit eosinophil infiltration.[6,7] Eosinophilia results in an excess of cationic proteins, eosinophil-derived neurotoxins, and eosinophil peroxidase.[6,8,9] Eosinophil peroxidase catalyzes the formation of hypobromous acid and is toxic to host cells in the presence of hydrogen peroxide and halide ions.[3] Eosinophil functions are regulated by IL-5, a T_H2 cytokine.[10–12] IL-5 regulates eosinophil proliferation, maturation, activation, and survival.[11,13] EoE pathogenesis appears to be mediated by the overexpression of IL-5 leading to a surplus of eosinophils and an inflammatory response. EoE is a chronic and relapsing condition with a constant need for disease monitoring.

Eosinophilic esophagitis is diagnosed by endoscopy and identification of eosinophilic infiltration in the esophagus.[14] EoE presents similar symptoms to gastroesophageal reflux disease (GERD), but patients with EoE do not respond to anti-reflux therapy.[1,15] The two are distinguished endoscopically by the number of esophageal eosinophils per high-powered field (HPF). Literature has suggested that patients with GERD have less than 5 per HPF whereas patients with EoE have more than 20 eosinophils per HPF.[3,7,14] The current consensus for diagnosing EoE requires 1 or more biopsy samples to contain 15 or more eosinophils per HPF.[16,17] Multiple tissue samples are suggested to be taken from the proximal and distal regions to account for variability along the length of the esophagus. Endoscopy with biopsy, despite being invasive and expensive, remains the only effective tool for diagnosis and the only way to

[a]Furman University, Chemistry Department, Greenville, SC, USA. E-mail: mac.gilliland@furman.edu

[b]Prisma Health, Greenville, SC, USA

† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d4ay00697f

differentiate EoE from GERD patients. Following diagnosis, patients with EoE must follow up with endoscopy with biopsy for treatment and disease monitoring. There are no current guidelines for the frequency of biopsies following diagnosis, but a biopsy is normally done after treatment to evaluate response or changes in symptoms.[18] However, esophageal findings often do not correlate with symptoms.[18] A noninvasive biomarker in serum proteins would provide a less invasive and expensive route for EoE patients.

Previous literature has suggested a correlation between inflammation and elevated levels of 3-bromotyrosine.[19,20] Biological bromination occurs from the production of hypobromous acid. Hypobromous acid, a product of eosinophil peroxidase, is an oxidant that converts L-tyrosine to 3-bromotyrosine in plasma proteins.[6,18,20,21] As eosinophils are activated by IL-5, eosinophil peroxidase utilizes hypobromous acid to catalyze the peroxidation of halides. Intermediates are formed from halogens already existing in the human body. Bromide is found in plasma and extracellular fluids at concentrations ranging from 20 to 120 μM. Activated eosinophils, in conjunction with the reactive halogen species, result in the incorporation of halogens into proteins.[20]

The stable and unique properties of 3-bromotyrosine make it a good candidate as a biomarker for eosinophil activation.[18,21] In previous work, urinary levels of 3-bromotyrosine were used to predict the presence of asthma.[22,23] Levels of 3-bromotyrosine in plasma proteins may be a useful biomarker for eosinophil activation in EoE patients. Determining a correlation between 3-bromotyrosine and active eosinophils could provide an advanced and less invasive form of disease monitoring. Further research on EoE could also provide insight into inflammatory-mediated diseases such as cardiovascular disease and asthma.

Precise serum analysis of protein residues generally requires analysis techniques such as liquid chromatography (LC) coupled to mass spectrometry (MS). This study used LC and triple quadrupole MS in multiple reaction monitoring (MRM) mode to accurately detect and quantify L-tyrosine and 3-bromotyrosine in human plasma. Liquid chromatography separation minimizes ion suppression while triple quadruple mass spectrometry analyzes ions with high selectivity and sensitivity. The two together provide a fast and effective method of biomarker analysis in plasma.

Plasma contains high concentrations of proteins, so a minimal amount of sample (10 μL) was required to achieve accurate quantification. Using a small volume of plasma reduces the amount of blood that needs to be taken from patients. Patients diagnosed with EoE undergo routine endoscopies for disease monitoring which requires an IV. Plasma was chosen over urine because endoscopy preparation requires patients to refrain from drinking liquids the morning of the procedure, so a urine sample is often difficult to obtain.

Herein, we propose a method with fewer sample preparation steps and faster analysis times than those previously reported.[18,19] Higher throughput of sample analysis both allows patients to obtain quicker results and saves time and money. This method can provide researchers with the means to investigate biomarkers in plasma using a small amount of sample. Further investigation into 3-bromotyrosine and inflammatory-mediated diseases could provide patients and providers with much less invasive forms of disease monitoring.

2 Materials and methods

2.1 Materials

L-Tyrosine and 3-bromotyrosine standards were purchased from Chem-Impex (Wood Dale, IL, USA). L-Tyrosine-$^{13}C_6$ and 3-bromotyrosine-$^{13}C_6$ were used as internal standards and purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). All solvents (water, methanol, acetonitrile, and formic acid) were of LC-MS grade. Water and formic acid were purchased from Fisher (Thermo Fisher Scientific, Pittsburgh, PA, USA); methanol and acetonitrile were purchased from Birch Biotech (Morgantown, PA, USA). HLB Oasis solid phase extraction (SPE) columns (200 mg, 30 μm) were purchased from Waters (Milford, MA, USA). Pierce 660 nm protein assay kit was purchased from Fisher.

2.2 Plasma sample preparation

Deidentified plasma samples were collected by Prisma Health-Upstate from 4 patients with a confirmed diagnosis of EoE. Samples were collected after IRB approval (IRB review #0000483, FWA #00001382) and stored at −80 °C until analysis.

Plasma samples were thawed to room temperature prior to analysis. The plasma sample (10 μL) was diluted up to 1 mL in 6 M HCl and hydrolyzed at 120 °C for 24 h. Internal standards 3-bromotyrosine-$^{13}C_6$ (10.4 μL, 38.4 μg mL^{-1}) and L-tyrosine-$^{13}C_6$ (231 μL, 64.8 μg mL^{-1}) were added to solution, and samples were loaded on an HLB Oasis SPE column. The following procedures were done under positive pressure using a custom multichannel SPE manifold printed with an ELEGOO Mars 4 3D-printer. After the sample was loaded and the loading volume eluted, the column was washed with 1 mL H_2O followed by 0.75 mL of 5% methanol in water. Analytes were collected in two 0.9 mL elutions of 100% acetonitrile and evaporated under vacuum with a Thermo Scientific (Waltham, MA, USA) Savant Speedvac concentrator equipped with a refrigerated vapor trap (RVT5105).

2.3 LC-MS/MS analysis

Dried samples were reconstituted in 1 mL 80/20/0.1% (water/methanol/formic acid, v/v/v) and filtered with a 13 mm PTFE syringe filter (0.2 μM membrane). The same sample was diluted 100× and 10× for analysis of L-tyrosine and 3-bromotyrosine, respectively. A volume of 10 μL was injected into an LC system consisting of an Agilent 1260 Infinity Binary Pump (Agilent, Santa Clara, CA, USA) and an Agilent reverse phase C_{18} column (Poroshell 120, 4.6 × 150 mm, 4 mm). The flow rate was set at 1 mL min^{-1} with a solvent composition of 20% methanol from 0 to 0.5 min. The methanol content was linearly increased from 20% to 100% from 0.5 to 4 min, then held at 100% methanol from 4 to 5 min. The gradient decreased linearly from 100% to 20% methanol from 5 to 7 min. The column was then allowed to equilibrate at the starting solvent composition from 7 to 13 min.

The effluent was subject to analysis by an Agilent 6470 triple quadrupole mass spectrometer equipped with an electrospray ionization source. MassHunter (version 12.0) software was used for instrument control and data analysis. The gas flow was set to 13.0 L min^{-1}, the nebulizer gas was set to 35.0 psi, and the sheath gas flow was set to 11.0 L min^{-1}. A +3.5 kV capillary voltage was applied along with a 300 °C gas temperature to obtain optimal desolvation. Quantitative analysis was conducted using selected reaction monitoring (MRM) in positive mode. The fragmentor was set to 95 V for 3-bromotyrosine and 3-bromotyrosine-$^{13}C_6$ and 80 V for L-tyrosine and L-tyrosine-$^{13}C_6$. The collision cell accelerator voltage (CAV) was set to 5 V and the dwell time was 10 ms for each analyte. MS/MS transitions for L-tyrosine and L-tyrosine-$^{13}C_6$ were *m*/*z* 182.0 to *m*/*z* 164.9 (CE 7 V) and *m*/*z* 188.0 to *m*/*z* 170.9 (CE 7 V), respectively. MS/MS transitions for 3-bromotyrosine and 3-bromotyrosine-$^{13}C_6$ were *m*/*z* 260.0 to *m*/*z* 213.9 (CE 11 V) and *m*/*z* 266.0 and *m*/*z* 248.9 (CE 11 V), respectively. Extracted ion chromatograms of the precursor ions were used for quantification.

For the calibration curve, stock solutions of L-tyrosine (183.6 μg mL^{-1}) and 3-bromotyrosine (219.6 μg mL^{-1}) were made up in 80/20 (water/methanol) and stored at −20 °C. Calibration curve solutions were prepared by serial dilution. L-Tyrosine calibration levels were made up in 80/20/0.1% (water/methanol/formic acid) at 50, 100, 200, 300, 400, and 500 ng mL^{-1} with 150 ng mL^{-1} internal standard. 3-Bromotyrosine calibration levels were made up in 80/20/0.1% (water/methanol/formic acid) at 10, 25, 50, 100, 200, and 300 ng mL^{-1} with 40 ng mL^{-1} internal standard.

2.4 Plasma digestion validation using 660 nm protein assay

For optimization of the protein digestion, we measured the amount of protein present in a sample before and after subject to hydrolysis. A calibration curve was made at the following concentrations of BSA: 2000, 1500, 1000, 750, 500, 250, 125, 25, 0 ng mL^{-1}. To measure total protein content in samples, 150 μL Pierce reagent followed by 10 μL sample was pipetted into a 96 well plate in 3 replicates. The samples were mixed and allowed to incubate for 10 min. Samples were read at 660 nm using a BioTek Epoch 2 microplate reader (BioTek, Winooski, VT, USA).

3 Results and discussion

3.1 Method development

An LC-MS/MS method was created to detect and quantify 3-bromotyrosine in human plasma. Our method workflow involves plasma sample preparation by protein hydrolysis and solid phase extraction, followed by analysis using high-pressure liquid chromatography and triple quadrupole mass spectrometry. The method is selective and sensitive and provides a faster analysis of 3-bromotyrosine in plasma than previous methods.[19,24]

3.1.1 Protein precipitation. The first step optimized in our plasma sample preparation method was protein precipitation. Precipitation is usually performed with organic solvents such as acetone. Doucette, *et al.* proposed a rapid precipitation method using 20 mM NaCl and 80% acetone resulting in a >98% recovery of proteins after only 2 minutes.[25] We attempted to apply this precipitation method to our sample preparation workflow. The precipitation of proteins was successful; however, we found the precipitated proteins to be difficult to resuspend in the digestion matrix (6 M HCl). Because of the harsh nature of the next step in the workflow (hydrolysis in 6 M HCl) and the difficulty of dissolution of precipitated proteins, we removed the protein precipitation step from the workflow.

3.1.2 Protein hydrolysis. Our target analytes were amino acid residues on whole proteins, so hydrolysis was necessary for quantification. We first optimized the digestion of our samples by hydrolyzing the proteins in 6 M HCl at 175 °C for 10 minutes in a microwave reactor. We were able to detect L-tyrosine and 3-bromotyrosine; however, quantification of L-tyrosine and 3-bromotyrosine was not reproducible. We hypothesized the lack of reproducibility was due to incomplete digestion of proteins, so we altered our method to a 24 hour digest at 120 °C. Using a 660 nm protein assay, we determined that there were no quantifiable proteins left following the 24 hour digestion. In addition, microwave digestion enabled faster hydrolysis for a single sample, but we found that digestion in an oven is more efficient for multiple samples. Microwave digestion was limited to 1 sample at a time with a specialized apparatus, while the overnight digestion in the oven only required centrifuge tubes and space in the oven for the samples. We digested as many as 24 samples simultaneously in the oven.

3.1.3 Solid phase extraction and recovery. The next step in our sample preparation workflow was solid phase extraction. We found that the Oasis hydrophilic lipophilic-balanced (HLB) cartridge provided the best extraction of our sample and was stable even with the low pH starting solution. This cartridge is made of two monomers, hydrophilic *N*-vinylpyrrolidone and lipophilic divinylbenzene, which provide a neutral polar "hook" retaining polar analytes.[26] Due to the large loading volume (1 mL), we chose a cartridge with 200 mg of packing material. Recovery decreased as we decreased the volume of packing material, likely due to a smaller loading capacity. We initially performed the extraction with just one wash step of water followed by elution with 1 mL of methanol. However, the recovery increased when we introduced an additional wash step of 0.75 mL 95/5 (water/methanol, v/v) and two 0.9 mL elutions of acetonitrile. The percent recovery for our internal standards was investigated at three quality control levels for both L-tyrosine-$^{13}C_6$ and 3-bromotyrosine-$^{13}C_6$. We used isotopic internal standards because L-tyrosine and 3-bromotyrosine are endogenous in plasma. The recovery for L-tyrosine-$^{13}C_6$ at 150, 250, and 450 ng mL^{-1} was 99% (±5%), 86% (±12%), and 82% (±6%), respectively, with relative standard deviations in parentheses. The recovery for 3-bromotyrosine-$^{13}C_6$ at 30, 75, 250 ng mL^{-1} was 91% (±7%), 85% (±5%), and 104% (±3%), respectively. We custom-built a positive-pressure SPE manifold and attached Teflon tubing maintaining an air pressure of <2 psi. We found that conducting SPE under positive pressure was more reproducible than negative pressure, so we used the manifold to process all samples. A design and description of the apparatus is included in the ESI.†

Following SPE, samples were dried down *via* vacuum centrifugation and reconstituted in 1 mL 80/20/0.1% (water/methanol/formic acid) for accurate quantification. The samples were filtered using a PTFE syringe filter with a 0.2 μM membrane to remove any large impurities that may clog LC tubing. The concentration of L-tyrosine was much higher than that of 3-bromotyrosine, so a larger dilution for L-tyrosine was required. The sample was split into two aliquots, one for 3-bromotyrosine and one for L-tyrosine. The aliquot for 3-bromotyrosine was diluted 10-fold, and the aliquot for L-tyrosine was diluted 100-fold.

3.1.4 LC separation and extracted ion chromatograms. Fig. 1 shows extracted ion chromatograms of L-tyrosine (Fig. 1A) and 3-bromotyrosine (Fig. 1B) with chromatograms of their respective internal standards below each (Fig. 1C and D). Both of our target analytes have the same retention time as their internal standard. L-Tyrosine elutes at 1.8 minutes and 3-bromotyrosine elutes at 2.6 minutes, demonstrating rapid separation of our analytes. We initially ran our separation at 0.75 mL min^{-1} but found that increasing the flow rate to 1 mL min^{-1} allowed for faster separation without meaningfully reducing resolution. We allowed 6 minutes for equilibration at the end of the separation to prevent variability in our retention times. Lastly, we determined that heating the column to 50 °C provided optimum efficiency of LC separation.

3.2 LC-MS/MS analysis

3.2.1 Multiple reaction monitoring. Fig. 2 shows sample mass spectra for L-tyrosine (Fig. 2A) and 3-bromotyrosine (Fig. 2B and C). The precursor ions ($[M + H]^+$) for tyrosine and 3-bromotyrosine were *m*/*z* 182.1, *m*/*z* 260.1, and *m*/*z* 261.9. The fragments used for quantification occurred at *m*/*z* 165.0 for tyrosine and *m*/*z* 213.9 for 3-bromotyrosine. The L-tyrosine fragmentation to *m*/*z* 165.0 likely corresponds to the loss of a hydroxyl group, $(M + H–OH)^+$ and the 3-bromotyrosine fragmentation to *m*/*z* 213.9 likely corresponds to the loss of H_2O and CO.[27]

3.2.2 Calibration, linearity, and lower limit of quantification. Calibration curves were constructed for L-tyrosine from 50 to 500 ng mL^{-1} with 150 ng mL^{-1} internal standard and for 3-bromotyrosine from 10 to 300 ng mL^{-1} with 40 ng mL^{-1} internal standard. Calibration curves are shown in Fig. 3. Each standard was injected for 6 replicates per day for 3 days. Fig. 3A shows that the area ratio (analyte area/IS area) for L-tyrosine was linear from 50 to 500 ng mL^{-1} with an R^2 value of 0.9995. The same was seen for 3-bromotyrosine with a linear fit from 10 to 300 ng mL^{-1} and an R^2 value of 0.9998 (Fig. 3B). Extracted ion chromatograms were used for detection and quantification. Under the optimized MRM conditions, the lower limit of quantification for L-tyrosine was 50 ng mL^{-1} and 10 ng mL^{-1} for 3-bromotyrosine.

3.3 Method validation

3.3.1 Precision and accuracy. The precision of this method was assessed by injecting each calibration level in 6 replicates over 3 separate days. The precision and accuracy data for L-tyrosine are shown in Table 1, and the precision and accuracy data for 3-bromotyrosine are shown in Table 2. The intraday variability (% RSD) for 3-bromotyrosine ranged from 0.46% to 4.9% and the interday variability (% RSD) for all samples injected ranged from 0.95% to 4.6%. The intraday variability for L-tyrosine ranged from 0.11% to 0.90%, and the interday variability ranged from 0.20% to 0.58%.

The accuracy of the assay was performed using 3 quality controls (QC) in 6 replicates over 3 separate days. The QC concentrations were 150, 250, and 450 ng mL^{-1} for L-tyrosine and 30, 75, and 250 ng mL^{-1} for 3-bromotyrosine. The bias of

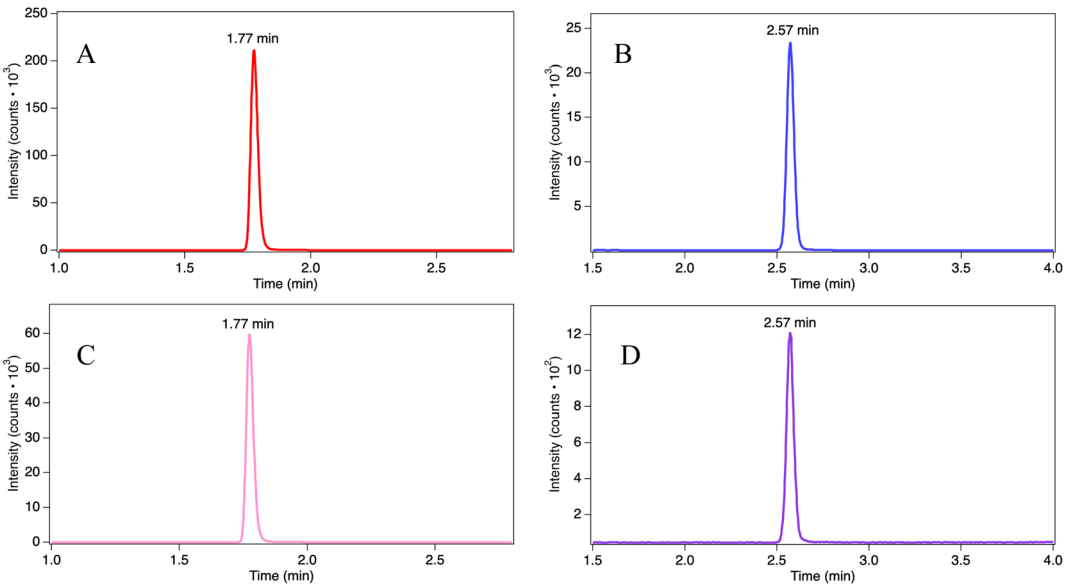

Fig. 1 (A) Extracted ion chromatogram (EIC) of L-tyrosine (300 ng mL^{-1}). (B) EIC of 3-bromotyrosine (50 ng mL^{-1}). (C) EIC of L-tyrosine-$^{13}C_6$ (150 ng mL^{-1}). (D) EIC of 3-bromotyrosine-$^{13}C_6$ (40 ng mL^{-1}).

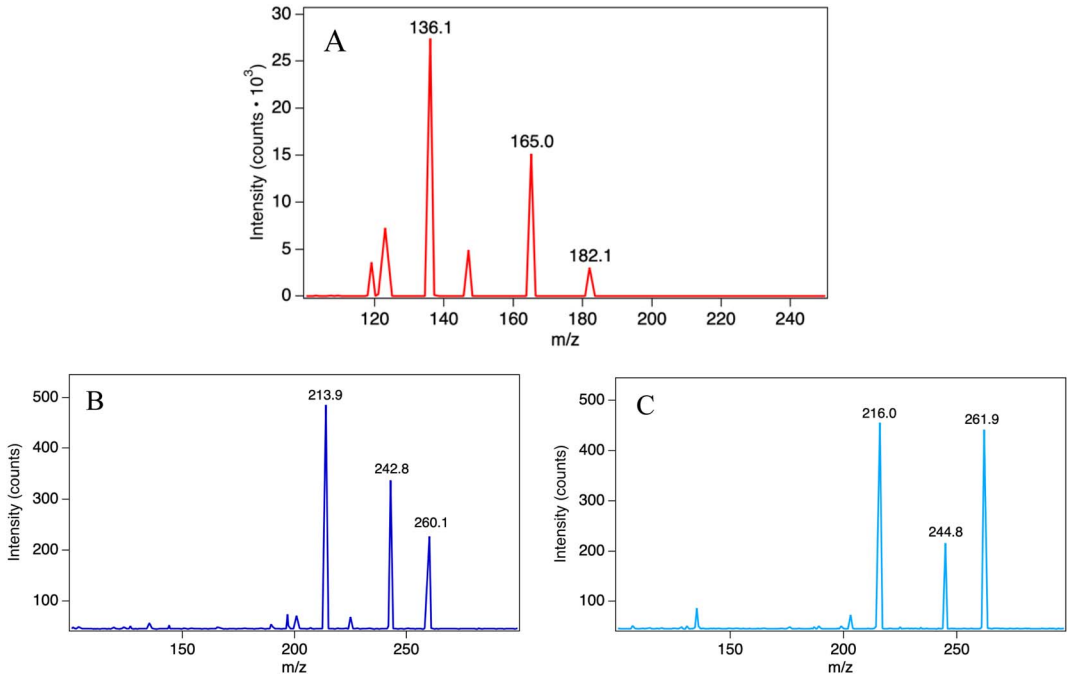

Fig. 2 MS/MS spectra of (A) L-tyrosine (*m/z* 182.1 → 165.0, 136.1), (B) 3-bromotyrosine with the lighter bromine isotope (260.1 → 242.8, 213.9), and (C) 3-bromotyrosine with the heavier bromine isotope (261.9 → 244.8, 216.0).

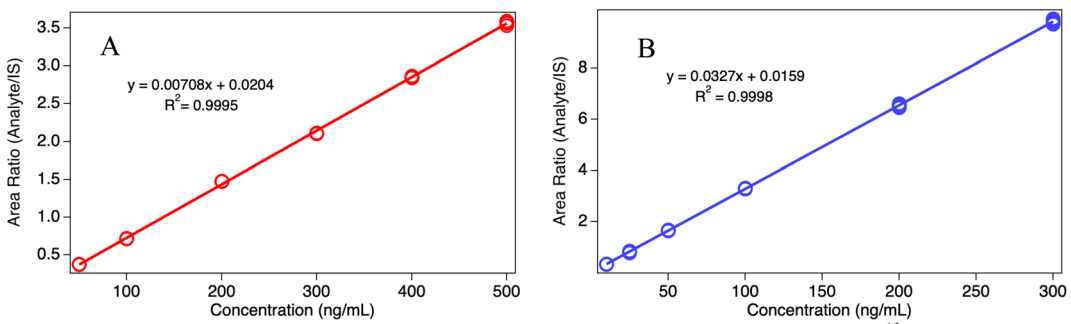

Fig. 3 Calibration curves of (A) L-tyrosine and (B) 3-bromotyrosine at 6 levels with $^{13}C_6$ internal standard (n = 6 injections/level).

Table 1 Precision and accuracy of L-tyrosine calibration curves[a]

Nominal conc. (ng mL^{-1})	Calculated conc. (ng mL^{-1}) day 1, n = 6	Calculated conc. (ng mL^{-1}) day 2, n = 6	Calculated conc. (ng mL^{-1}) day 3, n = 6	Bias (%) n = 18	Interday variation (% RSD) (n = 18)
50	50.1 (±0.5%)	50.2 (±0.3%)	50.1 (±0.5%)	2.5	0.41
100	98.3 (±0.5%)	98.5 (±0.3%)	98.3 (±0.2%)	−1.7	0.36
200	206 (±0.3%)	205 (±0.4%)	206 (±0.1%)	2.8	0.30
300	296 (±0.9%)	295 (±0.4%)	295 (±0.3%)	−1.7	0.58
400	398 (±0.2%)	399 (±0.3%)	400 (±0.3%)	−0.16	0.31
500	502 (±0.4%)	502 (±0.4%)	501 (±0.7%)	0.32	0.47
Low QC	143 (±0.7%)	144 (±0.2%)	144 (±0.2%)	−4.2	0.43
Med. QC	230 (±0.2%)	229 (±0.3%)	230 (±0.1%)	−8.1	0.20
High QC	409 (±0.5%)	410 (±0.2%)	411 (±0.3%)	−8.8	0.34

[a] The relative standard deviation (CV%) is shown in parentheses.

QCs was maintained between −8.8–2.8% for L-tyrosine and −5.7–0.47% for 3-bromotyrosine. These method validation parameters fall within FDA guidelines.[28]

3.3.2 Matrix effects, carryover, and stability. Matrix effects were evaluated by spiking low and high QCs (30 ng mL^{-1} and 250 ng mL^{-1}) of 3-bromotyrosine-$^{13}C_6$ into digested plasma and

Table 2 Precision and accuracy of 3-bromotyrosine calibration curves[a]

Nominal conc. (ng mL^{-1})	Calculated conc. (ng mL^{-1}) day 1, $n = 6$	Calculated conc. (ng mL^{-1}) day 2, $n = 6$	Calculated conc. (ng mL^{-1}) day 3, $n = 6$	Bias (%) $n = 18$	Interday variation (% RSD) ($n = 18$)
10	10.5 (±5%)	9.76 (±2%)	9.86 (±2%)	0.40	4.6
25	25.6 (±5%)	24.8 (±4%)	24.7 (±4%)	0.34	4.5
50	49.7 (±1%)	49.9 (±0.6%)	50.3 (±1%)	−0.06	0.95
100	100 (±3%)	101 (±0.9%)	101 (±0.5%)	0.37	1.9
200	198 (±2%)	199 (±1%)	200 (±0.8%)	−0.51	1.5
300	302 (±2%)	300 (±1%)	300 (±0.9%)	0.19	1.3
Low QC	30.3 (±2%)	29.9 (±2%)	30.1 (±2%)	0.33	1.6
Med. QC	75.4 (±0.9%)	74.9 (±0.9%)	75.8 (±0.8%)	0.47	0.98
High QC	235 (±2%)	236 (±0.5%)	236 (±0.7%)	−5.7	1.0

[a] The relative standard deviation (CV%) is shown in parentheses.

comparing the peak area to internal standards spiked into neat solution. The peak area for the matrix solution was 85% (±10%) compared to the neat solution for the low QC and 88% (±8%) for the high QC. Carryover was determined by running 3 blank injections of 80/20 (water/methanol) after 6 injections of the upper limit of quantification (ULOQ). Very little carryover was detected for L-tyrosine, resulting in 0.52% (±2%) of the ULOQ. 3-Bromotyrosine exhibited even less carryover, being 0.043% (±0%) of the ULOQ.

The stability of 3-bromotyrosine and L-tyrosine in 6 M HCl at 120 °C was investigated by spiking internal standards into plasma in 6 M HCl prior to oven digestion. Both internal standards were stable with a 99.0% (±8%) recovery of L-tyrosine-$^{13}C_6$ and a 94.0% (±7%) recovery of 3-bromotyrosine-$^{13}C_6$ after 24 hours in the oven.

3.4 Application of method in biological samples

Method applicability was investigated using plasma samples from 4 patients with a confirmed diagnosis of EoE. Sample extracted ion chromatograms are shown in Fig. 4. Due to individual samples having different protein concentrations, the quantity of 3-bromotyrosine was normalized by dividing by the total quantified L-tyrosine. Table 3 reports the patients' demographics and compares eosinophils to raw and normalized 3-bromotyrosine concentration. In our initial sample collection, all plasma samples came from male patients due to the more frequent occurrence of the disease in males than females.[29] The patient with 0 eosinophils per high-powered field (HPF) had a [BrY]/[Tyr] ratio of 0.236 which is greater than the patients with 55 and 100 eosinophils per HPF. These patients had a [BrY]/[Tyr] ratio of 0.154 and 0.158, respectively. If 3-bromotyrosine were correlated with eosinophil count, we would expect to see an increase in [BrY]/[Tyr] ratio as the eosinophil count increased. Interestingly, the patient with only 15 eosinophils per HPF had the highest normalized amount of 3-bromotyrosine, with a value of 0.313. This was a limited sample size, but no correlation was found between eosinophils and 3-bromotyrosine concentration in the plasma of patients diagnosed with EoE. While this study was not powered to make any statistical conclusions, these initial data suggest little to no correlation between eosinophil count and concentration of 3-bromotyrosine in plasma.

Eosinophils may not be correlated to plasma levels of 3-bromotyrosine because of the sporadic nature of the disease. Previous literature has suggested that eosinophilic esophagitis affects the esophagus in a patchy manner leading to higher concentrations of eosinophils in some areas of the esophagus than in other areas.[1] This can lead to sampling errors in routine biopsies. A more effective biomarker correlation could be 3-bromotyrosine compared to esophageal deformities. Current EoE endoscopic features for pediatric patients include white exudates, edematous mucosa, fixed rings, esophageal strictures, and longitudinal furrows.[1,30] Interestingly, we noticed that the patient with the highest [BrY]/[Tyr] ratio of 0.313 and only 15 eosinophils per HPF had active endoscopic deformities including fixed rings and esophageal strictures. However, the

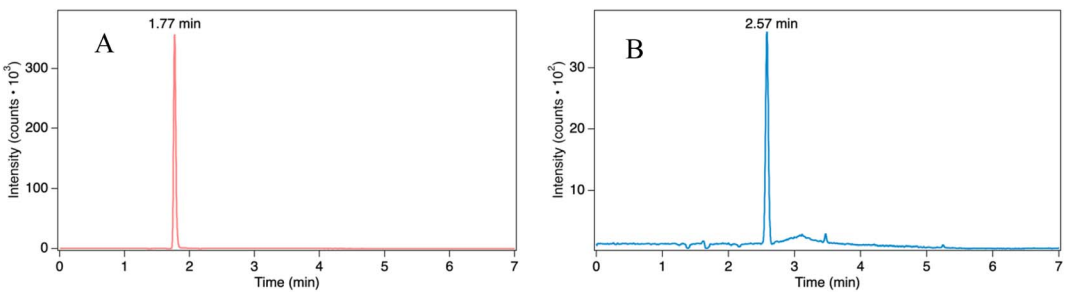

Fig. 4 EIC of (A) L-tyrosine and (B) 3-bromotyrosine in patient plasma with a confirmed diagnosis of EoE (patient 0001).

Table 3 Patient demographics, eosinophil count, and concentrations of 3-bromotyrosine and L-tyrosine[a]

Patient ID	3-Bromotyrosine (ng mL^{-1}) ($n = 9$)	L-Tyrosine (ng mL^{-1}) ($n = 9$)	[BrY]/[Tyr]	Eosinophils (HPF)	Age	Sex	Race
0001	87.7 (±19%)	371 (±2%)	0.236	0	2	Male	Other
0002	122 (±10%)	390 (±1%)	0.313	15	4	Male	White
0003	51.4 (±5%)	334 (±3%)	0.154	55	4	Male	White
0004	64.5 (±13%)	408 (±2%)	0.158	100	4	Male	Black/AA

[a] The relative standard deviation (CV%) is shown in parentheses.

patient with 100 eosinophils per HPF and a [BrY]/[Tyr] ratio of 0.158 had no active endoscopic features. These findings suggest there may be a relationship between 3-bromotyrosine and the effects of eosinophil production. The next step of this study is to expand the sample size and determine if a correlation exists between active symptoms and 3-bromotyrosine concentration or [BrY/Tyr] ratio in the plasma of pediatric patients diagnosed with EoE.

4 Conclusions

In this study, we present a fast and simple LC-MS/MS method to detect and quantify 3-bromotyrosine in plasma as a potential biomarker for eosinophilic esophagitis. We want to highlight the importance of analytical methodology in biomarker discovery. Biomarkers have the potential for less invasive and less expensive forms of disease diagnosis and monitoring. The data presented in this paper could provide researchers with the means to investigate other inflammation indicators in plasma and obtain results more rapidly than previous methods. We have proposed a sample preparation workflow consisting of protein digestion and solid phase extraction and an analysis method that successfully detects L-tyrosine and 3-bromotyrosine with low limits of quantification; we had to dilute our samples to fall within the calibration range. Our method is accurate and precise, falling within FDA guidelines. We applied our method to samples from patients diagnosed with EoE and compared normalized 3-bromotyrosine concentration to the number of eosinophils per HPF. We found no significant correlation between eosinophil count per HPF but were unable to make any conclusion regarding correlation due to small sample size. Nonetheless, the initial set of samples provides proof-of-principle for quantification of 3-bromotyrosine in patients with EoE using our method. We have proposed an additional study expanding the sample size and comparing active esophageal deformities to 3-bromotyrosine concentration and/or [BrY]/[Tyr] ratio.

Data availability

Data for this article are included in the main manuscript. STL files for the 3D-printed SPE manifold are included as a part of the ESI.† More detailed information regarding the human participants in the study are not available for confidentiality reasons.

Author contributions

Markowitz and Gilliland designed the study. Thomas performed the experimental work and analyzed the data. Germany performed some of the early experimental work. Arwood assisted in experiments for revisions. Gilliland and Thomas wrote the manuscript drafts.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We want to thank Prisma Health for funding from a SEED grant to work on this project. We also would like to thank the American Society for Mass Spectrometry for the Research at PUIs award used to fund some of this work. We want to thank Prisma Health-Upstate for providing the plasma samples. We want to thank Agilent Technologies for the generous donation of the mass spectrometer used in this study. We would also like to thank Furman University Chemistry Department for additional resources.

References

1 J. E. Markowitz and S. B. Clayton, *Gastrointest. Endosc. Clin. N. Am.*, 2018, **28**, 59–75.
2 A. J. Lucendo, J. Molina-Infante, Á. Arias, U. von Arnim, A. J. Bredenoord, C. Bussmann, J. Amil Dias, M. Bove, J. González-Cervera, H. Larsson, S. Miehlke, A. Papadopoulou, J. Rodríguez-Sánchez, A. Ravelli, J. Ronkainen, C. Santander, A. M. Schoepfer, M. A. Storr, I. Terreehorst, A. Straumann and S. E. Attwood, *United European Gastroenterology Journal*, 2017, **5**, 335–358.
3 C. A. Liacouras, *J. Pediatr. Gastroenterol. Nutr.*, 2003, **37**(suppl. 1), S23–S28.
4 H. P. Kim, R. B. Vance, N. J. Shaheen and E. S. Dellon, *Clin. Gastroenterol. Hepatol.*, 2012, **10**, 988–996.
5 J. Croese, S. K. Fairley, J. W. Masson, A. K. H. Chong, D. A. Whitaker, P. A. Kanowski and N. I. Walker, *Gastrointest. Endosc.*, 2003, **58**, 516–522.
6 J. S. Flier, L. H. Underhill and P. F. Weller, *N. Engl. J. Med.*, 1991, **324**, 1110–1118.
7 E. Ruchelli, W. Wenner, T. Voytek, K. Brown and C. Liacouras, *Pediatr. Dev. Pathol.*, 1999, **2**, 15–18.

8 S. Ryu, K. H. Lee, K. Tizaoui, S. Terrazzino, S. Cargnin, M. Effenberger, J. Il Shin and A. Kronbichler, *Int. J. Mol. Sci.*, 2020, **21**, 7253.
9 J. B. Wechsler, S. J. Ackerman, M. Chehade, K. Amsden, M. E. Riffle, M. Wang, J. Du, M. L. Kleinjan, P. Alumkal, E. Gray, K. A. Kim, B. K. Wershil and A. F. Kagalwalla, *Allergy*, 2021, **76**, 3755–3765.
10 J. J. O'Shea, M. Gadina and R. Siegel, in *Clinical Immunology*, Elsevier, 2013, pp. 108–135.
11 M. Stein, M. Collins, J. Villanueva, J. Kushner, P. Putnam, B. Buckmeier, A. Filipovich, A. Assaad and M. Rothenberg, *J. Allergy Clin. Immunol.*, 2006, **118**, 1312–1319.
12 P. Williamson and S. Aceves, *Curr. Gastroenterol. Rep.*, 2019, **21**, 56.
13 K. Takatsu and H. Nakajima, *Curr. Opin. Immunol.*, 2008, **20**, 288–294.
14 J. E. Markowitz and C. A. Liacouras, *Gastroenterol. Clin. N. Am.*, 2003, **32**, 949–966.
15 P. E. Putnam, *Gastrointest. Endosc. Clin. N. Am.*, 2008, **18**, 11–23.
16 C. A. Liacouras, G. T. Furuta, I. Hirano, D. Atkins, S. E. Attwood, P. A. Bonis, A. W. Burks, M. Chehade, M. H. Collins, E. S. Dellon, R. Dohil, G. W. Falk, N. Gonsalves, S. K. Gupta, D. A. Katzka, A. J. Lucendo, J. E. Markowitz, R. J. Noel, R. D. Odze, P. E. Putnam, J. E. Richter, Y. Romero, E. Ruchelli, H. A. Sampson, A. Schoepfer, N. J. Shaheen, S. H. Sicherer, S. Spechler, J. M. Spergel, A. Straumann, B. K. Wershil, M. E. Rothenberg and S. S. Aceves, *J. Allergy Clin. Immunol.*, 2011, **128**, 3–20.
17 A. Muir and G. W. Falk, *JAMA*, 2021, **326**, 1310.
18 K. M. Cunnion, L. K. Willis, H. B. Minto, T. C. Burch, A. L. Werner, T. A. Shah, N. K. Krishna, J. O. Nyalwidhe and K. M. Maples, *Ann. Allergy, Asthma, Immunol.*, 2016, **116**, 435–439.
19 H.-J. C. Chen and W.-L. Chiu, *Toxicol. Lett.*, 2008, **181**, 31–39.
20 W. Wu, Y. Chen, A. d'Avignon and S. L. Hazen, *Biochemistry*, 1999, **38**, 3538–3548.
21 B. Bathish, M. Paumann-Page, L. N. Paton, A. J. Kettle and C. C. Winterbourn, *J. Biol. Chem.*, 2020, **295**, 12697–12705.
22 S. H. Wedes, S. B. Khatri, R. Zhang, W. Wu, S. A. A. Comhair, S. Wenzel, W. G. Teague, E. Israel, S. C. Erzurum and S. L. Hazen, *Clin. Transl. Sci.*, 2009, **2**, 112–117.
23 Z. Wang, W. Xu, S. A. A. Comhair, X. Fu, Z. Shao, R. Bearden, J. G. Zein, E. R. Bleecker, M. Castro, L. C. Denlinger, J. V. Fahy, E. Israel, B. D. Levy, N. N. Jarjour, W. C. Moore, S. E. Wenzel, D. T. Mauger, B. Gaston, S. L. Hazen and S. C. Erzurum, *Am. J. Physiol.: Lung Cell. Mol. Physiol.*, 2022, **323**, L548–L557.
24 J. P. Gaut, J. Byun, H. D. Tran and J. W. Heinecke, *Anal. Biochem.*, 2002, **300**, 252–259.
25 J. L. Nickerson and A. A. Doucette, *J. Proteome Res.*, 2020, **19**, 2035–2042.
26 *Care and Use Manual: Oasis HLB Cartridges and 96-Well Plates*, **https://www.waters.com/webassets/cms/support/docs/715000109.pdf**, accessed 22 February 2024.
27 P. Zhao, T. White, R. Graham Cooks, Q. Chen, Y. Liu and H. Chen, *J. Am. Soc. Mass Spectrom.*, 2018, **29**, 2317–2326.
28 U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research and Center for Biological Evaluation and Research, *M10 Bioanalytical Method Validation and Study Sample Analysis*, 2022.
29 N. de Bortoli, P. Visaggi, R. Penagini, B. Annibale, F. Baiano Svizzero, G. Barbara, O. Bartolo, E. Battaglia, A. Di Sabatino, P. De Angelis, L. Docimo, M. Frazzoni, M. Furnari, A. Iori, P. Iovino, M. V. Lenti, E. Marabotto, G. Marasco, A. Mauro, S. Oliva, G. Pellegatta, M. Pesce, A. C. Privitera, I. Puxeddu, F. Racca, M. Ribolsi, E. Ridolo, S. Russo, G. Sarnelli, S. Tolone, P. Zentilin, F. Zingone, B. Barberio, M. Ghisa and E. V. Savarino, *Dig. Liver Dis.*, 2024, **56**, 951–963.
30 S. Miehlke, *Best Pract. Res., Clin. Gastroenterol.*, 2015, **29**, 739–748.

